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 4/29/91
Section II, Toxicology Branch II (HFAS)
Health Effects Division (H7509C)

TO: J.Coombs
Product Manager (23
Reregistration Division

THRU: K.Clark Swentzell, Section Head K. Clark Swentzell 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 IDENTIFICATIONS:

HT Project No. 0-1395 EPA ID # 030053; Record # 264,506;

MRID # 414981-01--- Caswell No. 315 AI
MRID # 414981-02--- Caswell No. 315 AS
MRID # 414981-03--- Caswell No. 315 U

ACTION REQUESTED: Review of data.

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.

2. Evaluation of a Formulation containing 2,4-Dichlorophenoxyacetic Acid Triisopropanolamine 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.

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

Page 1 of 61

Printed on Recycled Paper

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.416981-03

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 (84-4) for other genotoxic effects, Category III, other mutagenic mechanisms.

CONFIDENTIAL BUSINESS INFORMATION
[illegible]
NATIONAL SECURITY INFORMATION (EO 12065)

EPA No.: 68D80056
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: [signature]

Date: April 17 1991

Guideline Series 84: MUTAGENICITY

EPA No.: 68D80056
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(S):

I. Cecil Pfaffenberger, Ph.D.
Principal Reviewer
Dynamac Corporation
Signature: [signature]
Date: 4-16-91

Nancy K. McCarroll, B.S.
Independent Reviewer
Dynamac Corporation
Signature: [signature]
Date: 4-16-91

APPROVED BY:

Nicolas P. Hajjar, Ph.D.
Department Manager
Dynamac Corporation
Signature: [signature]
Date: April 17, 1991

Jean Rawlins, M.S.
EPA Reviewer, Section II
Toxicology Branch II
(H-7509C)
Signature: [signature]
Date: 4/26/91

K. Clark Swentzel
EPA Section Head, Section II
Toxicology Branch II
(H-7509C)
Signature: [signature]
Date: 5/1/91

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 MRID NUMBER: 414931-01.

FORMULA/CAS NUMBER: $C_{14}H_{18}Cl_2O_4$/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 Gollapudi, B.

STUDY NUMBER: TXT:X-007722-013.

REPORT ISSUED: May 10, 1990.

CONCLUSIONS - Executive Summary: Five doses of 2,4-dichlorophenoxyacetic acid butoxyethyl ester (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 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 (Hinze, 1989).[1]

2. Indicator Cells: Primary rat hepatocytes 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 µL/mL was used as the solvent control, and 2-acetylaminofluorene (2-AAF) at 2.333 µg/mL was used as the positive control.

4. Medium: WMEI: William's Medium E, supplemented with 2 mM L-glutamine, 1 µM 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. Hepatocyte harvest/culture preparation: Recovered cells were centrifuged at ≈50 x g for 2 minutes, resuspended in WME+, counted, and aliquoted (0.5×10^5 cells/3 mL WME+) onto plastic coverslips in a series of

[1] Hinze, C.A. Analytical Report Code GT-96-89, H&ES-Texas, Analytical Chemistry Laboratory, The Dow Chemical Company (unpublished).

culture dishes. The cultures were [illegible] humidified, 37°C, 5% incubator for a 4-hour [illegible] period. Nonviable cells were removed by washing [illegible] 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 WME containing 10 µCi/mL [^{3}H]thymidine. Treated monolayers were washed twice with WMEI; one of the four replicates for each treatment group was used to determine cytotoxicity. Those 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[2] and Williams, et al. 1982.[3]

b. Positive response: The assay was considered positive if the increase in the mean net nuclear grain count was ≥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 ≥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 FORMAT:

1. Analytical Determinations: Not presented in this report.

2. UDS Assay: The preliminary toxicity assay qualitative results showed that concentrations of 1167 and 5000 µg/mL were excessively cytotoxic to the hepatocytes, whereas concentrations of 500 and 555.7 µg/mL caused cell rounding without lethality, and 166.7 µg/mL gave slight toxicity. It is not clear to the reviewers whether the 555.7-µg/mL dose was actually used (see Appendix B) or if this was intended to indicate a different concentration, e.g., 259.7, 55.57 or other. Based on these results, a dose range of 5.0 to 500 µg/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-µg/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 500 µg/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 µg/mL). Cytoplasmic grain

[2]Williams, G.M. 1977. Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell cultures. Cancer Res. 37:1845-1851.

[3]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.

