



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

008372

MAY 20 1991

MEMORANDUM

OFFICE OF  
PESTICIDES AND TOXIC  
SUBSTANCES

SUBJECT:

2,4-Dichlorophenoxyacetic acid: Priority Review of  
Mutagenicity Data Identified in Registration  
Standard.

FROM:

Jess Rowland, M.S., Toxicologist *Jess Rowland 4/24/91*  
Section II, Toxicology Branch II (HFAS)  
Health Effects Division (H7509C)

TO:

J. Coombs  
Product Manager (23)  
Reregistration Division

THRU:

K. Clark Swartzel, Section Head *K. Clark Swartzel 5/2/91*  
Section II, Toxicology Branch II (HFAS)  
Health Effects Division (H7509C)  
and  
Marcia van Gemert, Ph.D., Chief  
Toxicology Branch II (HFAS)  
Health Effects Division (H7509C)

STUDY IDENTIFICATION:

MF Project No. 6-1395 EPA ID # 030653; Record # 264,566;

MRID # 414981-01--- Caswell No. 315 AI

MRID # 414981-02--- Caswell No. 315 AE

MRID # 414981-03--- Caswell No. 315 U

ACTION REQUESTED: Review of data.

1. Evaluation of 2,4-Dichlorophenoxyacetic Acid Butoxyethyl Ester (2,4-D BBE) in the Rat Hepatocyte Unscheduled DNA Synthesis (UDS) Assay. MRID No. 414981-01.
2. Evaluation of a Formulation containing 2,4-Dichlorophenoxyacetic Acid Triisopropylamine Salt (2,4-D TIPA) in the Rat Hepatocyte Unscheduled DNA Synthesis (UDS) Assay. MRID No. 414981-02.
3. Evaluation of a Formulation containing 2,4-Dichlorophenoxyacetic Acid Isopropylamine Salt (2,4-D IPA) in the Rat Hepatocyte Unscheduled DNA Synthesis (UDS) Assay. MRID No. 414981-03.

REFERENCE: A separate Data Evaluation Report (DER) for the above reference studies is attached. A summary of each study is as follows:

1361

1. Evaluation of 2,4-Dichlorophenoxyacetic Acid Butoxyethyl Ester (2,4-D BEE) in the Rat Hepatocyte Unscheduled DNA Synthesis (UDS) Assay. MRID No. 414981-01.

Five doses of 2,4-D BEE ranging from 5.0 to 500 µg/mL were reported as not inducing an appreciable increase in the net nuclear grain counts of treated rat hepatocytes in two independent unscheduled DNA (UDS) assays. Although the dose range tested and the protocol designed for the study appeared to be adequate, technical problems with the assay preclude an accurate assessment of the results. The excessively high background cytoplasmic and nuclear grain counts in all groups make it difficult to distinguish between a positive and negative genotoxic response. The mean net nuclear count was not determined from the triplicate coverslips (50 nuclei/coverslip) from each treatment condition.

Study Classification: Unacceptable; does not meet Guideline requirement (84-4) for other genotoxic effects, Category III, other mutagenic mechanisms.

2. Evaluation of a Formulation containing 2,4-Dichlorophenoxy acetic Acid Triisopropanolamine Salt (2,4-D TIPA) in the Rat Hepatocyte Unscheduled DNA Synthesis (UDS) Assay. MRID NO. 414981-02.

Five doses of 2,4-D TIPA ranging from 5.0 to 500 µg/mL were reported as not inducing an appreciable increase in the net nuclear grain counts of treated rat hepatocytes in two independent unscheduled DNA (UDS) assays. Although the dose range tested and the protocol designed for the study appeared to be adequate, technical problems with the assay preclude an accurate assessment of the results. The excessively high background cytoplasmic and nuclear grain counts in all groups make it difficult to distinguish between a positive and negative genotoxic response. The mean net nuclear count was not determined from the triplicate coverslips (50 nuclei/coverslip) from each treatment condition.

Study Classification: Unacceptable; does not meet Guideline requirement (84-4) for other genotoxic effects, Category III, other mutagenic mechanisms.

3. Evaluation of a Formulation containing 2,4-Dichlorophenoxy acetic Acid Isopropylamine Salt (2,4-D IPA) in the Rat Hepatocyte Unscheduled DNA Synthesis (UDS) Assay. MRID No. 414981-03

Five doses of 2,4-D IPA ranging from 5.0 to 500 µg/mL were reported as not inducing an appreciable increase in the net nuclear grain counts of treated rat hepatocytes in two independent unscheduled DNA (UDS) assays. Although the dose range tested and the protocol designed for the study appeared to be adequate, technical problems with the assay preclude an accurate assessment of the results. The excessively high background cytoplasmic and nuclear grain counts in all groups make it difficult to distinguish between a positive and negative genotoxic response. The mean net nuclear count was not determined from the triplicate coverslips (50 nuclei/coverslip) from each treatment condition.

Study Classification: Unacceptable; does not meet Guideline requirement (64-4) for other genotoxic effects, Category III, other mutagenic mechanisms.

CONFIDENTIAL BUSINESS INFORMATION  
NATIONAL SECURITY INFORMATION (EO 12065)

EPA No.: 68080056  
DYNAMAC No.: 310-D  
TASK No.: 3-10D  
April 17, 1991

008372

DATA EVALUATION RECORD

2,4-Dichlorophenoxyacetic Acid Butoxyethyl Ester (2,4-D BEE)

Mutagenicity--Unscheduled DNA Synthesis  
Assay in Primary Rat Hepatocytes

APPROVED BY:

Robert J. Weir, Ph.D.  
Program Manager  
Dynamac Corporation

Signature: *Robert J. Weir*  
Date: *April 17, 1991*



Guideline Series 84: MUTAGENICITY

EPA No.: 68DS0056  
DYNAMAC No.: 310-D  
TASK No.: 3-10D  
April 17, 1991

008372

DATA EVALUATION RECORD

2,4-Dichlorophenoxyacetic Acid Butoxyethyl Ester (2,4-D BEE)

Mutagenicity--Unscheduled DNA Synthesis  
Assay in Primary Rat Hepatocytes

REVIEWER IX:

I. Cecil Miller, Ph.D.  
Principal Reviewer  
Dynamac Corporation

Signature: I. Cecil Miller

Date: 4-16-91

Nancy R. McCerroll, B.S.  
Independent Reviewer  
Dynamac Corporation

Signature: Nancy R. McCerroll

Date: 4-16-91

APPROVED BY:

Nicolas P. Hajjar, Ph.D.  
Department Manager  
Dynamac Corporation

Signature: Nicolas P. Hajjar

Date: April 17, 1991

Jeane Rowland, M.S.  
EPA Reviewer, Section II  
Toxicology Branch II  
(H-7509C)

Signature: Jeane Rowland

Date: 4/20/91

K. Clark Swartzel  
EPA Section Head, Section II  
Toxicology Branch II  
(H-7509C)

Signature: K. Clark Swartzel

Date: 4/17/91

UDS

008372

DATA EVALUATION RECORD

Tox. Chem. No.:

EPA File Symbol:

CHEMICAL: 2,4-Dichlorophenoxyacetic acid butoxyethyl ester (2,4-D BEE).

