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HEALTH EFFECTS DIVISION  
SCIENTIFIC DATA REVIEWS  
EPA SERIES 361



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

WASHINGTON, D.C. 20460

OFF OFFICIAL RECORD  
HEALTH EFFECTS DIVISION  
SCIENTIFIC DATA REVIEWS  
EPA SERIES 361

MAY 31 1996

OFFICE OF  
PREVENTION, PESTICIDES AND  
TOXIC SUBSTANCES

MAY 31 1996

MEMORANDUM

**SUBJECT** 2,4-DICHLOROPHENOXYACETIC ACID - Triisopropanolamine salt (TIPA);  
Isopropylamine salt (IPA); and Butoxyethyl ester (BEE): Review of Mutagenicity  
Studies As Requested by the Agency in a Letter Dated 10/30/93.

**FROM:** Jess Rowland, M.S., Toxicologist *Jess Rowland 5/23/96*  
Section I, Toxicology Branch II, Health Effects Division (7509C)

**TO:** Walter Waldrop / Judy Coombs  
Product Manager 71  
Reregistration Division

**THRU:** Yiannakis Ioannou, Ph.D., Head *J.M. Ioannou 5/23/96*  
Section I, Toxicology Branch II, Health Effects Division (7509C)

and

Stephanie Irene, Ph.D., Acting Chief *Stephanie R. Irene 5/30/96*  
Toxicology Branch II, Health Effects Division (7509C)

**DATA PACKAGE**

**IDENTIFICATIONS:** Submission: S472693

DP Barcode: D207071

<u>Chemical</u>	<u>PC Code</u>	<u>Caswell No</u>	<u>MRID No.</u>
IPA	030025	315 U	43327303 & 43327304
TIPA	030035	315 AE	43327301 & 43327302
BEE	030053	315 AI	43327305

**ACTION REQUESTED:** Review of three *in vitro* mammalian chromosomal aberration in rat lymphocyte assays for IPA, TIPA, and BEE and two CHO/HGPRT forward mutation assays for IPA and TIPA salts to satisfy the 1991 Guideline requirement §84-2(2).

**RESPONSE:** In the attached letter dated September 30, 1993, the Agency accepted the Registrant's (Dow Elanco) commitment to perform CHO/HGPRT forward gene mutation assays and *in vitro* chromosomal aberration assays in rat lymphocytes for IPA, TIPA and BEE of 2,4-D to satisfy the 1991 mutagenicity guidelines §84-(2). In this submission, the Registrant has submitted these studies. A Data Evaluation Record for each of the five studies cited above are attached. The Executive Summaries are provided below.

- I. **"Evaluation of 2,4-D Triisopropanolamine Salt in an In Vitro Chromosomal Aberration Assay Utilizing Rat Lymphocytes".** Health and Environmental Sciences Toxicology Research Laboratory, Dow Chemical Company, Midland, Michigan. Study ID K-008866-017, 1/13/94. MRID No. 43327301.

**EXECUTIVE SUMMARY:** In a mammalian cell chromosome aberration assay (MRID 43327301), rat lymphocyte cultures were exposed to a 2,4-D TIPA (70.9% a.i.), in deionized distilled water for 4 hours at concentrations of 78, 156, 313, 625, 1,250, 2,500, and 5,000  $\mu\text{g}/\text{mL}$  with or without metabolic activation. Preparations for metabolic activation were made from induced rat livers. Cells were harvested at 24 or 48 hours post-treatment.

2,4-D TIPA was tested to the limit concentration, 5,000  $\mu\text{g}/\text{mL}$ , to determine cytotoxicity levels. For metaphase analysis, the highest concentration produced cytotoxicity (mitotic index 39-57% of negative control without S9 activation or 59-86% with S9 activation). Two lower concentrations were 50% and 25% of the high concentration. Positive controls induced the appropriate response. There were no statistically significant increases in the proportion of aberrant cells over negative control values.

This study is classified as acceptable and satisfies the requirements for the 1991 Guideline § 84-2(2) for an *in vitro* cytogenetic mutagenicity assay.

- 
- II. **"Evaluation of 2,4-D triisopropanolamine salt in the Chinese Hamster Ovary Cell /Hypoxanthine-guanine-phosphoribosyl Transferase (CHO/HGPRT) Forward Mutation Assay".** Health and Environmental Sciences, The Toxicology Research Laboratory, Midland, MI. Laboratory Project Study ID K-008866-018. 1/31/94. MRID 43327302.

**EXECUTIVE SUMMARY:** In an *in vitro* mammalian cell gene forward mutation assay, CHO/HGPRT, (MRID 43327302) Chinese hamster ovary (CHO) cells cultured *in vitro* were exposed to a 2,4-D TIPA salt (70.9%) at concentrations of 800, 1,000, 1,250, 2,500, and 5,000  $\mu\text{g}/\text{mL}$  with and without S9 activation. The S9 fraction was derived from Aroclor-induced rat liver. The test material was delivered to the test system in water.

The test material was cytotoxic at the limit dose of 5,000  $\mu\text{g}/\text{mL}$  without S9 activation. There was no evidence of mutagenic effect at any dose level with or without activation. Findings with the positive controls confirmed the sensitivity of the test system to detect mutagenesis.

This study is classified as acceptable and satisfies the requirements for the 1991 Guideline § 84-2(2) for *in vitro* mutagenicity (mammalian forward gene mutation) assay.

- III. **"Evaluation of 2,4-Dichlorophenoxyacetic Acid Isopropylamine Salt in an In Vitro Chromosomal Aberration Assay Utilizing Rat Lymphocytes"**. Health and Environmental Sciences Toxicology Research Laboratory, Dow Chemical Company, Midland, Michigan. Study ID M-004725-016, 5/27/94. MRID No. 43327303.

**EXECUTIVE SUMMARY:** In a mammalian cell chromosome aberration assay (MRID 43327303), rat lymphocyte cultures were exposed to a 2,4-D IPA (50.2% a.i.), in deionized distilled water for 4 hours at concentrations of 96, 192, 384, 767, 1,534, 3,068, and 6,137  $\mu\text{g}/\text{mL}$  with or without metabolic activation. Preparations for metabolic activation were made from induced rat livers. Cells were harvested at 24 or 48 hours post-treatment.

2,4-D IPA was tested to 6,137  $\mu\text{g}/\text{mL}$ , to determine cytotoxicity levels. For metaphase analysis, the highest concentration produced cytotoxicity (mitotic index 28-43% of negative control without S9 activation or 48-53% with S9 activation). Two lower concentrations were 50% and 25% of the high concentration. Positive controls induced the appropriate response. The statistically significant increase in the proportion of aberrant cells at the highest concentration over the negative control value was determined not to be biologically significant since the background aberration frequency for rat lymphocytes can range from 0- 5.5% and the statistical significance was seen only because the concurrent solvent control value was 0%. Therefore, it was concluded that 2,4-D IPA at upto 3000  $\mu\text{g}/\text{mL}$  was not clastogenic in cultured rat lymphocytes harvested 48 hours after treatment.

This study is classified as acceptable and satisfies the requirements for the 1991 Guideline § 84-2(2) for *in vitro* cytogenetic mutagenicity data.

- 
- IV. **"Evaluation of 2,4- Dichlorophenoxyacetic acid isopropylamine salt in the Chinese Hamster Ovary Cell/Hypoxanthine-guanine-phosphoribosyl Transferase (CHO/HGPRT) Forward mutation Assay"**. Health and Environmental Sciences, The Toxicology Research Laboratory, Midland, MI. Laboratory Project Study ID M-004725-017. 5/27/94. MRID 43327304.

**EXECUTIVE SUMMARY:** In an *in vitro* mammalian cell gene forward mutation assay, CHO/HGPRT, (MRID 43327304) Chinese hamster ovary (CHO) cells cultured *in vitro* were exposed to a 2,4-D IPA salt (50.2%) at concentrations of 500, 1,000, 1,500, 2,000, and 3,000  $\mu\text{g}/\text{mL}$  with and without S9 activation. The S9 fraction was derived from Aroclor-induced rat liver. The test material was delivered to the test system in water.

The test material was cytotoxic at a dose of 2,500  $\mu\text{g}/\text{mL}$  with and without S9 activation. There was no evidence of a mutagenic effect at any dose level with or without activation. Findings with the positive controls confirmed the sensitivity of the test system to detect mutagenesis.

This study is classified as acceptable and satisfies the requirements for FIFRA Test Guideline 84-2 for *in vitro* mutagenicity (mammalian forward gene mutation) data.

- V. "Evaluation of 2,4-Dichlorophenoxyacetic Acid Butoxyethyl Ester in an *In Vitro* Chromosomal Aberration Assay Utilizing Rat Lymphocytes". Health and Environmental Sciences, Toxicology Research Laboratory, Dow Chemical Company, Midland, Michigan. Study ID K-007722-022, Study dates: 5/27/94. MRID No. 43327305.

**EXECUTIVE SUMMARY:** In a mammalian cell chromosome aberration assay (MRID 43327305), rat lymphocyte cultures were exposed to 2,4-D BEE (94.6% a.i.), in dimethyl sulfoxide for 4 hours at concentrations of 87.5, 175, 350, 700, and 1,400  $\mu\text{g/mL}$  with or without metabolic activation. Preparations for metabolic activation were made from induced rat livers. Cells were harvested at 24 or 48 hours post-treatment.

2,4-D BEE was tested to the limit of solubility, 1.4 mg/mL, to determine cytotoxicity levels. For metaphase analysis, the highest concentration produced cytotoxicity (mitotic index 31-80% of negative control without S9 activation or 61-67% with S9 activation). Two lower concentrations were 50% and 25% of the high concentration. Positive controls induced the appropriate response.