TABLE 1: Representative Results of the Unscheduled DNA Synthesis Rat Hepatocyte Assay with 2,4-Dichlorophenoxyacetic Acid Butoxyethyl Ester (2,4-D BEE)

Treatment	Dose/mL	Cells Scored	Average Cytoplasmic Grain Count[a]	Mean Net Nuclear Grain Count[a] ± S.D.	Average Percent Nuclei with ≥5 Grains[a]
Solvent Control					
Dimethyl-sulfoxide	10 µL[b]	100	34.1	-16.6 ± 7.1	--
	10 µL[c]	100	34.1	-13.0 ± 6.2	--
Positive Control					
2-Acetyl-aminofluorene	2.225 µg[b]	50	35.6	66.4 ± 8.0[d]	--
	2.225 µg[c]	50	31.0	69.0 ± 7.3[d]	--
Test Material					
2,4-D BEE	500 µg[b]	100	36.2	-14.6 ± 7.8	--
	500 µg[c]	100	31.5	-14.4 ± 7.0	--
	166.7 µg[b,e]	100	34.9	-15.1 ± 6.9	--
	166.7 µg[c,e]	100	33.6	-13.9 ± 6.5	--

[a] derived from counting cells on duplicate coverslips.

[b] ults from assay No. 1.

[c] ults from assay No. 2.

[d] ults reporting laboratory's criteria for a positive effect; nuclear grains were too high to be accurately ntedly hence nuclear grain counts were considered only good estimates.

[e] ults for the low dose (5.0 µg/mL) and intermediate doses (16.7 and 50 µg/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 2-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. REVIEWER'S 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 BEE (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 555.7 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).

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.

X Sales or other commercial/financial information.

_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

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

008372

DATA EVALUATION RECORD

2,4-Dichlorophenoxyacetic Acid Triisopropanolamine 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 L. McLellan for

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 Triisopropanolamine 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. McCartoll, B.S.
Independent Reviewer
Dynamac Corporation
Signature: Nancy E. McCartoll
Date: 4-16-91

APPROVED BY:

Nicolas P. Hajjar, Ph.D.
Department Manager
Dynamac Corporation
Signature: Nicolas P. Hajjar for
Date: April 17, 1991

Jean Rowland, M.S.
EPA Reviewer, Section II
Toxicology Branch II
(H-7509C)
Signature: Jean Rowland
Date: 9/25/91

K. Clark Swentzel
EPA Section Head, Section II
Toxicology Branch II
(H-7509C)
Signature: K. Clark Swentzel
Date: 9/25/91

009372

DATA EVALUATION RECORD

Tox. Chem. No.:

EPA File Symbol:

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

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

ACCESSION OR MRID NUMBER: 42498I-02.

FORMULA/CAS NUMBER: $C_{17}H_{27}Cl_2NO_5$/18584-79-7.

SPONSOR: DowElanco, Indianapolis, IN.

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

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

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

STUDY NUMBER: TX:K-008866-008

REPORT ISSUED: May 3, 1990

M.N. 24

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.

A. MATERIALS:

1. Test Material:

Name:	2,4-Dichlorophenoxyacetic acid triisopropanolamine salt (2,4-D TIPA)
Description:	Amber liquid
Batch, Lot No.:	AGR 276428
Purity:	72.2% TIPA salt (Formulation in CBI Table 1; s.: Appendix B, Chemical Analysis)
Contaminants:	See (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 344 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.

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 mg/mL (390 to 630 units/mL) collagenase. Livers were removed and disrupted 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 ≈50 x *g* for 2 minutes, resuspended in [illegible], counted, and aliquoted (3×10^6 cells) onto plastic coverslips in a series of culture dishes. The cultures were placed in a humidified, 37°C, CO_2 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 fed 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 viability 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. 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 (2-AAF) for 18 to 20 hours in WMEI containing 10 µCi/mL [^{3}H]thymidine. One of the four replicates for each treatment group was used to determine cytotoxic *p* 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.

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 ≈50 x g for 2 minutes, resuspended in WME+, counted, and aliquoted (5 x 10^5 cells) onto plastic coverslips in a series of culture dishes. The cultures were placed in a humidified, 37°C, CO_2 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 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 µg/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 (2-AAF) for 18 to 20 hours in WMEI containing 10 µCi/mL [^{3}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 ± SD.