STUDY TYPE: Unscheduled DNA synthesis assay in primary rat hepatocytes.

ACCESSION OR NID NUMBER: 414931-01.

FORMULA/CAS NUMBER:  $C_{14}H_{15}Cl_2O_4$ /1929-73-3.

SPONSOR: DowElanco, Indianapolis, IN.

TESTING FACILITY: Health and Environmental Sciences-Texas, Lake Jackson Research Center, The Dow Chemical Company, Freeport, TX.

TITLE OF REPORT: Evaluation of 2,4-Dichlorophenoxyacetic Acid Butoxyethyl Ester (2,4-D BEE) in the Rat Hepatocyte Unscheduled DNA Synthesis (UDS) Assay.

AUTHORS: McClintock, M.L. and Bhaskar Gollegudi, B.

STUDY NUMBER: TXN:K-007722-013.

REPORT ISSUED: May 10, 1990.

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CONCLUSIONS - Executive Summary: Five doses of 2,4-dichlorophenoxyacetic acid butoxyethyl ester (2,4-D BEP) ranging from 5.0 to 500 µg/mL were reported as not inducing an appreciable increase in the net nuclear grain counts of treated rat hepatocytes in two independent unscheduled DNA synthesis (UDS) assays. Although the dose range tested and the protocol designed for the study appeared to be adequate, technical problems with the assay preclude us from making an accurate assessment of the results. The excessively high background cytoplasmic and nuclear grain counts in all groups make it difficult to distinguish between a positive and negative genotoxic response. The mean net nuclear count was not determined from the triplicate coverslips (50 nuclei/coverslip) from each treatment condition. The study does not satisfy the Guideline requirements for genetic effects Category III, Other Mutagenic Mechanisms.

Study Classification: The study is unacceptable.

A. MATERIALS:

1. Test Material:  
 Name: 2,4-Dichlorophenoxyacetic acid butoxyethyl ester (2,4-D BEE).  
 Description: Amber liquid.  
 Lot No.: AGR 276426.  
 Purity: 94.6-95.6%.  
 Contaminants: Not listed; however, see study TXT:K-008866-009 on 2,4-D BEE.  
 Solvent used: Dimethyl sulfoxide (DMSO).  
 Other comments: The test material was reported to be stable in DMSO (Minze, 1989).
2. Indicator Cells: Primary rat hepocytes were obtained by the *in situ* perfusion of the liver of male CDF Fischer 344 rats aged 15-20 weeks (weight: 258-292 g) purchased from Charles River, Wilmington, MI.
3. Control Substances: DMSO at a concentration of 10  $\mu$ L/mL was used as the solvent control, and 2-acetylaminofluorene (2-AAF) at 2.233  $\mu$ g/mL was used as the positive control.
4. Media: WMEI: William's Medium E, supplemented with 2 mM L-glutamine, 1  $\mu$ M dexamethasone, and antibiotics; WME+: As above with 10% fetal bovine serum.

B. STUDY DESIGN:

1. Cell Preparation:
  - a. Perfusion techniques: Livers were perfused with Hanks' balanced salts containing 0.5 mM EGTA and HEPES buffer, pH 7.3, for 4 to 5 minutes and with WMEI containing 0.5 mg/mL (390 to 630 units/mL) collagenase for 10 minutes. Livers were removed and dispersed in 50 mL of WMEI and collagenase; an equal volume of WME+ was added to the cell suspension.
  - b. Harvest via harvest/culture preparation: Recovered cells were centrifuged at 450 x g for 2 minutes, resuspended in WME+, counted, and aliquoted (0.5 x 10<sup>6</sup> cells/3 mL WME+) onto plastic coverslips in a series of

<sup>1</sup>Minze, C.A. Analytical Report Code GF-96-89, H&ES-Texas, Analytical Chemistry Laboratory, The Dow Chemical Company (unpublished).

culture dishes. The cultures were placed in a humidified, 37°C, 5% incubator for a 4-hour attachment period. Nonviable cells were removed by washing with 3 mL of WMEI after the attachment period; viable cells were refed and established as monolayer cultures.

2. Dose Selection: Initially, five concentrations of the test material (166.7 to 5000 µg/mL) were assayed. When the viability estimate was obtained (18-20 hours after treatment initiation), doses were chosen for analysis of nuclear labeling. The five doses chosen for the UDS assay were 5.0, 16.7, 50, 166.7 and 500.0 µg/mL.
3. UDS Assay:
  - a. Treatment: Two separate assays were conducted with hepatocytes derived from two different rats (one rat/assay). Four replicate monolayer cultures were exposed to the selected doses of the test material, negative (DMSO) control, or positive control (2-AAF) for 18-20 hours in WMEI containing 10 µCi/mL [<sup>3</sup>H]thymidine. Treated monolayers were washed twice with WMEI; one of the four replicates for each treatment group was used to determine cytotoxicity. These cultures were refed, reincubated, and monitored for cytotoxicity at 18-20 hours posttreatment by trypan blue exclusion.
  - b. UDS slide preparation: Treated hepatocytes, attached to coverslips, were exposed to 1.4% sodium citrate for 10 minutes, fixed in acetic acid:ethanol (1:3), dried, and mounted.
  - c. Preparation of autoradiographs/grain development: Slides were coated with Kodak NTB-2 emulsion, stored in a refrigerator for 10 days, developed in Kodak D-19, fixed, stained with Giemsa, coverslipped, coded, and counted.
  - d. Grain counting: The nuclear grains of 100 morphological normal cells (50/coverslip) for each test dose and for the negative control and 25 cells/slide (total of 50/treatment) positive controls were counted by projection on a television screen using a high-resolution camera mounted on a microscope. Net nuclear grain counts (NNG) were determined by subtracting the average cytoplasmic grain counts from the nuclear grain count of each cell. Grain counts were reported as the mean per cell ± SD.