There were no statistically significant increases in the proportion of aberrant cells over negative control values. However, cytotoxicity of 2,4-D BEE in the presence of S9 activation was marginal at the highest dose levels selected for metaphase analysis. In addition, the highest dose level selected for metaphase analysis in the initial assay produced marginal cytotoxicity, while this same dose level was too cytotoxic for metaphase analysis in the confirmatory assay. Therefore, a decision regarding the clastogenicity of 2,4-D BEE in the presence of metabolic activation, based on the results of this study, cannot be made. It is concluded that a new study should be conducted and the chemical be evaluated at up to a reproducible cytotoxic level.

This study is classified as unacceptable and does not satisfy the requirements for the 1991 Guideline, 84-2 for *in vitro* cytogenetic mutagenicity data.



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY  
WASHINGTON, D.C. 20460

9-30-93

OFFICE OF  
PREVENTION, PESTICIDES  
AND TOXIC SUBSTANCES

RECEIVED

**CERTIFIED MAIL P322-239**

Larry E. Hammond  
Product Registration Manager  
DowElanco  
9002 Purdue Rd.  
Indianapolis, IN 46268-1189

OCT 04 1993

Registration

Dear Mr. Hammond:

Subject: 2,4-D TIPA, IPA and BEE Mouse Micronucleus Assays

The Agency has reviewed supplemental information submitted by DowElanco to upgrade the 2,4-D IPA and BEE Mouse Bone Marrow Micronucleus Test to acceptable, provided a new study is completed for mammalian cells in culture; forward gene mutation assay. A copy of the data review is enclosed for your information.

With your commitment to do a CHO/HGPRT forward gene mutation assay and an in vitro chromosomal aberration assay in rat lymphocytes to satisfy the new 84-2(2) requirement for all three compounds, the Agency has upgraded the classifications for the Ames assays (guideline 84-2a) for 2,4-D TIPA (41388202, 41797901), IPA (41388203, 41797902), and BEE (41388204, 41797903) to acceptable. The Agency has also concluded that sufficient information has been provided to reclassify the 2,4-D IPA and 2,4-D BEE studies to acceptable for satisfying the 84-2(3) data requirement. (Review of 5/4/93 accepted the 2,4-D TIPA study.)

In your letter dated March 25, 1993, you committed to do a CHO/HGPRT forward gene mutation assay and an in vitro chromosomal aberration assay in rat lymphocytes for all three compounds. These studies must be submitted to the Agency within 12 months of the date of receipt of this letter. Failure to provide these data within the time provided may result in the issuance of a Notice of Intent to Suspend affecting your registrations containing 2,4-D TIPA, IPA and BEE.



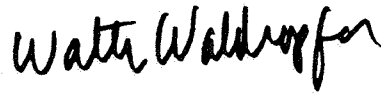
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Printed on paper that contains  
at least 75% recycled fiber

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If you have any further questions concerning this review, please refer them to Judith Coombs, the Case Review Manager for 2,4-D at (703) 308-8046.

Sincerely,

A handwritten signature in black ink that reads "Walter Waldrop". The signature is written in a cursive, slightly slanted style.

Lois Rossi, Chief  
Reregistration Branch  
Special Review and  
Reregistration Division (H7508W)

Enclosure

## DATA EVALUATION RECORD

2,4-D; triisopropylamine salt (2,4-D TIPA)

Study Type: 84-2; *In vitro* Mammalian Chromosome Aberrations in Rat Lymphocytes

Work Assignment No. 1-16A (MRID 43327301)

Prepared for

Health Effects Division  
Office of Pesticide Programs  
U.S. Environmental Protection Agency  
1921 Jefferson Davis Highway  
Arlington, VA 22202

Prepared by

Pesticide Health Effects Group  
Sciences Division  
Dynamac Corporation  
2275 Research Boulevard  
Rockville, MD 20850-3268

Primary Reviewer:  
Steven Brecher, Ph.D.

Signature: Steven Brecher  
Date: 4/15/96

Secondary Reviewer:  
William Spangler, Ph.D.

Signature: William J. Spangler  
Date: 4/15/96

Project Manager:  
William Spangler, Ph.D.

Signature: William J. Spangler  
Date: 4/15/96

Quality Assurance:  
Reto Engler, Ph.D.

Signature: Reto Engler  
Date: 4/15/96

### Disclaimer

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by Dynamac Corporation personnel.



2,4-D TIPA

IN VITRO CHROM. ABERRATION (84-2)

EPA Reviewer: Jess Rowland, M.S., Toxicologist *Jess Rowland*  
Review Section I, Toxicology Branch II (7509C)

Date 5/23/96

EPA Secondary Reviewer: Yiannakis Ioannou, Ph.D., Head *Yiannakis Ioannou*  
Review Section I, Toxicology Branch II (7509C)

Date 5/23/96

DATA EVALUATION RECORD

STUDY TYPE: *In vitro* mammalian chromosome aberrations in rat lymphocytes

Guideline: S84-2(2)

DP BARCODE: D207071

SUBMISSION: S472693

P.C. CODE: 030035

TOX. CHEM. NO.: 315AE

TEST MATERIAL: 2,4-dichlorophenoxyacetic acid triisopropanolamine salt

SYNONYMS: 2,4-D TIPA

CITATION: Linscombe, V.A. and S.J. Lick (1994) Evaluation of 2,4-D Triisopropanolamine Salt in an In Vitro Chromosomal Aberration Assay Utilizing Rat Lymphocytes. Health and Environmental Sciences Toxicology Research Laboratory, Dow Chemical Company, Midland, Michigan. Study ID K-008866-017, Study dates: 6/3/93-1/13/94. MRID No. 43327301. Unpublished.

SPONSOR: DowElanco, Indianapolis, IN 46268

EXECUTIVE SUMMARY: In a mammalian cell chromosome aberration assay (MRID 43327301), rat lymphocyte cultures were exposed to a 2,4-D TIPA formulation (70.9% a.i.), in deionized distilled water for 4 hours at concentrations of 78, 156, 313, 625, 1,250, 2,500, and 5,000 µg/mL with or without metabolic activation. Preparations for metabolic activation were made from induced rat livers. Cells were harvested at 24 or 48 hours post-treatment.

2,4-D TIPA was tested to the limit concentration, 5,000 µg/mL, to determine cytotoxicity levels. For metaphase analysis, the highest concentration produced cytotoxicity (mitotic index 39-57% of negative control without S9 activation or 59-86% with S9 activation). Two lower concentrations were 50% and 25% of the high concentration. Positive controls induced the appropriate response. There were no statistically significant increases in the proportion of aberrant cells over negative control values.

This study is classified as acceptable and satisfies the requirements for the 1991 Guideline 84-2(2) for *in vitro* cytogenetic mutagenicity data.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality provided.

## I. MATERIALS AND METHODS

## A. MATERIALS

1. Test Material: 2,4-D TIPA

Description: amber liquid

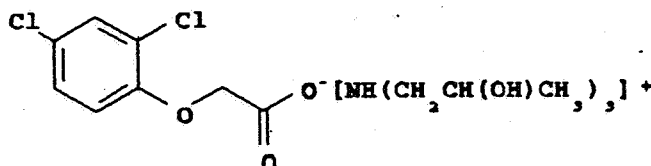
Lot/Batch #: AGR 295711

Purity: The test material is a formulation intermediate containing 70.9% 2,4-D TIPA salt. The acid (2,4-D) equivalent = 38.0%, TIPA equivalent = 38.6%

Stability of compound: Not addressed

CAS #: 18584-79-7

Structure:



Solvent used: Deionized distilled water

Other comments: Dosing solutions analyzed for actual concentration

2. Control Materials:

Negative: Solvent control

Solvent/final concentration: Deionized distilled water/1%

Positive: Nonactivation: Mitomycin C (0.5 µg/mL)

Activation: Cyclophosphamide (6 µg/mL)

3. Activation: S9 derived from

<input checked="" type="checkbox"/> Aroclor 1254	<input checked="" type="checkbox"/> induced	<input checked="" type="checkbox"/> rat	<input checked="" type="checkbox"/> liver
<input type="checkbox"/> phenobarbital	<input type="checkbox"/> non-induced	<input type="checkbox"/> mouse	<input type="checkbox"/> lung
<input type="checkbox"/> none		<input type="checkbox"/> hamster	<input type="checkbox"/> other
<input type="checkbox"/> other			<input type="checkbox"/> other

S9 purchased from Sitek Research Laboratories, Rockville, Maryland

S9 mix composition: S9 fraction (10% v/v), MgCl<sub>2</sub> (10mM), Na<sub>2</sub>HPO<sub>4</sub> buffer (50mM), pH 8.0, glucose-6-phosphate (5mM), NADP (4mM), CaCl<sub>2</sub> (10mM), KCl (30mM)

4. Test compound concentrations used: Preliminary cytotoxicity test not performed; cytotoxicity assessed concurrently with cytogenetic assay

Nonactivated conditions:

Assay 1: 4 hour treatment (78, 156, 313, 625, 1,250, 2,500, 5,000  $\mu\text{g/mL}$ ), harvest at 24 hours

Assay 2: 4 hour treatment (1,250, 2,500, 5,000  $\mu\text{g/mL}$ ), harvest at 24 and 48 hours

Activated conditions:

Assay 1: 4 hour treatment (78, 156, 313, 625, 1,250, 2,500, 5,000  $\mu\text{g/mL}$ ), harvest at 24 hours

Assay 2: 4 hour treatment (1,250, 2,500, 5,000  $\mu\text{g/mL}$ ), harvest at 24 and 48 hours

5. Test cells: Sprague-Dawley rat lymphocytes in culture. Whole blood grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, antibiotics, antimycotics, and phytohemagglutinin (20  $\mu\text{g/mL}$ ).