4: Evaluation Criteria:

- a. Assay validity: Not presented, but assumed by the reviewers to be that of Williams, 1977<sup>2</sup> and Williams, et al. 1982.<sup>3</sup>
- b. Positive Response: The assay was considered positive if the increase in the mean net nuclear grain count was  $\geq 5$  grains/nucleus over the negative control value, and at the same time significantly greater ( $p < 0.05$ ) than the negative control value. Statistical analyses were performed only when the net nuclear grain count of a treatment group was  $\geq 5$ . Kruskal-Wallis one-way analysis of variance and Wilcoxon's Rank Sum Test with Bonferroni's correction were used in a computer program on the grain count data.

C. REPORT RE UDS:

- 1. Analytical Determinations: Not presented in this report.
- 2. UDS Assay: The preliminary toxicity assay qualitative results showed that concentrations of 1167 and 5000  $\mu\text{g}/\text{ml}$  were excessively cytotoxic to the hepatocytes, whereas concentrations of 500 and 335.7  $\mu\text{g}/\text{ml}$  caused cell rounding without lethality, and 166.7  $\mu\text{g}/\text{ml}$  gave slight toxicity. It is not clear to the reviewers whether the 335.7- $\mu\text{g}/\text{ml}$  dose was actually used (see Appendix B) or if this was intended to indicate a different concentration, e.g., 255.7, 55.57 or other. Based on these results, a dose range of 5.0 to 500  $\mu\text{g}/\text{ml}$  was chosen for the UDS assays. The results from the two assays are presented in Table 1. As shown, net nuclear grain counts for the 500- and 166.7- $\mu\text{g}/\text{ml}$  dose groups in both assays were not appreciably higher than those in the solvent control. The report indicated that moderate cytotoxicity was seen at the 5.0  $\mu\text{g}/\text{ml}$  level in assay No. 1. Results similar to the solvent control were seen for the three lower concentrations of test material (5.0, 16.7, and 50  $\mu\text{g}/\text{ml}$ ). Cytoplasmic grain

<sup>2</sup>Williams, G.N. 1977. Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell cultures. Cancer Res. 37:1845-1851.

<sup>3</sup>Williams, G.N., Laspia, M.F., and Dunkel, V.C. 1982. Reliability of the hepatocyte primary culture/DNA repair test in testing of coded carcinogens and noncarcinogens. Mutat. Res. 97:359-370.



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UDS

TABLE 1: Representative Results of the Unconjugated DNA Synthesis Rat Hepatocyte Assay with 2,4-Dichlorophenoxyacetic Acid Butylsilyl Ester (2,4-D DBE)

Treatment	Dose/ml	Cells Scored	Average Cytoplasmic Grain Count <sup>a</sup>	Mean Net Nuclear Grain Count <sup>b</sup> ± S.D.	Average Percent Nuclei with ≥5 Grains <sup>c</sup>
Solvent Control					
Dimethyl-sulfoxide	10 $\mu$ l <sup>b</sup>	100	34.1	-16.6 ± 7.1	--
	10 $\mu$ l <sup>c</sup>	100	34.1	-13.0 ± 6.2	--
Positive Control					
2-Acetyl-aminofluorene	2.225 $\mu$ g <sup>b</sup>	50	35.6	66.4 ± 8.0 <sup>d</sup>	--
	2.225 $\mu$ g <sup>c</sup>	50	31.0	69.0 ± 7.3 <sup>e</sup>	--
Test Material					
2,4-D DBE	500 $\mu$ g <sup>b</sup>	100	36.2	-14.6 ± 7.8	--
	50.7 $\mu$ g <sup>f</sup>	100	31.5	-14.4 ± 7.0	--
	16.7 $\mu$ g <sup>h, g</sup>	100	34.9	-15.1 ± 6.9	--
	166.7 $\mu$ g <sup>d, e</sup>	100	33.6	-13.0 ± 6.5	--

undervived from counting cells on duplicate coverslips.

data from assay No. 1.

data from assay No. 2.

fills regarding laboratory's criteria for a positive effect; nuclear grains were too high to be accurately read; hence nuclear grain counts were considered only good estimates.

data for the low dose (5.0  $\mu$ g/ml) and intermediate doses (16.7 and 50  $\mu$ g/ml) did not indicate a genotoxic effect.

counts were high in all test groups suggesting a technical problem with the staining procedure. By contrast, the positive control (2.233 µg/mL 3-AAF) induced a marked increase in net nuclear counts and 100% of the cells had ≥5 grains; however, even these results were not quantitative owing to the high cytoplasmic grain counts, which resulted in inaccurate scoring.

Based on the overall results, the study authors concluded: "that the test material did not elicit a positive UDS response in the rat hepatocyte cultures. Hence under the experimental conditions used, the test material was judged negative in the rat hepatocyte UDS assay."

D. REVIEWERS' DISCUSSION/INTERPRETATION OF STUDY RESULTS:

We assess that the study protocol used by the authors to conduct these assays was adequate, but we cannot verify their interpretation of the results. The highest dose of 2,4-D BBE (500.0 µg/mL) affected the cell morphology (rounding up) in the preliminary toxicity assay and caused a moderate cytotoxicity response in assay No. 1, indicating that the dose level was sufficient; however, cytotoxicity was not confirmed in UDS assay No. 2. Further, our reviewers have concern over the high cytoplasmic and nuclear grain counts reported; the high background counts for the cytoplasmic grains and net nuclear grains make it difficult to determine whether a positive response has been obtained. In fact, these counts are at least 3-fold higher than expected based on our past experience. As indicated from the reported results, even the positive control could not be accurately assessed because of the excessive cytoplasmic and nuclear grains. It is our assessment that there may have been technical difficulties with the staining procedures, making it difficult to score the slides. We, therefore, are unable to determine whether the test material was negative for genotoxicity over the dose range tested.

- E. QUALITY ASSURANCE MEASURES: A quality assurance statement was signed and dated May 10, 1990. We have concern about this statement because there are apparent errors, including the dose of 535.7 µg/mL (see Appendix B) cited in the preliminary assay and the lack of concern over high cytoplasmic and nuclear grain counts.

- F. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 9-13; Appendix B, Table 1. Quantitative Assessment of the Toxicity of the Test Material. Results of Preliminary Toxicity Assay (CBI p. 16).

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APPENDIX A

Materials and Methods  
(CBI pp. 9-13)

RIN 2465-01

2,4-D DER

Page      is not included in this copy.

Pages 15 through 21 are not included.

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.