Properly maintained? Yes

Cell line or strain periodically checked for Mycoplasma contamination? N/A

Cell line or strain periodically checked for karyotype stability? N/A

B. TEST PERFORMANCE

1. Preliminary Cytotoxicity Assay: Performed concurrently with cytogenetic assay. Duplicate cultures per dose level. Percentage of metaphase cells per 1000 cells in each culture determined. The highest dose level was that causing depression of mitotic index 30-70% with adequate scorable cells when compared with the negative control or 5,000  $\mu\text{g/mL}$ , whichever is lower. Intermediate and low concentrations approximated to 50% and 25% of highest dose level were selected for subsequent metaphase analysis at 24-hour harvest (only highest dose level and negative control selected for subsequent metaphase analysis at 48-hour harvest).

2. Cytogenetic Assay: Two assays were run; activated and nonactivated sets were used in each assay; approximately 48 hours after initiation of cell cultures, cells were exposed to test chemical or positive control for a selected time interval, then the chemical was removed and cells were continued in culture until time of harvest
- a. Cell treatment:  
Cells exposed to test compound, solvent, or positive control for 4 hours (nonactivated), or 4 hours (activated)
- Assay 1: Set 1: 4 hr exposure, -S9, harvest at 24 hrs  
Set 2: 4 hr exposure, +S9, harvest at 24 hrs
- Assay 2: Set 1: 4 hr exposure, -S9, harvest at 24 hrs  
Set 2: 4 hr exposure, +S9, harvest at 24 hrs  
Set 3: 4 hr exposure, -S9, harvest at 48 hrs  
Set 4: 4 hr exposure, +S9, harvest at 48 hrs
- b. Spindle inhibition  
Inhibitor used/concentration: Colcemid (0.2  $\mu\text{g}/\text{mL}$ )  
Administration time: 3 hours (before cell harvest)
- c. Cell harvest:  
Cells exposed to test material, or solvent harvested 24 or 48 hours after termination of treatment (nonactivated), or 24 or 48 hours after termination of treatment (activated). Positive control cultures harvested 24 hours after termination of treatment. Cells swollen by hypotonic treatment (0.075 M KCl) and fixed with methanol:glacial acetic acid (3:1).
- d. Details of slide preparation:  
Cells suspended in fixative dropped onto microscopic slides and stained with Giemsa.

## e. Metaphase analysis

No. of cells examined per dose: 200Solvent control: 200Positive control: 100Scored for structural: **Yes**Scored for numerical: **No**Coded prior to analysis: **Yes**

f. Evaluation criteria: Only metaphases with 42 centromeres were scored with the exception of cells with multiple aberrations. Gaps were not included in calculations of total cytogenetic aberrations. The test was considered acceptable if chromosomal aberration frequency in the positive control was significantly higher than the negative control. Aberration frequency in the negative control should be within reasonable limits of laboratory historical values. Positive response was claimed if a significant treatment-related and reproducible increase in frequency of cells with chromosome aberrations was observed.

g. Statistical analysis: Data evaluated for statistical significance at  $\alpha=0.01$ , using Chi-square statistics from two way contingency table.

**II. REPORTED RESULTS**

Analysis of dose formulations by HPLC indicated that concentrations were between 93 and 103% of target concentrations for Assay 1 and between 100 and 106% for Assay 2.

A. Preliminary cytotoxicity assay: Cytotoxicity, as indicated by effects on the mitotic index, was assessed concurrently with the cytogenetic assay. In Assay 1, Set 1, without S9 activation, the 5,000  $\mu\text{g}/\text{mL}$  concentration reduced the mitotic index to 57% of the negative control value. In Set 2, with S9 activation, 5,000  $\mu\text{g}/\text{mL}$  reduced the mitotic index to 86% of the negative control value (first replicate was 54% of control, second replicate was 119% of control).

In Assay 2, Set 1, without S9 activation, 5,000  $\mu\text{g}/\text{mL}$  reduced the mitotic index to 39% of the negative control value. In Set 2, with S9 activation, 5,000  $\mu\text{g}/\text{mL}$  reduced the mitotic index to 59% of the negative control value. By the 48-hour harvest (Sets 3 and 4), the mitotic indices were similar to negative control values.

- B. Cytogenetic assay: Results are presented in Attachments 1 and 2 (study report pages 23, 24, 27, 29).

In Assay 1, cultures treated with 1,250, 2,500, or 5,000  $\mu\text{g}/\text{mL}$  of the test compound were analyzed for chromosomal aberration frequencies at the 24-hour harvest (Attachment 1). 2,4-D TIPA caused no statistically significant increases in the proportion of aberrant cells, in either the presence or absence of S9 activation. The frequency of abnormal cells in the negative controls was within the historical range of the laboratory. Significant increases in the frequency of cells with aberrations were observed in cultures treated with the positive control compounds (mitomycin C, without S9, cyclophosphamide, with S9).

In confirmatory Assay 2, cultures treated with 1,250, 2,500, or 5,000  $\mu\text{g}/\text{mL}$  of the test compound were analyzed for chromosomal aberration frequencies at the 24-hour harvest (Attachment 2). Cultures treated with 5,000  $\mu\text{g}/\text{mL}$  of the test compound were analyzed for chromosomal aberration frequencies at the 48-hour harvest. 2,4-D TIPA caused no statistically significant increases in the proportion of aberrant cells, either in the presence or absence of S9 activation. The frequency of abnormal cells in the negative controls was within the historical range of the laboratory. Significant increases in the frequency of cells with aberrations were observed at the 24-hour harvest in cultures treated with positive control compounds.

### III. REVIEWER'S DISCUSSION/CONCLUSIONS:

- A. The reviewer agrees with the study authors that 2,4-D TIPA, at up to 5,000  $\mu\text{g}/\text{mL}$ , was not clastogenic in cultured rat lymphocytes harvested 24 or 48 hours after treatment (1-2 cell cycles). The negative controls had comparable low frequencies of chromosome aberrations. In addition, the sensitivity of the assay system to detect damage to chromosomes was adequately demonstrated by the results obtained with the positive controls (mitomycin C, -S9, and cyclophosphamide, +S9). The reviewer concludes that the results of this study provided sufficient evidence to consider 2,4-D-TIPA negative in this *in vitro* test system.

**B. Study deficiencies**

The following deficiencies would not be expected to alter the conclusions of the study:

1. Cytotoxicity of 2,4-D TIPA at the limit concentration (5,000  $\mu\text{g/mL}$ ) was marginal; 43-61% reduction in mitotic activity versus negative control at the 24-hour harvest without S9 activation, and 14-41% reduction in mitotic activity with S9 activation (14% reduction was mean of duplicate cultures; one exhibited a 46% reduction, the other, a 19% increase).

2. The report indicated that a summary of characterization data for 2,4-D TIPA was included as Table 8. However, Table 8 was not provided.

**ATTACHMENTS**



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*TR Review 11942*

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Pages 16 through 19 are not included in this copy.

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The material not included contains the following type of information:

- Identity of product inert ingredients.
  - Identity of product impurities.
  - Description of the product manufacturing process.
  - Description of quality control procedures.
  - Identity of the source of product ingredients.
  - Sales or other commercial/financial information.
  - A draft product label.
  - The product confidential statement of formula.
  - Information about a pending registration action.
  - FIFRA registration data.
  - The document is a duplicate of page(s) \_\_\_\_\_.
  - The document is not responsive to the request.
- 

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

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## DATA EVALUATION RECORD

2,4-D; triisopropylamine salt (2,4-D TIPA)

Study Type: 84-2; Mammalian Cells in Culture Gene Mutation Assay in  
Chinese Hamster Ovary Cells (CHO/HGPRT)

Work Assignment No. 1-16B (MRID 43327302)

Prepared for

Health Effects Division  
Office of Pesticide Programs  
U.S. Environmental Protection Agency  
1921 Jefferson Davis Highway  
Arlington, VA 22202

Prepared by

Pesticide Health Effects Group  
Sciences Division  
Dynamac Corporation  
2275 Research Boulevard  
Rockville, MD 20850-3268

Primary Reviewer:  
Ann Foster, Ph.D.

Signature: Steven Boehm  
Date: 4/15/96

Secondary Reviewer:  
William Spangler, Ph.D.

Signature: William J. Spangler  
Date: 4/15/96

Project Manager:  
William Spangler, Ph.D.

Signature: William J. Spangler  
Date: 4/15/96

Quality Assurance:  
Reto Engler, Ph.D.

Signature: Reto Engler  
Date: 4/15/96

### Disclaimer

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by Dynamac Corporation personnel.

2,4-D TIPA Salt

MAMMALIAN CELLS IN CULTURE; GENE MUTATION (84-2)

EPA Reviewer: Jess Rowland, M.S., Toxicologist *Jess Rowland*  
Review Section I, Toxicology Branch II (7509C)

Date 5/23/96

EPA Secondary Reviewer: Yiannakis Ioannou, Ph.D., Head *YIoannou*  
Review Section I, Toxicology Branch II(7509C)

Date 5/23/96

DATA EVALUATION RECORD

STUDY TYPE: Mammalian cells in culture gene mutation assay in Chinese hamster ovary cells.

Guideline Number: 584-2(2)

DP BARCODE: D207071

SUBMISSION: S472693

P.C. CODE: 030035

TOX. CHEM. NO.: 315AE

TEST MATERIAL: 2,4-Dichlorophenoxyacetic acid triisopropanolamine salt

SYNONYMS: 2,4-D TIPA

CITATION: Linscombe, V.A. and S.J. Lick (1994) Evaluation of 2,4-D triisopropanolamine salt in the Chinese Hamster Ovary Cell/Hypoxanthine-guanine-phosphoribosyl Transferase (CHO/HGPRT) Forward Mutation Assay. Health and Environmental Sciences, The Toxicology Research Laboratory, Midland, MI. Laboratory Project Study ID K-008866-018. 1/13/94. MRID 43327302.

SPONSOR: DowElanco, Indianapolis, IN 46268

EXECUTIVE SUMMARY: In an *in vitro* mammalian cell gene forward mutation assay, CHO/HGPRT, (MRID 43327302) Chinese hamster ovary (CHO) cells cultured *in vitro* were exposed to a 2,4-D TIPA salt formulation (70.9%) at concentrations of 800, 1,000, 1,250, 2,500, and 5,000  $\mu\text{g}/\text{mL}$  with and without S9 activation. The S9 fraction was derived from Aroclor-induced rat liver. The test material was delivered to the test system in water.

The test material was cytotoxic at the limit dose of 5,000  $\mu\text{g}/\text{mL}$  without S9 activation. There was no evidence of mutagenic effect at any dose level with or without activation. Findings with the positive controls confirmed the sensitivity of the test system to detect mutagenesis.

This study is classified as **Acceptable** and satisfies the requirements for the 1991 Guideline 84-2(2) for *in vitro* mutagenicity (mammalian forward gene mutation) data.

COMPLIANCE: Signed and dated GLP, Quality Assurance, Data Confidentiality, and Flagging statements were provided.

I. MATERIALS AND METHODS

## I. MATERIALS AND METHODS

## A. MATERIALS

1. Test Material: 2,4-Dichlorophenoxyacetic acid triisopropanolamine (TIPA) salt

Description: amber liquid

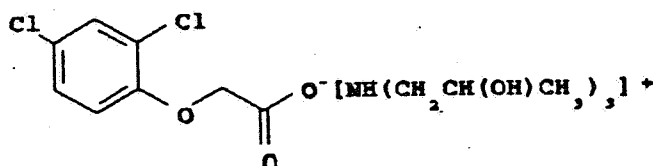
Lot/Batch #: AGR 295711

Purity: 70.9% a.i.

Stability of compound: Not reported

CAS #: 18584-79-7

Structure:



Solvent used: Water

Other comments: None

2. Control Materials:

Solvent/final concentration: culture medium

Positive: Non-activation (concentrations, solvent):

Ethylnethanesulfonate (EMS)/621  $\mu\text{g}/\text{mL}$  in culture medium

Activation (concentrations, solvent):

20-Methylcholanthrene (20-MCA)/4  $\mu\text{g}/\text{mL}$  in 1% DMSO/culture medium3. Activation: S9 derived from

<input checked="" type="checkbox"/> Aroclor 1254	<input checked="" type="checkbox"/> induced	<input checked="" type="checkbox"/> rat	<input checked="" type="checkbox"/> liver
<input type="checkbox"/> phenobarbital	<input type="checkbox"/> non-induced	<input type="checkbox"/> mouse	<input type="checkbox"/> lung
<input type="checkbox"/> none		<input type="checkbox"/> hamster	<input type="checkbox"/> other
<input type="checkbox"/> other		<input type="checkbox"/> other	

Describe S9 mix composition (if purchased, give details): 10 mM  $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$ , 5 mM glucose-6-phosphate, 4 mM NADP, 10 mM  $\text{CaCl}_2$ , 30 mM KCl, and 50 mM sodium phosphate (pH 8.0)

4. Test Cells: Chinese hamster ovary (CHO) cellsProperly maintained? **Yes**Periodically checked for Mycoplasma contamination? **Yes**Periodically checked for karyotype stability? **Not reported**Periodically "cleansed" against high spontaneous background? **Not reported**

Media: Ham's F-12 nutrient mix supplemented with 5% heat-activated fetal calf serum; 25 mM HEPES; Fungizone; penicillin G; and streptomycin sulfate.

5. Locus Examined: thymidine kinase (TK)

Selection agent: \_\_\_\_\_

bromodeoxyuridine (BrdU)

fluorodeoxyuridine (FdU)

trifluorothymidine (TFT)

 hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)

Selection agent: \_\_\_\_\_

8-azaguanine (8-AG)

10  $\mu$ M 6-thioguanine (6-TG) Na<sup>+</sup>/K<sup>+</sup> ATPase

Selection agent: \_\_\_\_\_ ouabain

(give concentration)

 other (locus and/or selection agent; give details):6. Test compound concentrations used:

Non-activated conditions:

Cytotoxicity assay: 150, 300, 600, 800, 1,000, 1,250, 2,500, and 5,000  $\mu$ g/mL  $\mu$ g/mLGene mutation Assay 1: 800, 1,000, 1,250, 2,500, and 5,000  $\mu$ g/mLGene mutation Assay 2: 800, 1,000, 1,250, 2,500, and 5,000  $\mu$ g/mL

Activated conditions:

Cytotoxicity assay: 150, 300, 600, 800, 1,000, 1,250, 2,500, and 5,000  $\mu$ g/mL  $\mu$ g/mLGene mutation Assay 1: 800, 1,000, 1,250, 2,500, and 5,000  $\mu$ g/mLGene mutation Assay 2: 800, 1,000, 1,250, 2,500, and 5,000  $\mu$ g/mL

**B. TEST PERFORMANCE****1. Cell treatment:**

a. Cells exposed to test compound, negative/solvent or positive controls for: 4 hours (non-activated) 4 hours (activated)

b. After washing, cells cultured for 6-8 days (expression period) before cell selection:

c. After expression,  $2 \times 10^5$  cells/dish (10 dishes/ group) were cultured for 7-9 days in selection medium to determine numbers of mutants and 200 cells/dish (3 dishes/group) were cultured for 7-9 days without selective agent to determine cloning efficiency.

**2. Statistical Methods:** The frequency of mutants per  $10^6$  cells was evaluated using weighted analysis of variance. Treated groups were compared to the vehicle control using a linear trend test and lack of fit test ( $\alpha=0.05$ ). In the event of a significantly increasing trend or significant lack of fit, a Dunnett's t-test was conducted, and additional comparison of the positive control to the negative control was conducted using a linear contrast statement.

**3. Evaluation Criteria:** An assay was considered acceptable if the mutation frequency in the positive controls was significantly higher than the negative controls and if the negative controls were within reasonable limits of the laboratory historical control and literature values.

The test chemical was considered positive if it induced a statistically significant, dose related, reproducible increase in mutation frequency compared to the vehicle control.

**II. REPORTED RESULTS**

**A. Preliminary cytotoxicity assay:** The cytotoxicity test (Table 1, study report page 20) was conducted with seven concentrations of 2,4-D TIPA salt ranging from 150 to 5,000  $\mu\text{g/mL}$  with or without S9 activation. In the non-activated cultures, toxicity (~ 20% relative cell survival, RCS) was observed at the 5,000  $\mu\text{g/mL}$  dose levels. In the presence of S9, RCS was 56% at the highest dose level. Based on these results, dose levels of 800-5,000  $\mu\text{g/mL}$  were chosen for the test with and without S9 activation.

**B. Mutagenicity assay:** Analyses (HPLC) of the test material stock solutions from 800-5,000  $\mu\text{g/mL}$  indicated that the actual concentrations were 96-104% of the target concentrations.

The mutagenicity assay results are presented in Tables 2A, 2B, 3A, and 3B, (study report pages 21-24). The mutation frequencies in cultures treated with the test material in the absence and presence of S9 were not significantly different from the concurrent negative controls and were within the laboratory historical background range of 0-2.6 (minimum) and 8.0-27.9 (maximum) TG<sup>r</sup> mutants per 10<sup>6</sup> cells over a 9-year period. In all assays, the positive control chemicals EMS (non-activated assay) and 20-MCA caused significant increases in mutation frequencies. Based on these results, the study author concluded that 2,4-D TIPA salt was not mutagenic in this *in vitro* mammalian cell test system.

### III. REVIEWER'S DISCUSSION/CONCLUSIONS:

- A. The reviewer agrees with the study author, that the 2,4-D TIPA salt formulation did not induce mutation in this CHO/HGPRT mammalian forward gene mutation assay when tested to the limit dose of 5,000  $\mu\text{g}/\text{mL}$ . The sensitivity of the test system to detect a mutagenic response was clearly demonstrated by the significant results obtained with the positive control substances, 621  $\mu\text{g}/\text{mL}$  EMS in the non-activated system and 4  $\mu\text{g}/\text{mL}$  20-MCA in the S9 activated system. We conclude that the 2,4-D TIPA salt formulation is not mutagenic in this *in vitro* forward gene mutation system.
- B. Study deficiencies: None

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*Td Review 11942*

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Pages 26 through 30 are not included in this copy.

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The material not included contains the following type of information:

- Identity of product inert ingredients.
  - Identity of product impurities.
  - Description of the product manufacturing process.
  - Description of quality control procedures.
  - Identity of the source of product ingredients.
  - Sales or other commercial/financial information.
  - A draft product label.
  - The product confidential statement of formula.
  - Information about a pending registration action.
  - FIFRA registration data.
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  - The document is not responsive to the request.
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The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

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**DATA EVALUATION RECORD**

2,4-D; isopropylamine salt (2,4-D IPA)

Study Type: 84-2; *In vitro* Mammalian Chromosome Aberrations in Rat Lymphocytes

Work Assignment No. 1-16C (MRID 43327303)

Prepared for

Health Effects Division  
Office of Pesticide Programs  
U.S. Environmental Protection Agency  
1921 Jefferson Davis Highway  
Arlington, VA 22202

Prepared by

Pesticide Health Effects Group  
Sciences Division  
Dynamac Corporation  
2275 Research Boulevard  
Rockville, MD 20850-3268

Primary Reviewer:  
Steven Brecher, Ph.D.

Signature: Steven Brecher  
Date: 4/15/96

Secondary Reviewer:  
William Spangler, Ph.D.

Signature: William J. Spangler  
Date: 4/15/96

Project Manager:  
William Spangler, Ph.D.

Signature: William J. Spangler  
Date: 4/15/96

Quality Assurance:  
Reto Engler, Ph.D.

Signature: Reto Engler  
Date: 4/15/96

**Disclaimer**

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by Dynamac Corporation personnel.