CONFIDENTIAL BUSINESS INFORMATION  
DOES NOT CONTAIN  
NATIONAL SECURITY INFORMATION (EQ 12045)

EPA No.: 68D80056  
DYNAMAC No.: 310-E  
TASK No.: 3-10E  
April 17, 1991

008372

DATA EVALUATION RECORD

2,4-Dichlorophenoxyacetic Acid Triisopropanol-  
amine Salt (2,4-D TIPA)

Mutagenicity--Unscheduled DNA Synthesis  
Assay in Primary Rat Hepatocytes

APPROVED BY:

Robert J. Weir, Ph.D.  
Program Manager  
Dynamac Corporation

Signature: William J. McSweeney

Date: April 17, 1991



Guideline Series 84: MUTAGENICITY

EPA No.: 68D80056  
DYNAMAC No.: 310-E  
TASK No.: 3-10E  
April 17, 1991

008372

DATA EVALUATION RECORD

2,4-Dichlorophenoxyacetic Acid Triisopropylammonium Salt (2,4-D TIPA)

Mutagenicity--Unscheduled DNA Synthesis Assay in Primary Rat Hepatocytes

REVIEWED BY:

I. Cecil Felkner, Ph.D.  
Principal Reviewer  
Dynamac Corporation

Signature: I. Cecil Felkner  
Date: 4-16-91

Nancy E. McCarroll, B.S.  
Independent Reviewer  
Dynamac Corporation

Signature: Nancy E. McCarroll  
Date: 4-16-91

APPROVED BY:

Nicolas P. Hajjar, Ph.D.  
Department Manager  
Dynamac Corporation

Signature: Nicolas P. Hajjar  
Date: April 17, 1991

Jess Rowland, M.S.  
EPA Reviewer, Section II  
Toxicology Branch II  
(H-7509C)

Signature: Jess Rowland  
Date: 4/22/91

K. Clark Swentzell  
EPA Section Head, Section II  
Toxicology Branch II  
(H-7509C)

Signature: K. Clark Swentzell  
Date: 4/22/91

009372

DATA EVALUATION RECORD

Tox. Chem. No.:

EPA File Symbol:

CHEMICAL: 2,4-Dichlorophenoxyacetic acid trisopropanolamine salt  
(2,4-D TIPA).

STUDY TYPE: Unscheduled DNA synthesis assay in primary rat  
hepatocytes.

ACCESSION OR NRTD NUMBER: 674981-02.

FORMULA/CAS NUMBER:  $C_{17}H_{27}Cl_2NO_2$ /18584-79-7.

SPONSOR: DowDance, Indianapolis, IN.

TESTING FACILITY: Health and Environmental Sciences-Texas, Jack  
Jackson Research Center, The Dow Chemical Company, Freeport, TX.

TITLE OF REPORT: Evaluation of a Formulation Containing 2,4-Di-  
chlorophenoxyacetic acid Trisopropanolamine Salt (2,4-D TIPA) in  
the Rat Hepatocyte Unscheduled DNA Synthesis Assay.

AUTHORS: McClintock, M.L., and Bhaskar Gollapudi, S.

STUDY NUMBER: TXR-K-008666-008

REPORT ISSUED: May 3, 1980

UDS

**CONCLUSIONS - Executive Summary:** Five doses of 2,4-Dichlorophenoxyacetic acid triisopropanolamine salt (2,4-D TIPA) ranging from 5.0 to 500.0 µg/mL were reported as not inducing an appreciable increase in the net nuclear grain counts of treated rat hepatocytes in two independent unscheduled DNA synthesis (UDS) assays. Although the dose range tested and the protocol designed for the study appeared to be adequate, technical problems with the assay preclude us from making an accurate assessment of the results. The excessively high cytoplasmic and nuclear grain counts in all groups make it difficult to distinguish between a positive and negative genotoxic response. The mean net nuclear count was not determined from the triplicate coverslips (50 nuclei/coverslip) from each treatment condition. The study does not satisfy the Guideline requirements for genetic effects Category III, Other Mutagenic Mechanisms.

**Study Classification:** The study is unacceptable.

**4. MATERIALS:**

1. **Test Material:**

**Name:** 2,4-Dichlorophenoxyacetic acid triisopropanolamine salt (2,4-D TIPA)  
**Description:** Amber liquid  
**Batch, Lot No.:** AGA 276428  
**Purity:** 72.28 TIPA salt (Formulation in CBI Table 1; S. Appendix B, Chemical Analysis)  
**Contaminants:** None (Chemical Analysis) Appendix B  
**Solvent used:** Williams Medium E (WME) supplemented with 2 mM L-glutamine, 1 µM dexamethasone, and antibiotics.  
**Other comments:** The test material was dissolved in WME; the frequency of test solution preparation was not reported.

2. **Indicator Cells:** Primary rat hepatocytes were obtained by the in situ perfusion of the liver of male CDF Fischer F44 rats aged 13 to 20 weeks (weight: 204-292 g) purchased from Charles River, Wilmington, NY.

3. **Control Substances:** WME was used as the solvent control, and 2-acetylaminofluorene (2-AAF) at 2.233 µg/mL was used as the positive control.

4. **Medium:** WME: William's Medium E, supplemented with 2 mM L-glutamine, 1 µM dexamethasone, and antibiotics; WME+: As above with 10% fetal bovine serum.

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**B. STUDY DESIGN:****1. Cell Preparation:**

- a. Perfusion techniques: Livers were perfused with Hanks' balanced salts containing 0.5 mM EGTA and HEPES buffer, pH 7.3, for 4 to 5 minutes and with WMEI containing 0.5 mg/mL (390 to 630 units/mL) collagenase. Livers were removed and dispersed in 50 mL of WMEI and collagenase; an equal volume of WMEI+ was added to the cell suspension.
- b. Hepatocyte harvest/culture preparation: Recovered cells were centrifuged at  $450 \times g$  for 2 minutes, resuspended in WMEI, counted, and aliquoted ( $5 \times 10^6$  cells) onto plastic coverslips in a series of culture dishes. The cultures were placed in a humidified, 37°C, CO<sub>2</sub> 5% incubator for a 4-hour attachment period. Nonviable cells were removed by washing with 3 mL of WMEI after the attachment period; viable cells were refed and established as monolayer cultures.

2. Dose Selection: Initially, 5 concentrations of the test material (166.7 to 5000.0 µg/mL) were assayed. When the visibility estimate was obtained (16-20 hours after treatment initiation), doses were chosen for analysis of nuclear labeling. The five doses chosen for the UDS assay were 5.0, 16.7, 50.0, 166.7 and 500.0 µg/mL.