2,4-D IPA

IN VITRO CHROM. ABERRATION (84-2)

EPA Reviewer: Jess Rowland, M.S., Toxicologist *Jess Rowland*  
Review Section I, Toxicology Branch II (7509C)

Date 7/22/96

EPA Secondary Reviewer: Yiannakis Ioannou, Ph.D., Head *YIoannou*  
Review Section I, Toxicology Branch II (7509C)

Date 5/23/96

DATA EVALUATION RECORD

STUDY TYPE: *In vitro* mammalian chromosome aberrations in rat lymphocytes

OPP Guideline Number: 84-2(2)

DP BARCODE: D207071

SUBMISSION: S472693

P.C. CODE: 030025

TOX. CHEM. NO.: 315U

TEST MATERIAL: 2,4-dichlorophenoxyacetic acid isopropylamine salt;

SYNONYMS: 2,4-D IPA

CITATION: Linscombe, V.A. and S.J. Lick (1994) Evaluation of 2,4-Dichlorophenoxyacetic Acid Isopropylamine Salt in an In Vitro Chromosomal Aberration Assay Utilizing Rat Lymphocytes. Health and Environmental Sciences Toxicology Research Laboratory, Dow Chemical Company, Midland, Michigan. Study ID M-004725-016, Study dates: 5/19/93-5/27/94. MRID No. 43327303. Unpublished.

SPONSOR: DowElanco, Indianapolis, IN 46268

EXECUTIVE SUMMARY: In a mammalian cell chromosome aberration assay (MRID 43327303), rat lymphocyte cultures were exposed to a 2,4-D IPA formulation (50.2% a.i.), in deionized distilled water for 4 hours at concentrations of 96, 192, 384, 767, 1,534, 3,068, and 6,137  $\mu\text{g}/\text{mL}$  with or without metabolic activation. Preparations for metabolic activation were made from induced rat livers. Cells were harvested at 24 or 48 hours post-treatment.

2,4-D IPA was tested to 6,137  $\mu\text{g}/\text{mL}$ , to determine cytotoxicity levels. For metaphase analysis, the highest concentration produced cytotoxicity (mitotic index 28-43% of negative control without S9 activation or 48-53% with S9 activation). Two lower concentrations were 50% and 25% of the high concentration. Positive controls induced the appropriate response. The statistically significant increase in the proportion of aberrant cells at the highest concentration over the negative control value was determined not to be biologically significant since the background aberration frequency for rat lymphocytes can range from 0-5.5% and statistical significance was seen only because the concurrent solvent control value was 0%. Therefore it was concluded that 2,4-D IPA at upto 3000  $\mu\text{g}/\text{mL}$  was not clastogenic in cultured rat lymphocytes harvested 48 hours after treatment.

This study is classified as **Acceptable** and does satisfy the requirements for the 1991 Guideline 84-2(2) for *in vitro* cytogenetic mutagenicity data.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality provided

## I. MATERIALS AND METHODS

## A. MATERIALS

1. Test Material: 2,4-D IPA

Description: amber liquid

Lot/Batch #: AGR 276461

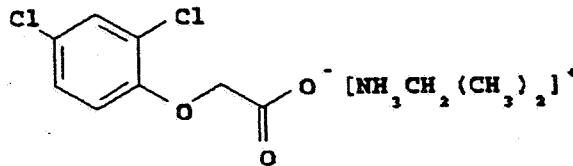
Purity: The test material is a formulation intermediate containing 50.2% 2,4-D IPA salt.

The acid (2,4-D) equivalent = 40.9%

Stability of compound: Not addressed

CAS #: 5742-17-6

Structure:



Solvent used: Deionized distilled water

Other comments: Dosing solutions analyzed for actual concentration

2. Control Materials:

Negative: Solvent control

Solvent/final concentration: Deionized distilled water/1%

Positive: Nonactivation: Mitomycin C (0.5 µg/mL)

Activation: Cyclophosphamide (6 µg/mL)

3. Activation: S9 derived from

<input checked="" type="checkbox"/> Aroclor 1254	<input checked="" type="checkbox"/> induced	<input checked="" type="checkbox"/> rat	<input checked="" type="checkbox"/> liver
<input type="checkbox"/> phenobarbital	<input type="checkbox"/> non-induced	<input type="checkbox"/> mouse	<input type="checkbox"/> lung
<input type="checkbox"/> none		<input type="checkbox"/> hamster	<input type="checkbox"/> other
<input type="checkbox"/> other			<input type="checkbox"/> other

S9 purchased from Sitek Research Laboratories,  
Rockville, MarylandS9 mix composition: S9 fraction (10% v/v),  
MgCl<sub>2</sub> (10mM), Na<sub>2</sub>HPO<sub>4</sub> buffer (50mM), pH 8.0, glucose-6-  
phosphate (5mM), NADP (4mM), CaCl<sub>2</sub> (10mM), KCl (30mM)

4. Test compound concentrations used: Preliminary cytotoxicity test not performed; cytotoxicity assessed concurrently with cytogenetic assay

Nonactivated conditions:

Assay 1\*: 4 hour treatment (96, 192, 384, 767, 1,534, 3,068, 6,137  $\mu\text{g/mL}$ ), harvest at 24 hours

Assay 2: 4 hour treatment (750, 1,500, 3,000  $\mu\text{g/mL}$ ), harvest at 24 and 48 hours

Activated conditions:

Assay 1\*: 4 hour treatment (96, 192, 384, 767, 1,534, 3,068, 6,137  $\mu\text{g/mL}$ ), harvest at 24 hours

Assay 2: 4 hour treatment (375, 750, 1,500  $\mu\text{g/mL}$ ), harvest at 24 and 48 hours

\*The laboratory indicated that the concentrations used were approximately 23% higher than targeted concentrations because the purity of the test material (40.9%), as originally provided by the sponsor, was actually the acid equivalent of 2,4-D. The purity of the salt was later reported to be 50.2%.

5. Test cells: Sprague-Dawley rat lymphocytes in culture. Whole blood grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, antibiotics, antimycotics, and phytohemagglutinin (20  $\mu\text{g/mL}$ ).

Properly maintained? **Yes**

Cell line or strain periodically checked for Mycoplasma contamination? **N/A**

Cell line or strain periodically checked for karyotype stability? **N/A**

**B. TEST PERFORMANCE**

1. Preliminary Cytotoxicity Assay: Performed concurrently with cytogenetic assay. Duplicate cultures per dose level. Percentage of metaphase cells per 1000 cells in each culture determined. The highest dose level was that causing depression of mitotic index 30-70% with adequate scorable cells when compared with the negative control or 5 mg/mL, whichever is lower. Intermediate and low concentrations approximated to 50% and 25% of highest dose level were selected for subsequent metaphase analysis at 24-hour harvest (only highest dose level and negative control selected initially for subsequent metaphase analysis at 48-hour harvest).

2. Cytogenetic Assay: Two assays were run; activated and nonactivated sets were used in each assay; approximately 48 hours after initiation of cell cultures, cells were exposed to the test chemical or positive control for a selected time interval, then the chemical was removed and the cells continued in culture until time of harvest

a. Cell treatment:

Cells exposed to test compound, solvent, or positive control for 4 hours (nonactivated), or 4 hours (activated)

Assay 1: 4 hr exposure, -S9, harvest  
at 24 hrs  
4 hr exposure, +S9, harvest  
at 24 hrs

Assay 2: 4 hr exposure, -S9, harvest  
at 24 hrs  
4 hr exposure, +S9, harvest  
at 24 hrs  
4 hr exposure, -S9, harvest  
at 48 hrs  
4 hr exposure, +S9, harvest  
at 48 hrs

b. Spindle inhibition

Inhibitor used/concentration: Colcemid (0.2  $\mu\text{g}/\text{mL}$ )  
Administration time: 3 hours (before cell harvest)

c. Cell harvest:

Cells exposed to test material, or solvent harvested 24 or 48 hours after termination of treatment (nonactivated), or 24 or 48 hours after termination of treatment (activated). Positive control cultures harvested 24 hours after termination of treatment. Cells swollen by hypotonic treatment (0.075 M KCl) and fixed with methanol:glacial acetic acid (3:1).

d. Details of slide preparation:

Cells suspended in fixative dropped onto microscopic slides and stained with Giemsa.

e. Metaphase analysis

No. of cells examined per dose: 200  
Solvent control: 200  
Positive control: 100

Scored for structural: **Yes**

Scored for numerical: **No**

Coded prior to analysis: **Yes**

- f. Evaluation criteria: Only metaphases with 42 centromeres were scored with the exception of cells with multiple aberrations. Gaps were not included in calculations of total cytogenetic aberrations. The test was considered acceptable if chromosomal aberration frequency in the positive control was significantly higher than the negative control. Aberration frequency in the negative control should be within reasonable limits of laboratory historical values. Positive response was claimed if a significant treatment-related and reproducible increase in frequency of cells with chromosome aberrations was observed.
- g. Statistical analysis: Data evaluated for statistical significance at  $\alpha=0.01$ , using Chi-square statistics from two way contingency table.

## II. REPORTED RESULTS

Analysis of dose formulations by HPLC indicated that concentrations were between 115 and 129% of target concentrations for Assay 1 and between 100 and 107% for Assay 2.

### A. Preliminary cytotoxicity assay:

Cytotoxicity, as indicated by effects on the mitotic index, was assessed concurrently with the cytogenetic assay. In Assay 1, both with and without S9 activation, the 6,137  $\mu\text{g/mL}$  concentration reduced the mitotic index to 0%. At 3,068  $\mu\text{g/mL}$  without S9 activation the mitotic index was 43% of the negative control value. With S9 activation, only one of two cultures survived treatment at 3068  $\mu\text{g/mL}$ . At 1,534  $\mu\text{g/mL}$ , the mitotic index was 53% of the negative control value.

In Assay 2, without S9 activation, 3,000  $\mu\text{g/mL}$  reduced the mitotic index to 28% of the negative control value 24 hours after treatment. By 48 hours the mitotic index was still 33% of the negative control. With S9 activation, 1,500  $\mu\text{g/mL}$  reduced the mitotic index to 48% of the negative control value 24 hours after treatment. By 48 hours the mitotic index returned to 90% of the negative control.