**3. UDS Assay:**

- a. Treatments: Two separate assays were conducted with hepatocytes derived from two different rats (1 rat/assay). Four replicate monolayer cultures were exposed to the selected doses of the test material, negative control (culture medium), or positive control (2-AMP) for 18 to 20 hours in WMEI containing 10 µCi/mL [<sup>3</sup>H]thymidine. One of the four replicates for each treatment group was used to determine cytotoxicity by trypan blue exclusion. The remaining monolayers were washed with phosphate buffered saline and used to prepare UDS slides.
- b. UDS slide preparation: Treated hepatocytes, attached to coverslips, were exposed to 1.4% sodium citrate for 10 minutes, fixed in acetic acid:ethanol (1:3), dried, and mounted.
- c. Preparation of autoradiographs/grain development: Slides were coated with Kodak NTB-2 emulsion, stored in a refrigerator for 10 days, developed in Kodak D-19, fixed, stained with Giemsa, coverslipped, coded, and counted.



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### 1. Cell Preparation:

- a. Perfusion techniques: Livers were perfused with Hanks' balanced salts containing 0.5 mM EGTA and HEPES buffer, pH 7.3, for 4 to 5 minutes and with WMEI containing 0.5 mg/mL (390 to 630 units/mL) collagenase. Livers were removed and dispersed in 50 mL of WMEI and collagenase; an equal volume of WME+ was added to the cell suspension.
- b. Hepatocyte harvest/culture preparation: Recovered cells were centrifuged at  $\approx 50 \times g$  for 2 minutes, resuspended in WME+, counted, and aliquoted ( $5 \times 10^5$  cells) onto plastic coverslips in a series of culture dishes. The cultures were placed in a humidified, 37°C, CO<sub>2</sub> 5% incubator for a 4-hour attachment period. Nonviable cells were removed by washing with 3 mL of WMEI after the attachment period; viable cells were refed and established as monolayer cultures.

2. Dose Selection: Initially, 5 concentrations of the test material (166.7 to 5000.0  $\mu\text{g/mL}$ ) were assayed. When the viability estimate was obtained (18-20 hours after treatment initiation), doses were chosen for analysis of nuclear labeling. The five doses chosen for the UDS assay were 5.0, 16.7, 50.0, 166.7 and 500.0  $\mu\text{g/mL}$ .

### 3. UDS Assay:

- a. Treatment: Two separate assays were conducted with hepatocytes derived from two different rats (1 rat/assay). Four replicate monolayer cultures were exposed to the selected doses of the test material, negative control (culture medium), or positive control ( $^3\text{-H}$ -AAF) for 18 to 20 hours in WMEI containing 10  $\mu\text{Ci/mL}$  [ $^3\text{H}$ ]thymidine. One of the four replicates for each treatment group was used to determine cytotoxicity by trypan blue exclusion. The remaining monolayers were washed with phosphate buffered saline and used to prepare UDS slides.
- b. UDS slide preparation: Treated hepatocytes, attached to coverslips, were exposed to 1.4% sodium citrate for  $\approx 10$  minutes, fixed in acetic acid:ethanol (1:3), dried, and mounted.
- c. Preparation of autoradiographs/grain development: Slides were coated with Kodak NTB-2 emulsion, stored in a refrigerator for 10 days, developed in Kodak D-19, fixed, stained with Giemsa, coverslipped, coded, and counted.

- d. Grain counting: The nuclear grains of 100 morphological normal cells (50/coverslip) for each test dose and for the negative control and 25 cells/slide (total of 50/treatment) from the positive control group were counted by projection on a television screen using a high resolution camera mounted on a microscope. Net nuclear grain counts (NNG) were determined by subtracting the average cytoplasmic grain count from the nuclear grain count of each cell. Grain counts were reported as the mean per cell  $\pm$  SD.

4. Evaluation Criteria:

- a. Assay validity: Not presented, but assumed by our reviewers to be that of Williams, 1977<sup>a</sup> and Williams, et al. 1982<sup>b</sup>.
- b. Positive response: The assay was considered positive if the increase in the mean net nuclear grain counts was  $\geq 5$  grains/nucleus over the negative control value, and significantly greater ( $p < 0.05$ ) than the negative control value. Statistical analyses were only performed when the net nuclear grain count of a treatment group was  $\geq 5$ . Kruskal-Wallis one-way analysis of variance and Wilcoxon's Rank Sum Test with Bonferroni's correction were used in a computer program on the grain count data.

C. REPORT RESULTS:

1. Analytical Determinations: Not presented in this report.
2. UDS Assay: The preliminary toxicity assay qualitative results (see Appendix C) showed that concentrations of 1167.0 and 5000.0  $\mu\text{g/mL}$  were excessively cytotoxicity to the hepatocytes, whereas concentrations of 500.0 and 555.7  $\mu\text{g/mL}$  caused cell rounding without lethality, and 166.7  $\mu\text{g/mL}$  gave no toxicity. It is not clear to our reviewers whether the

---

<sup>a</sup>Williams, G.M. 1977. Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell cultures. Cancer Res. 37:1845-1851.

<sup>b</sup>Williams, G.M., Laspia, M.F., and Dunkel, V.C. 1982. Reliability of the hepatocyte primary culture/DNA repair test in testing of coded carcinogens and noncarcinogens. Mutat. Res. 97:359-370.



555.7  $\mu\text{g}/\text{mL}$  dose was actually used or if this was intended to indicate a different concentration, (e.g., 255.7 or 55.57  $\mu\text{g}/\text{mL}$ ). Based on these results, a dose range of 5.0 to 500.0  $\mu\text{g}/\text{mL}$  was chosen for the UDS assays. The results from the two assays are presented in Table 1. As shown, net nuclear grain counts for the 500.0 and 166.7- $\mu\text{g}/\text{mL}$  dose groups in both assays were not appreciably higher than those in the solvent control. The cytotoxicity seen at the 500.0  $\mu\text{g}/\text{mL}$  level in the preliminary assay was not repeated in UDS assays #1 or #2. Results similar to the solvent control were seen for the three lower concentrations of test material (5.0, 16.7 and 50.0  $\mu\text{g}/\text{mL}$ ). Cytoplasmic grain counts were high in all test groups suggesting a technical problem with the staining procedure. Similarly two of the three slides prepared for the UDS evaluation at each treatment level were reported to be "unsuitable" for scoring because of a technical problem. By contrast, the positive control (2.233  $\mu\text{g}/\text{mL}$  2-AAF) induced a marked increase in net nuclear counts and 100% of the cells had  $\geq 5$  grains. However, even these results were not quantitative; owing to the high cytoplasmic and nuclear grain counts, net nuclear counts for the positive control were "only good estimates".

Based on the overall results, the study authors concluded that the test material did not elicit a positive UDS response in two separate experiments. Hence under the experimental conditions used, the test material was considered negative in the rat hepatocyte UDS assay."