### B. Cytogenetic assay: Results are presented in Attachments 1-3 (study report pages 23, 24, 27-30).

In Assay 1, cultures treated with 767, 1,534, and 3,068  $\mu\text{g/mL}$  of the test compound without S9 activation, or 384, 767, and 1,534  $\mu\text{g/mL}$  of the test compound with S9 activation, were analyzed for chromosomal aberration frequencies at the 24-hour harvest (Attachment 1).

2,4-D IPA caused no statistically significant increases in the proportion of aberrant cells, in either the presence or absence of S9 activation. The aberration frequencies of the treated cultures in the presence of S9 activation were outside the historical range of the laboratory; however, in the confirmatory study (Assay 2) they were within the historical range. The frequency of abnormal cells in the negative controls was within the historical range of the laboratory. Significant increases in the frequency of cells with aberrations were observed in cultures treated with the positive control compounds (mitomycin C, without S9, cyclophosphamide, with S9).

In confirmatory assay 2, cultures treated with 750, 1,500, and 3,000  $\mu\text{g}/\text{mL}$  of the test compound without S9 activation, or 375, 750, and 1,500  $\mu\text{g}/\text{mL}$  of the test compound with S9 activation were analyzed for chromosomal aberration frequencies at the 24-hour harvest (Attachment 2). The 2,4-D IPA formulation caused no statistically significant increases in the proportion of aberrant cells, either in the presence or absence of S9 activation. The frequency of abnormal cells in the negative controls was within the historical range of the laboratory. Significant increases in the frequency of cells with aberrations were observed at the 24-hour harvest in cultures treated with positive control compounds. At the 48-hour harvest only cultures from the negative control and highest treatment level were initially analyzed for chromosomal aberration frequencies. The number of aberrant cells in cultures treated at 3,000  $\mu\text{g}/\text{mL}$  without S9 activation was significantly higher than the negative control value (6% vs 0%). Subsequent analysis of the two lower concentrations did not show significant increases over control values (Attachment 3).

### III. REVIEWER'S DISCUSSION/CONCLUSIONS:

- A. The study authors suggest that the statistically identified increase in aberrant cells at the 48-hour harvest in cultures treated with 3,000  $\mu\text{g}/\text{mL}$  without S9 activation is due to the occurrence of 0% aberrant cells in the concurrent negative controls. They further state that the incidence of aberrant cells among negative control cultures of this study ranged from 0-5.5% and the laboratory historical negative control values ranged from 0-6.5%. The reviewers concur with the study authors and conclude that 2,4-D IPA at upto 3000  $\mu\text{g}/\text{mL}$  is not clastogenic in cultured rat lymphocytes harvested 48 hours after treatment.

- B. Study deficiencies None.

**ATTACHMENTS**



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*Td Review 11942*

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Pages 39 through 45 are not included in this copy.

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- Identity of product inert ingredients.
  - Identity of product impurities.
  - Description of the product manufacturing process.
  - Description of quality control procedures.
  - Identity of the source of product ingredients.
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  - The product confidential statement of formula.
  - Information about a pending registration action.
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011942

# DATA EVALUATION RECORD

2,4-D; isopropylamine salt (2,4-D IPA)

Study Type: 84-2; Mammalian Cells in Culture Gene Mutation Assay in Chinese Hamster Ovary Cells (CHO/HGPRT)

Work Assignment No. 1-16D (MRID 43327304)

Prepared for

Health Effects Division  
Office of Pesticide Programs  
U.S. Environmental Protection Agency  
1921 Jefferson Davis Highway  
Arlington, VA 22202

Prepared by

Pesticide Health Effects Group  
Sciences Division  
Dynamac Corporation  
2275 Research Boulevard  
Rockville, MD 20850-3268

Primary Reviewer:  
Ann Foster, Ph.D.

Signature: Steven Beech  
Date: 4/15/96

Secondary Reviewer:  
William Spangler, Ph.D.

Signature: William J. Spangler  
Date: 4/15/96

Project Manager:  
William Spangler, Ph.D.

Signature: William J. Spangler  
Date: 4/15/96

Quality Assurance:  
Reto Engler, Ph.D.

Signature: Reto Engler  
Date: 4/15/96

### Disclaimer

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by Dynamac Corporation personnel.

EPA Reviewer: Jess Rowland, M.S., Toxicologist. *Jess Rowland*  
Review Section I, Toxicology Branch II (7509C)

Date 7/23/96

EPA Secondary Reviewer: Yiannakis Ioannou, Ph.D., Head *Y.I.*  
Review Section I, Toxicology Branch II (7509C)

Date 5/23/96

DATA EVALUATION RECORD
------------------------

**STUDY TYPE:** Mammalian cells in culture gene mutation assay in Chinese hamster ovary cells.

**Guideline Number:** 584-2(2)

**DP BARCODE:** D206005

**SUBMISSION:** S472693

**P.C. CODE:** 030025

**TOX. CHEM. NO.:** 315U

**TEST MATERIAL:** 2,4-Dichlorophenoxyacetic acid isopropylamine salt

**SYNONYMS:** 2,4-D IPA

**CITATION:** Linscombe, V.A. and S.J. Lick (1994) Evaluation of 2,4- Dichlorophenoxyacetic acid isopropylamine salt in the Chinese Hamster Ovary Cell/Hypoxanthine-guanine-phosphoribosyl Transferase (CHO/HGPRT) Forward mutation Assay. Health and Environmental Sciences, The Toxicology Research Laboratory, Midland, MI. Laboratory Project Study ID M-004725-017. May 27, 1994. MRID 43327304.

**SPONSOR:** DowElanco, Indianapolis, IN 46268

**EXECUTIVE SUMMARY:** In an *in vitro* mammalian cell gene forward mutation assay, CHO/HGPRT, (MRID 43327304) Chinese hamster ovary (CHO) cells cultured *in vitro* were exposed to a 2,4-D IPA salt formulation (50.2%) at concentrations of 500, 1,000, 1,500, 2,000, and 3,000  $\mu\text{g}/\text{mL}$  with and without S9 activation. The S9 fraction was derived from Aroclor-induced rat liver. The test material was delivered to the test system in water.

The test material was cytotoxic at a dose of 2,500  $\mu\text{g}/\text{mL}$  with and without S9 activation. There was no evidence of a mutagenic effect at any dose level with or without activation. Findings with the positive controls confirmed the sensitivity of the test system to detect mutagenesis.

This study is classified as acceptable and satisfies the requirements for FIFRA Test Guideline 84-2 for *in vitro* mutagenicity (mammalian forward gene mutation) data.

**COMPLIANCE:** Signed and dated GLP, Quality Assurance, Data Confidentiality, and Flagging statements were provided.

## I. MATERIALS AND METHODS

## A. MATERIALS

1. Test Material: 2,4-Dichlorophenoxyacetic acid isopropylamine (IPA) salt

Description: amber liquid

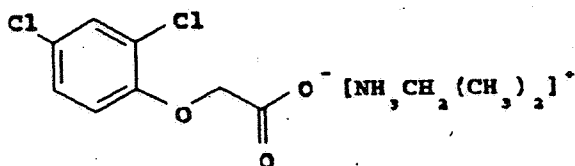
Lot/Batch #: AGR 276461

Purity: 50.2% a.i.

Stability of compound: Not reported

CAS #: 5742-17-6

Structure:



Solvent used: Water

Other comments: None

2. Control Materials:

Solvent/final concentration: culture medium

Positive: Non-activation (concentrations, solvent):

Ethylmethanesulfonate (EMS)/621  $\mu\text{g/mL}$  in culture medium

Activation (concentrations, solvent):

20-Methylcholanthrene (20-MCA)/4  $\mu\text{g/mL}$  in 1% DMSO/culture medium3. Activation: S9 derived from

<input checked="" type="checkbox"/> Aroclor 1254	<input checked="" type="checkbox"/> induced	<input checked="" type="checkbox"/> rat	<input checked="" type="checkbox"/> liver
<input type="checkbox"/> phenobarbital	<input type="checkbox"/> non-induced	<input type="checkbox"/> mouse	<input type="checkbox"/> lung
<input type="checkbox"/> none			<input type="checkbox"/> hamster
<input type="checkbox"/> other			<input type="checkbox"/> other

Describe S9 mix composition (if purchased, give details): 10 mM  $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$ , 5 mM glucose-6-phosphate, 4 mM NADP, 10 mM  $\text{CaCl}_2$ , 30 mM KCl, and 50 mM sodium phosphate (pH 8.0)

4. Test Cells: Chinese hamster ovary (CHO) cellsProperly maintained? **Yes**Periodically checked for Mycoplasma contamination? **Yes**Periodically checked for karyotype stability? **Not reported**Periodically "cleansed" against high spontaneous background? **Not reported**

Media: Ham's F-12 nutrient mix supplemented with 5% heat-activated fetal calf serum; 25 mM HEPES; Fungizone; penicillin G; and streptomycin sulfate.

5. Locus Examined: thymidine kinase (TK)

Selection agent:

 bromodeoxyuridine (BrdU) fluorodeoxyuridine (FdU) trifluorothymidine (TFT) hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)

Selection agent:

 8-azaguanine (8-AG) 10  $\mu$ M 6-thioguanine (6-TG) Na<sup>+</sup>/K<sup>+</sup> ATPase

Selection agent:

 ouabain

(give concentration)

 other (locus and/or selection agent; give details):6. Test compound concentrations used:

Non-activated conditions:

Cytotoxicity assay: 150, 300, 600, 800, 1,000, 1,250, 2,500, and 5,000  $\mu$ g/mL  $\mu$ g/mLGene mutation Assay: 500, 1,000, 1,500, 2,000, and 3,000  $\mu$ g/mL

Activated conditions:

Cytotoxicity assay: 150, 300, 600, 800, 1,000, 1,250, 2,500, and 5,000  $\mu$ g/mLGene mutation Assay: 500, 1,000, 1,500, 2,000, and 3,000  $\mu$ g/mL

**B. TEST PERFORMANCE****1. Cell treatment:**

- a. Cells exposed to test compound, negative/solvent or positive controls for:  
4 hours (non-activated) 4 hours (activated)
- b. After washing, cells cultured for 6-8 days (expression period) before cell selection:
- c. After expression,  $2 \times 10^5$  cells/dish (10 dishes/ group) were cultured for 7-9 days in selection medium to determine numbers of mutants and 200 cells/dish (3 dishes/group) were cultured for 7-9 days without selective agent to determine cloning efficiency.

**2. Statistical Methods:** The frequency of mutants per  $10^6$  cells was evaluated using weighted analysis of variance. Treated groups were compared to the vehicle control using a linear trend test and lack of fit test ( $\alpha=0.05$ ). In the event of a significantly increasing trend or significant lack of fit, a Dunnett's t-test was conducted, and additional comparison of the positive control to the negative control was conducted using a linear contrast statement.

**3. Evaluation Criteria:** An assay was considered acceptable if the mutation frequency in the positive controls was significantly higher than the negative controls and if the negative controls were within reasonable limits of the laboratory historical control and literature values.

The test chemical was considered positive if it induced a statistically significant, dose related, reproducible increase in mutation frequency compared to the vehicle control.

**II. REPORTED RESULTS**

**A. Preliminary cytotoxicity assay:** The cytotoxicity test was conducted with seven concentrations of a 2,4-D IPA salt formulation ranging from 150 to 5,000  $\mu\text{g/mL}$  with or without S9 activation (Table 1, study report page 20). The report stated that the actual concentrations were 23% higher than the targets owing to initial information from the sponsor that the purity of the test article was 40.9% a.i. instead of 50.2%. In the non-activated cultures, toxicity (1.6% relative cell survival, RCS) was observed at the 2,500  $\mu\text{g/mL}$  dose level. In the presence of S9, RCS was 0.8% at the 5,000  $\mu\text{g/mL}$  dose level and 5.4% at 2,500  $\mu\text{g/mL}$ . Based on these results, dose levels of 500-3,000  $\mu\text{g/mL}$  were chosen for the mutagenicity test with and without S9 activation.

- B. Mutagenicity assay: Analyses (HPLC) of the test material stock solutions from 500-3,000  $\mu\text{g}/\text{mL}$  indicated that the actual concentrations were 84-107% of the target concentrations.

The mutagenicity assay results are presented in Tables 2A, 2B, 3A, and 3B (study report pages 21-24). The mutation frequencies in cultures treated with the test material in the absence and presence of S9 were not significantly different from the concurrent negative controls and were within the laboratory historical background range of 0-2.6 (minimum) and 8.0-27.9 (maximum) TG<sup>+</sup> mutants per  $10^6$  cells over a 9-year period. In all assays, the positive control chemicals EMS (non-activated assay) and 20-MCA caused significant increases in mutation frequencies. Based on these results, the study author concluded that 2,4-D IPA salt was not mutagenic in this *in vitro* mammalian cell test system.

### III. REVIEWER'S DISCUSSION/CONCLUSIONS:

- A. The reviewer agrees with the study author, that the 2,4-D IPA salt formulation did not induce mutation in this CHO/HGPRT mammalian forward gene mutation assay when tested to the limit dose of 5,000  $\mu\text{g}/\text{mL}$ . The sensitivity of the test system to detect a mutagenic response was clearly demonstrated by the significant results obtained with the positive control substances, 621  $\mu\text{g}/\text{mL}$  EMS in the non-activated system and 4  $\mu\text{g}/\text{mL}$  20-MCA in the S9 activated system. We conclude that 2,4-D IPA salt is not mutagenic in this *in vitro* forward gene mutation system.
- B. Study deficiencies: None

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*Tf Review 11942*

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Pages 52 through 56 are not included in this copy.

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# DATA EVALUATION RECORD

2,4-D; butoxyethyl ester (2,4-D BEE)

Study Type: 84-2; *In vitro* Mammalian Chromosome Aberrations in Rat Lymphocytes

Work Assignment No. 1-16E (MRID 43327305)

Prepared for

Health Effects Division  
Office of Pesticide Programs  
U.S. Environmental Protection Agency  
1921 Jefferson Davis Highway  
Arlington, VA 22202

Prepared by

Pesticide Health Effects Group  
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Signature: Steven Brecher  
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Date: 4/15/96

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Signature: William Spangler  
Date: 4/15/96

Quality Assurance:  
Reto Engler, Ph.D.

Signature: Reto Engler  
Date: 4/15/96

### Disclaimer

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by Dynamac Corporation personnel.

EPA Reviewer: Jess Rowland, M.S., Toxicologist  
Review Section I, Toxicology Branch II (7509C)

*Jess Rowland*

Date 5/23/96

EPA Secondary Reviewer: Yiannakis Ioannou, Ph.D., Head  
Review Section I, Toxicology Branch II (7509C)

*Yiannakis Ioannou*

Date 5/23/96

DATA EVALUATION RECORD

**STUDY TYPE:** *In vitro* mammalian chromosome aberrations in rat lymphocytes

**Guideline Number:** §84-2(2)

**DP BARCODE:** D207071

**SUBMISSION:** S472693

**P.C. CODE:** 030053

**TOX. CHEM. NO.:** 315AE

**TEST MATERIAL:** 2,4-dichlorophenoxyacetic acid butoxyethyl ester

**SYNONYMS:** 2,4-D BEE

**CITATION:** Linscombe, V.A. and S.J. Lick (1994) Evaluation of 2,4-Dichlorophenoxyacetic Acid Butoxyethyl Ester in an In Vitro Chromosomal Aberration Assay Utilizing Rat Lymphocytes. Health and Environmental Sciences, Toxicology Research Laboratory, Dow Chemical Company, Midland, Michigan. Study ID K-007722-022, Study dates: 5/19/93-5/27/94. MRID No. 43327305. Unpublished.

**SPONSOR:** DowElanco, Indianapolis, IN 46268

**EXECUTIVE SUMMARY:** In a mammalian cell chromosome aberration assay (MRID 43327305), rat lymphocyte cultures were exposed to 2,4-D BEE (94.6% a.i.), in dimethyl sulfoxide for 4 hours at concentrations of 87.5, 175, 350, 700, and 1,400 µg/mL with or without metabolic activation. Preparations for metabolic activation were made from induced rat livers. Cells were harvested at 24 or 48 hours post-treatment.

2,4-D BEE was tested to the limit of solubility, 1.4 mg/mL, to determine cytotoxicity levels. For metaphase analysis, the highest concentration produced cytotoxicity (mitotic index 31-80% of negative control without S9 activation or 61-67% with S9 activation). Two lower concentrations were 50% and 25% of the high concentration. Positive controls induced the appropriate response.

There were no statistically significant increases in the proportion of aberrant cells over negative control values. However, cytotoxicity of 2,4-D BEE in the presence of S9 activation was marginal at the highest dose levels selected for metaphase analysis. In addition, the highest dose level selected for metaphase analysis in the initial assay produced marginal cytotoxicity, while this same dose level was too cytotoxic for metaphase analysis in the confirmatory assay.

2,4-D BEE

IN VITRO CHROM. ABERRATION (84-2)

Therefore, a decision regarding the clastogenicity of 2,4-D BEE in the presence of metabolic activation, based on the results of this study, cannot be made at this time. It is critical that this chemical be evaluated up to a reproducible cytotoxic level. It is concluded that a new study should be conducted.

This study is classified as **unacceptable** and does not satisfy the requirements for the 1991 Guideline, 84-2 for *in vitro* cytogenetic mutagenicity data.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality provided.

## I. MATERIALS AND METHODS

## A. MATERIALS

1. Test Material: 2,4-D BEE

Description: amber liquid

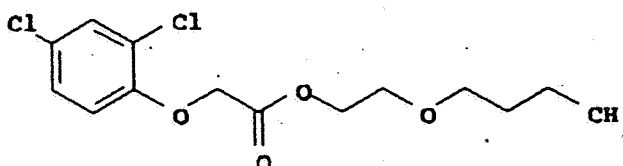
Lot/Batch #: AGR 276426

Purity: 94.6%

Stability of compound: Not addressed

CAS #: 1929-73-3

Structure:



Solvent used: Dimethyl sulfoxide (DMSO)

Other comments: Dosing solutions analyzed for actual concentration

2. Control Materials:

Negative: Solvent control

Solvent/final concentration: Dimethyl sulfoxide/1%

Positive: Nonactivation: Mitomycin C (MMC, 0.5  $\mu\text{g}/\text{mL}$ )Activation: Cyclophosphamide (CP, 6  $\mu\text{g}/\text{mL}$ )

Other comments: DMSO not used as solvent for positive controls. MMC and CP dissolved directly in treatment medium

3. Activation: S9 derived from

Aroclor 1254     induced  
 phenobarbital     non-induced  
 none  
 other

rat     liver  
 mouse     lung  
 hamster     other  
 other

S9 purchased from Sitek Research Laboratories, Rockville, Maryland

S9 mix composition: S9 fraction (10% v/v),  $\text{MgCl}_2$  (10mM),  $\text{Na}_2\text{HPO}_4$  (50mM), pH 8.0, glucose-6-phosphate (5mM), NADP (4mM),  $\text{CaCl}_2$  (10mM), KCl (30mM)

2,4-D BEE

IN VITRO CHROM. ABERRATION (84-2)

4. Test compound concentrations used: Preliminary cytotoxicity test not performed; cytotoxicity assessed concurrently with cytogenetic assay

Nonactivated conditions:

Assay 1: 4 hour treatment (87.5, 175, 350, 700, 1,400  $\mu\text{g/mL}$ ), harvest at 24 hours

Assay 2: 4 hour treatment (350, 700, 1,400  $\mu\text{g/mL}$ ), harvest at 24 and 48 hours

Activated conditions:

Assay 1: 4 hour treatment (87.5, 175, 350, 700, 1,400  $\mu\text{g/mL}$ ), harvest at 24 hours

Assay 2: 4 hour treatment (175, 350, 700  $\mu\text{g/mL}$ ), harvest at 24 and 48 hours

5. Test cells: Sprague-Dawley rat lymphocytes in culture. Whole blood grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, antibiotics, antimycotics, and phytohemagglutinin (20  $\mu\text{g/mL}$ ).