D. REVIEWERS' DISCUSSION/INTERPRETATION OF STUDY RESULTS:

We assess that the study protocol used by the authors to conduct these studies was adequate, but we cannot verify the interpretation of the results. The highest dose of 2,4-D TIPA (500.0  $\mu\text{g}/\text{mL}$ ) affected the cell morphology (rounding up) in the preliminary toxicity assay, indicating that the dose level was sufficient; however, this toxicity was not confirmed in UDS assays #1 or #2. Further, our reviewers have concern over the high cytoplasmic and nuclear grain counts that were reported. The high counts for the cytoplasmic grains and nuclear grains make it difficult to determine whether a positive response

TABLE 1. Representative Results of the Unscheduled DNA Synthesis Rat Hepatocyte Assay with 2,4-Dichlorophenoxyacetic Acid Triisopropylamine Salt (2,4-D TIPA)

Treatment	Dose/mL	Cells Scored	Average Cytoplasmic Grain Count <sup>a</sup>	Mean Net Nuclear Grain Count $\pm$ S.D. <sup>a</sup>	Average Percent Nuclei with $\geq 5$ Grains <sup>a</sup>
<u>Solvent Control</u>					
Culture Medium	--	100	35.5	-12.4 $\pm$ 6.4	--
	--	100	36.5	-11.9 $\pm$ 6.4	--
<u>Positive Control</u>					
2-Acetyl-aminofluorene	2.223 $\mu\text{g}^b$	50	56.5	49.5 $\pm$ 7.3 <sup>d</sup>	--
	2.223 $\mu\text{g}^c$	50	45.1	55.8 $\pm$ 8.6 <sup>d</sup>	--
<u>Test Material</u>					
2,4-D TIPA	500.0 $\mu\text{g}^b$	100	29.4	-9.2 $\pm$ 5.6	--
	500.0 $\mu\text{g}^c$	100	36.9	-10.6 $\pm$ 6.3	--
	166.7 $\mu\text{g}^{b,c}$	100	40.5	-15.3 $\pm$ 7.4	--
	166.7 $\mu\text{g}^{c,e}$	100	42.0	-10.9 $\pm$ 6.7	--

<sup>a</sup> Values calculated from 100 cells/slide in the solvent and test groups and 50 cells/slide in the positive control group. Owing to a technical problem only one slide/group was scored.

<sup>b</sup> Results from assay #1.

<sup>c</sup> Results from assay #2.

<sup>d</sup> Values reporting laboratory's criteria for a positive effect; nuclear grain counts were too high to be accurately counted; hence, nuclear grain counts were only considered grid cell counts.

<sup>e</sup> Results for the low dose (2.0  $\mu\text{g}/\text{mL}$ ) and intermediate doses (16.7 and 50.0  $\mu\text{g}/\text{mL}$ ) in both assays did not indicate a genotoxic effect.

has been obtained. In fact, these counts are at least 3-fold higher than expected based on our past experience. As indicated from the reported results, even the positive control could not be accurately assessed due to the excessive nuclear grains. It is our assessment that there may have been technical difficulties with the staining procedures, making it difficult to score the slides. We, therefore, are unable to determine whether the test material was negative for genotoxicity over the dose range tested.

- E. QUALITY ASSURANCE MEASURES: A quality assurance statement was signed and dated May 2, 1990. We have concern about this statement because there are apparent errors, including the dose of 555.7 µg/ml cited in the preliminary assay (see Appendix C) and the lack of concern over high cytoplasmic and nuclear grain counts.
- F. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 9-13; Appendix B, Liquid Chromatographic Analysis of 2,4-D TIPA, AGR 276426, CBI p. 16.; Appendix C, Quantitative Assessment of the Toxicity of the Test Material. Results of Preliminary Toxicity Assay, CBI p. 17.

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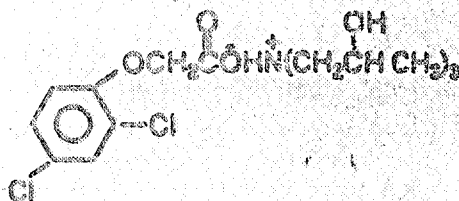
APPENDIX A  
Materials and Methods  
(CBI pp. 9-13)

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MATERIALS AND METHODS

Test Material

1. Chemical Name: 2,4-dichlorophenoxyacetic acid triisopropanolamine salt
2. Synonym: 2,4-D TIPA
3. C.A.S. Number: 18504-79-7
4. Chemical Formula:  $C_{17}H_{27}Cl_2NO_6$
5. Chemical Structure:



6. Molecular weight: 412.3
7. Physical State: Amber liquid
8. Source: The Dow Chemical Company  
Midland, MI
9. Lot Number: AGR 276428
10. Purity: The test material is a formulation containing 72.2% 2,4-D TIPA salt (see Table 1).

RIN 2465-01

2,4-D DER

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Pages 34 through 41 are not included.

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.



EPA No.: 68DS0056  
DYNAMAC No.: 310-F  
TASK No.: 3-10F  
April 17, 1991

008372

DATA EVALUATION RECORD

2,4-Dichlorophenoxyacetic Acid Isopropylamine Salt (2,4-D IPA)

Mutagenicity--Unscheduled DNA Synthesis  
Assay in Primary Rat Hepatocytes

APPROVED BY:

Robert J. Weir, Ph.D.  
Program Manager  
Dynamac Corporation

Signature: William L. McLellan Jr  
Date: April 19, 1991

Guideline Series 84: MUTAGENICITY

EPA No.: 68D90056  
DYNAMAC No.: 310-F  
TASK No.: 3-10F  
April 17, 1991

008372

DATA EVALUATION RECORD

2,4-Dichlorophenoxyacetic Acid Isopropylamine Salt (2,4-D IPA)

Mutagenicity--Unscheduled DNA Synthesis  
Assay in Primary Rat Hepatocytes

REVIEWED BY:

I. Cecil Felkner, Ph.D.  
Principal Reviewer  
Dynamac Corporation

Signature: I. Cecil Felkner  
Date: 4-16-91

Nancy E. McCarroll, B.S.  
Independent Reviewer  
Dynamac Corporation

Signature: Nancy E. McCarroll  
Date: 4-16-91

APPROVED BY:

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Department Manager  
Dynamac Corporation

Signature: Nicolas P. Hajjar  
Date: April 17, 1991

Jess Rowland, M.S.  
EPA Reviewer, Section II  
Toxicology Branch II  
(R-7509C)

Signature: Jess Rowland  
Date: 4/16/91

K. Clark Svontzel  
EPA Section Head, Section II  
Toxicology Branch II  
(H-7509C)

Signature: K. Clark Svontzel  
Date: 5/2/91

08372

## DATA EVALUATION RECORD

T. X. Chem. No.:EPA File Symbol:

CHEMICAL: 2,4-Dichlorophenoxyacetic acid isopropylamine salt (2,4-D IPA).

STUDY TYPE: Unscheduled DNA synthesis assay in primary rat hepatocytes.

ACCESSION OR FILE NUMBER: 414981-03.

FORMULA/CAS NUMBER: C<sub>11</sub>H<sub>15</sub>Cl<sub>2</sub>NO<sub>3</sub>/5742-17-6.

SPONSOR: DowElanco, Indianapolis, IN.

TESTING FACILITY: Health and Environmental Sciences-Texas, Lake Jackson Research Center, The Dow Chemical Company, Freeport, TX.

TITLE OF REPORT: Evaluation of a Formulation Containing 2,4-Dichlorophenoxyacetic Acid Isopropylamine Salt (2,4-D IPA) in the Rat Hepatocyte Unscheduled DNA Synthesis Assay.

AUTHORS: M.L. McClintock and B. Bhaskar Collapudi.

STUDY NUMBER: TXT:M-004725-006.

REPORT ISSUED: May 3, 1990.

CONCLUSIONS - Executive Summary: Five doses of 2,4-dichlorophenoxyacetic acid isopropylamine salt (2,4-D IPA) ranging from 5.0 to 500.0 µg/mL were reported as not inducing an appreciable increase in the net nuclear grain counts of treated rat hepatocytes in two independent unscheduled DNA synthesis (UDS) assays. Although the dose range tested and the protocol designed for the study appeared to be adequate, technical problems with the assay preclude us from making an accurate assessment of the results. The excessively high cytoplasmic and nuclear grain counts in all groups make it difficult to distinguish between a positive and negative genotoxic response. The mean net nuclear count was not determined from the triplicate coverslips (50 nuclei/coverslip) for each treatment condition. The study does not satisfy the Guideline requirements for genetic effects, Category III, Other Mutagenic Mechanisms.

Study Classification: The study is unacceptable.

**A. MATERIALS:**

**1. Test Material:**

**Name:** 2,4-Dichlorophenoxyacetic acid iso-propylamine salt (2,4-D IPA).  
**Description:** Amber liquid.  
**Lot No.:** AGR 276461.  
**Purity:** 50.2% of IPA salt (formulation as in CBI Table 1; see Appendix B, CBI p. 16).  
**Contaminants:** See CBI Table 1 (Appendix B, CBI p. 16).  
**Solvent used:** Williams Medium E (WMEI) supplemented with 2 mM L-glutamine, 1  $\mu$ M dexamethasone, and antibiotics.  
**Other comments:** The test material was dissolved in WMEI; the frequency of test solution preparation was not reported.

**2. Indicator Cells:** Primary rat hepatocytes were obtained by the *in situ* perfusion of the liver of male CDF Fischer 344 rats aged 13 to 20 weeks (weight, 204-292 g) purchased from Charles River, Wilmington, MI.

**3. Control Substances:** WMEI was used as the solvent control, and 2-acetylaminofluorene (2-AAF) at 2.233  $\mu$ g/mL was used as the positive control.

**4. Medium:** WMEI: William's Medium E, supplemented with 2 mM L-glutamine, 1  $\mu$ M dexamethasone, and antibiotics; WME+: As above with 10% fetal bovine serum.

**B. STUDY DESIGN:**

**1. Cell Preparation:**

**a. Perfusion technique:** Livers were perfused with Hanks' balanced salts containing 0.5 mM EGTA and HEPES buffer, pH 7.3, for 4 to 5 minutes and with WMEI containing 0.5 ng/mL (390 to 630 units/mL) collagenase. Livers were removed and dispersed in 50 mL of WMEI and collagenase; an equal volume of WME+ was added to the cell suspension.

**b. Hepatocyte harvest/culture preparation:** Recovered cells were centrifuged at  $\approx 50 \times g$  for 2 minutes, resuspended in WME+, counted, and aliquoted ( $5 \times 10^6$  cells) onto plastic coverslips in a series of culture dishes. The cultures were placed in a humidified, 5% CO<sub>2</sub> incubator maintained at 37°C for a 4-hour

attachment period. Nonviable cells were removed by washing with 3 mL of WMEI after the attachment period; viable cells were refed and established as monolayer cultures.

2. Dose Selection: Initially, five concentrations of the test material (166.7 to 5000.0  $\mu\text{g/mL}$ ) were assayed. When the viability estimate was obtained (18-20 hours after treatment initiation), doses were chosen for analysis of nuclear labeling. The five doses chosen for the UDS assay were 5.0, 16.7, 50.0, 166.7, and 500.0  $\mu\text{g/mL}$ .
3. UDS Assay:
  - a. Treatment: Two separate assays were conducted with hepatocytes derived from two different rats (one rat/assay). Four replicate monolayer cultures were exposed to the selected doses of the test material, negative control (culture medium), or positive control (2-AAF) for 18 to 20 hours in WMEI containing 10  $\mu\text{Ci/mL}$  [ $^3\text{H}$ ]thymidine. One of the four replicates for each treatment group was used to determine cytotoxicity by trypan blue exclusion. The remaining monolayers were washed with phosphate buffered saline and used to prepare UDS slides.
  - b. UDS slide preparation: Treated hepatocytes, attached to coverslips, were exposed to 1.4% sodium citrate for 10 minutes, fixed in acetic acid:ethanol (1:3), dried, and mounted.
  - c. Preparation of autoradiographs/grain development: Slides were coated with Kodak NTB-2 emulsion, stored in a refrigerator for 10 days, developed in Kodak D-19, fixed, stained with Giemsa, coverslipped, coded, and counted.
  - d. Grain counting: The nuclear grains of 100 morphological normal cells (50/coverslip) for each test dose and for the negative control and 25 cells/slide (total of 50/treatment) from the positive control group were counted by projection on a television screen using a high-resolution camera mounted on a microscope. Net nuclear grain counts (NNG) were determined by subtracting the average cytoplasmic grain count from the nuclear grain count of each cell. Grain counts were reported as the mean per cell  $\pm$  SD.

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#### 4. Evaluation Criteria:

- a. Assay validity: Not presented, but assumed by our reviewers to be that of Williams, 1977<sup>1</sup> and Williams, et al. 1982<sup>2</sup>.
- b. Positive response: The assay was considered positive if the increase in the mean net nuclear grains was  $\geq 5$  grains/nucleus over the negative control value, and significantly greater ( $p < 0.05$ ) than the negative control value. Statistical analyses were performed only when the net nuclear grain count of a treatment group was  $\geq 5$ . Krushal-Wallis one-way analysis of variance and Wilcoxon's Rank Sum Test with Bonferroni's correction were used in a computer program on the grain count data.