Properly maintained? **Yes**

Cell line or strain periodically checked for Mycoplasma contamination? **N/A**

Cell line or strain periodically checked for karyotype stability? **N/A**

**B. TEST PERFORMANCE**

1. Preliminary Cytotoxicity Assay: Performed concurrently with cytogenetic assay. Duplicate cultures per dose level. Percentage of metaphase cells per 1000 cells in each culture determined. The highest dose level for metaphase analysis was that causing depression of mitotic index 30-70% with adequate scorable cells when compared with the negative control or 5 mg/mL, whichever was lower. Intermediate and low concentrations approximated to 50% and 25% of highest dose level were selected for subsequent metaphase analysis at 24-hour harvest (only highest dose level and negative control selected for subsequent metaphase analysis at 48-hour harvest).
2. Cytogenetic Assay: Two assays were run; activated and nonactivated sets were used in each assay; approximately 48 hours after initiation of cell cultures, cells were exposed to the test chemical or positive control for a selected time interval, then the chemical was removed and the cells were continued in culture until time of harvest

- a. Cell treatment:  
Cells exposed to test compound, solvent, or positive control for 4 hours (nonactivated), or 4 hours (activated)
- Assay 1: 4 hr exposure, -S9, harvest at 24 hrs  
4 hr exposure, +S9, harvest at 24 hrs
- Assay 2: 4 hr exposure, -S9, harvest at 24 hrs  
4 hr exposure, +S9, harvest at 24 hrs  
4 hr exposure, -S9, harvest at 48 hrs  
4 hr exposure, +S9, harvest at 48 hrs
- b. Spindle inhibition  
Inhibitor used/concentration: Colcemid (0.2  $\mu\text{g}/\text{mL}$ )  
Administration time: 3 hours (before cell harvest)
- c. Cell harvest:  
Cells exposed to test material, or solvent harvested 24 or 48 hours after termination of treatment (nonactivated), or 24 or 48 hours after termination of treatment (activated). Positive control cultures harvested 24 hours after termination of treatment. Cells swollen by hypotonic treatment (0.075 M KCl) and fixed with methanol:glacial acetic acid (3:1).
- d. Details of slide preparation:  
Cells suspended in fixative dropped onto microscopic slides and stained with Giemsa.
- e. Metaphase analysis  
No. of cells examined per dose: 200  
Solvent control: 200  
Positive control: 100
- Scored for structural: **Yes**
- Scored for numerical: **No**
- Coded prior to analysis: **Yes**

- f. Evaluation criteria: Only metaphases with 42 centromeres were scored with the exception of cells with multiple aberrations. Gaps were not included in calculations of total cytogenetic aberrations. The test was considered acceptable if chromosomal aberration frequency in the positive control was significantly higher than the negative control. Aberration frequency in the negative control should be within reasonable limits of laboratory historical values. Positive response was claimed if a significant treatment-related and reproducible increase in frequency of cells with chromosome aberrations was observed.
- g. Statistical analysis: Data evaluated for statistical significance at  $\alpha=0.01$ , using Chi-square statistics from two way contingency table.

## II. REPORTED RESULTS

Analysis of dose formulations by HPLC indicated that concentrations were between 87 and 115% of target concentrations for Assay 1 and between 104 and 107% for Assay 2.

### A. Preliminary cytotoxicity assay:

Cytotoxicity, as indicated by effects on the mitotic index, was assessed concurrently with the cytogenetic assay. In Assay 1, without S9 activation, the 1,400  $\mu\text{g}/\text{mL}$  concentration reduced the mitotic index to 80% of the negative control value. With S9 activation, 1,400  $\mu\text{g}/\text{mL}$  reduced the mitotic index to 18% of the negative control value. The next highest dose (700  $\mu\text{g}/\text{mL}$ ) with S9 activation gave a relative mitotic index of 61%.

In Assay 2, without S9 activation, 1,400  $\mu\text{g}/\text{mL}$  reduced the mitotic index to 31% of the negative control value 24 hours after treatment and to 52%, 48 hours after treatment. With S9 activation, the highest dose (700  $\mu\text{g}/\text{mL}$ ) was very toxic resulting in the presence of sparse numbers of cells. The mitotic index was reduced to 27% (based on 500 cells per replicate) of the negative control value 24 hours after treatment. High toxicity was also observed 48 hours after treatment. The next highest dose (350  $\mu\text{g}/\text{mL}$ ) gave a relative mitotic index of 67%, 24 hours after treatment and 64%, 48 hours after treatment.

- B. Cytogenetic assay: Results are presented in Attachments 1 and 2 (study report pages 23, 24, 27-30).

In Assay 1, cultures treated with 350, 700, or 1,400  $\mu\text{g}/\text{mL}$  of the test compound in the absence of S9, and 175, 350, or 700  $\mu\text{g}/\text{mL}$  in the presence of S9 were analyzed for chromosomal aberration frequencies at the 24-hour harvest (Attachment 1). 2,4-D BEE caused no statistically significant increases in the proportion of aberrant cells, in either the presence or absence of S9 activation. The frequency of abnormal cells in the negative controls was within the historical range of the laboratory. Significant increases in the frequency of cells with aberrations were observed in cultures treated with the positive control compounds (mitomycin C, without S9, cyclophosphamide, with S9).

In confirmatory Assay 2, cultures treated with 350, 700, or 1,400  $\mu\text{g}/\text{mL}$  of the test compound in the absence of S9, and 175 or 350  $\mu\text{g}/\text{mL}$  in the presence of S9 were analyzed for chromosomal aberration frequencies at the 24-hour harvest. Cultures treated with 1,400  $\mu\text{g}/\text{mL}$  of the test compound in the absence of S9 or 350  $\mu\text{g}/\text{mL}$  in the presence of S9 were analyzed for chromosomal aberration frequencies at the 48-hour harvest (Attachment 2).

2,4-D BEE caused no statistically significant increases in the proportion of aberrant cells, either in the presence or absence of S9 activation at either harvest time. The frequency of abnormal cells in the negative controls was within the historical range of the laboratory. Significant increases in the frequency of cells with aberrations were observed at the 24-hour harvest in cultures treated with positive control compounds.

### III. REVIEWER'S DISCUSSION/CONCLUSIONS:

- A. The reviewer agrees with the study authors that 2,4-D BEE up to 1,400  $\mu\text{g}/\text{mL}$  in the absence of S9 was not clastogenic in cultured rat lymphocytes harvested 24 or 48 hours after treatment. Although cytotoxicity at 1,400  $\mu\text{g}/\text{mL}$  was minimal (relative mitotic index, 80%) in Assay 1, cytotoxicity was adequate in confirmatory Assay 2 (relative mitotic index, 31%). The negative controls had comparable low frequencies of chromosome aberrations. In addition, the sensitivity of the assay system to detect damage to chromosomes was adequately demonstrated by the results obtained with the positive controls (mitomycin C, -S9, and cyclophosphamide, +S9).



In Assay 1, in the presence of S9, as shown in Attachment 3 (study report page 22), cytotoxicity at 1,400  $\mu\text{g}/\text{mL}$  was high (relative mitotic index, 18%), and consequently, this dose level was not selected for metaphase analysis. However, because the dose level selected (700  $\mu\text{g}/\text{mL}$ ) had marginal cytotoxicity (relative mitotic index, 61%), the observation of a low frequency of chromosomal aberrations at the cytotoxic 1,400  $\mu\text{g}/\text{mL}$  dose level would have provided more convincing evidence that 2,4-D BEE is not clastogenic in this test system.

In Assay 2, in the presence of S9, as shown in Attachment 4 (study report page 26), cytotoxicity at 700  $\mu\text{g}/\text{mL}$  was high resulting in too few cells for metaphase analysis. The high dose level selected for metaphase analysis (350  $\mu\text{g}/\text{mL}$ ) had marginal cytotoxicity (relative mitotic index, 67%). A low frequency of chromosomal aberrations in the presence of marginal cytotoxicity at 700  $\mu\text{g}/\text{mL}$  (Assay 1) or 350  $\mu\text{g}/\text{mL}$  (Assay 2) is not convincing evidence that 2,4-D BEE is not clastogenic in this test system. In addition, high cytotoxicity at 700  $\mu\text{g}/\text{mL}$  in Assay 2 and only marginal cytotoxicity at the same dose level in Assay 1, suggests some variation in the test system or procedure between the two assays. Therefore, a decision regarding the clastogenicity of 2,4-D BEE in the presence of S9 activation, based on the results of this study, cannot be made.

The reviewer concludes that the results of this study do not provide sufficient evidence to consider 2,4-D BEE negative in this *in vitro* test system. It is also concluded that a new study must be conducted and the chemical be evaluated up to a reproducible cytotoxic level.

#### B. Study deficiencies

The following deficiencies would be expected to alter the conclusions of the study:

1. Cytotoxicity of 2,4-D BEE in the presence of S9 activation was marginal at the highest dose levels selected for metaphase analysis.
2. The highest dose level selected for metaphase analysis in the initial assay produced marginal cytotoxicity; this same dose level was too cytotoxic for metaphase analysis in the confirmatory assay. This suggests some variation in the test system or procedure between the two assays.

Chemical analysis of the 2,4-D BEE was not included in the study report, but this deficiency would not be expected to alter the conclusions of the study.

**ATTACHMENTS**

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