#### C. REPORT RESULTS:

1. Analytical Determinations: Not presented in this report.
2. UDS Assay: The preliminary toxicity assay qualitative results (see Appendix C) showed that concentrations of 1167.0 and 5000.0  $\mu\text{g/mL}$  were excessively cytotoxic to the hepatocytes, whereas concentrations of 100.0 and 555.7  $\mu\text{g/mL}$  caused cell rounding without lethality, and 166.7  $\mu\text{g/mL}$  gave no toxicity. It is not clear to our reviewers whether the 555.7  $\mu\text{g/mL}$  dose was actually used or if this was intended to indicate a different concentration (e.g., 255.7 or 55.57  $\mu\text{g/mL}$ ). Based on these results, a dose range of 5.0 to 500.0  $\mu\text{g/mL}$  was chosen for the UDS assays. The results from the two assays are presented in Table 1. As shown, net nuclear grain counts for the 500.0- and 166.7- $\mu\text{g/mL}$  dose groups in both assays were not appreciably higher than those in the solvent control. Results similar to the solvent control were seen for the three lower concentrations of test material (5.0, 16.7, and 50.0  $\mu\text{g/mL}$ ). Cytoplasmic grain counts were high in all test groups,

<sup>1</sup>Williams, G.M. 1977. Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell cultures. Cancer Res. 37:1845-1851.

<sup>2</sup>Williams, G.M., Iaspia, M.F., and Dunkel, V.C. 1982. Reliability of the hepatocyte primary culture/DNA repair test in testing of coded carcinogens and noncarcinogens. Mut. Res. 97:359-370.



TABLE 1. Representative Results of the Unscheduled DNA Synthesis Rat Hepatocyte Assays with 2,4-Dichlorophenoxyacetic Acid Isopropylamine Salt (2,4-D IPA)

Treatment	Dose/ $\mu$ l	Cells Scored	Average Cytoplasmic Grain Count <sup>a</sup>	Mean Net Nuclear Grain Count <sup>a</sup> $\pm$ S.D.	Average Percent Nuclear with $\geq 5$ Grains <sup>a</sup>
<b>Solvent Control</b>					
Culture medium	-- <sup>b</sup>	100	37.6	-13.7 $\pm$ 6.6	--
	-- <sup>c</sup>	100	38.0	-14.5 $\pm$ 7.2	--
<b>Positive Control</b>					
2-Acetylaminofluorene	2.233 $\mu$ g <sup>b</sup>	50	50.9	49.5 $\pm$ 7.3 <sup>d</sup>	--
	2.235 $\mu$ g <sup>c</sup>	50	51.0	55.8 $\pm$ 8.8 <sup>d</sup>	--
<b>Test Material</b>					
2,4-D IPA	500.0 $\mu$ g <sup>b</sup>	100	35.5	-11.6 $\pm$ 7.2	--
	500.0 $\mu$ g <sup>c</sup>	100	33.7	-11.7 $\pm$ 6.7	--
	166.7 $\mu$ g <sup>b,e</sup>	100	41.2	-15.1 $\pm$ 7.4	--
	166.7 $\mu$ g <sup>c,e</sup>	100	42.5	-16.3 $\pm$ 7.5	--

<sup>a</sup> Calculated from 100 cells/slides in the solvent and test groups and 50 cells/slides in the positive control group. Owing to a technical problem, only one slide/group was scored.

<sup>b</sup> Data from assay No. 1.

<sup>c</sup> Data from assay No. 2.

<sup>d</sup> This reporting laboratory's criteria for a positive effect; nuclear grains were too high to be accurately scored; hence, nuclear grain counts were considered only good estimates.

<sup>e</sup> Data for the low dose (5.0  $\mu$ g/ml) and intermediate doses (16.7 and 50.0  $\mu$ g/ml) in both assays did not indicate a toxic effect.

UDS

suggesting a technical problem with the staining procedure. Similarly, two of the three slides prepared for the UDS evaluation at each treatment level were reported to be "unsuitable for scoring because of a technical problem." By contrast, the positive control (2.233 µg/mL 2-AAF) induced a marked increase in net nuclear counts, and 100% of the cells had ≥5 grains. However, these results were not quantitative; owing to the high cytoplasmic and nuclear grain counts, net nuclear counts for the positive control group were "only good estimates."

Based on the overall results, the study authors concluded: "the test material did not elicit a positive UDS response in the rat hepatocyte cultures. Hence under the experimental conditions used, the test material was judged negative in the rat hepatocyte UDS assay."

D. REVIEWERS' DISCUSSION/INTERPRETATION OF STUDY RESULTS:

We assess that the study protocol used by the authors to conduct these assays was adequate, but we cannot verify the interpretation of the results. The highest dose of 2,4-D IPA (500.0 µg/mL) affected the cell morphology (rounding up) in the preliminary toxicity assay. However, this toxicity was not confirmed in UDS assays No. 1 and No. 2. Further, our reviewers have concern over the high cytoplasmic and nuclear grain counts reported. The high counts for the cytoplasmic and nuclear grains make it difficult to determine whether a positive response has been obtained. In fact, these counts are at least threefold higher than expected based on our past experience. As indicated from the reported results, even the positive control could not be accurately assessed because of the excessive nuclear grains. It is our assessment that there may have been technical difficulties with the staining procedures, making it difficult to score the slides. We, therefore, are unable to determine whether the test material was negative for genotoxicity over the dose range tested.

E. QUALITY ASSURANCE MEASURES: A quality assurance statement was signed and dated May 3, 1990. We have concern about this statement because there are apparent errors, including the dose of 555.7 µg/mL cited in the preliminary assay and the lack of concern over high cytoplasmic and nuclear grain counts.

F. CB APPENDIX: Appendix A, Materials and Methods, CBI pp. 9-13; Appendix B, Liquid Chromatographic Analysis of 2,4-D IPA, AGR

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UDS

276461, CBI p. 16.; Appendix C, Quantitative Assessment of the Toxicity of the Test Material. Results of Preliminary Toxicity Assay (CBI p. 17).

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APPENDIX A  
Materials and Methods  
(CBI pp. 9-13)

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2,4-D DER

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Pages 53 through 61 are not included.

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- Identity of product inert ingredients.
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- Description of the product manufacturing process.
- Description of quality control procedures.
- Identity of the source of product ingredients.
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