4. Evaluation Criteria:

a. Assay validity: Not presented, but assumed by our reviewers to be that of Williams, 1977[a] and Williams, et al. 1982[b].

b. Positive response: The assay was considered positive if the increase in the mean net nuclear grain counts was ≥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 ≥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 µg/mL were excessively cytotoxicity to the hepatocytes, whereas concentrations of 500.0 and 555.7 µg/mL caused cell rounding without lethality, and 166.7 µg/mL gave no toxicity. It is not clear to our reviewers whether the

---

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

[b]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 µg/mL dose was actually used or if this was intended to indicate a different concentration, (e.g., 255.7 or 55.57 µg/mL). Based on these results, a dose range of 5.0 to 500.0 µg/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-µg/mL dose groups in both assays were not appreciably higher than those in the solvent control. The cytotoxicity seen at the 500.0 µg/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 µg/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 µg/mL 2-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 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 µg/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 Triisopropanolamine Salt (2,4-D TIPA)

Treatment	Dose/mL	Cells Scored	Average Cytoplasmic Grain Count[a]	Mean Net Nuclear Grain Count ± S.D.[a]	Average Percent Nuclei with ≥5 Grains[a]
Solvent Control					
Culture Medium	--	100	35.5	-12.4 ± 6.4	--
	--	100	36.5	-11.9 ± 6.4	--
Positive Control					
2-Acetyl-aminofluorene	2.223 μg[b]	50	56.5	49.5 ± 7.3[d]	--
	2.223 μg[c]	50	45.1	55.8 ± 8.8[d]	--
Test Material					
2,4-D TIPA	900.0 μg[b]	100	29.4	-9.2 ± 5.4	--
	900.0 μg[c]	100	34.9	-10.8 ± 6.3	--
	166.7 μg[b,e]	100	40.9	-13.3 ± 7.4	--
	166.7 μg[c,e]	100	42.0	-10.9 ± 6.7	--

[a] Are 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.

[b] Results from assay #1.

[c] Results from assay #2.

[d] Fulfills reporting laboratory's criteria for a positive effect; nuclear grains were too high to be accurately counted; hence, nuclear grain counts were only considered good estimates.

[e] Results for the low dose (9.0 μg/mL) and intermediate doses (16.7 and 90.0 μg/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.

APPENDIX A

Materials and Methods
(CBI pp. 9-13)

008372

MATERIALS AND METHODS

Test Material

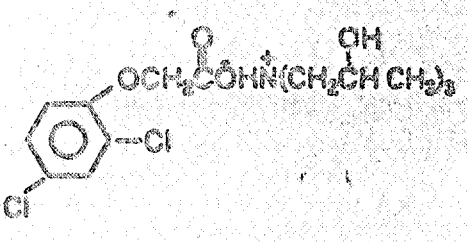
1. Chemical Name: 2,4-dichlorophenoxyacetic acid triisopropanolamine salt

2. Synonym: 2,4-D TIPA

3. C.A.S. Number: 18564-79-7

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).

RD 2465-01

2,4-D DER

Page_____ is not included in this copy.

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.

X Sales or other commercial/financial information.

____ A draft product label.

____ The product confidential statement of formula.

____ Information about a pending registration action.

X 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.: 68D80056
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 J. McLaughlin Jr.

Date: April 17, 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:

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: 6/26/91

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

Signature: K. Clark Swentzel
Date: 7/2/91

DATA EVALUATION RECORD

Tox. 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 MRID NUMBER: 414981-03.

FORMULA/CAS NUMBER: $C_{11}H_{15}Cl_2NO_3$/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 Gollapudi.

STUDY NUMBER: TXT:M-004725-008.

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 isopropylamine 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 µm 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 µg/mL was used as the positive control.

4. Media: WMEI: William's Medium E, supplemented with 2 mM L-glutamine, 1 µM 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. 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 ≈50 x g for 2 minutes, resuspended in WME+, counted, and aliquoted (5×10^5 cells) onto plastic coverslips in a series of culture dishes. The cultures were placed in a humidified, 5% CO_2 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 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, 50.0, 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 control (culture medium), or positive control (2-AAF) for 18 to 20 hours in WMEI containing 10 µCi/mL [^{3}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 ± SD.

4. Evaluation Criteria:

a. Assay validity: Not presented, but assumed by our reviewers to be that of Williams, 1977[1] and Williams, et al. 1982[2].

b. Positive response: The assay was considered positive if the increase in the mean net nuclear grains was ≥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 ≥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 µg/mL were excessively cytotoxic to the hepatocytes, whereas concentrations of [illegible]0.0 and 555.7 µg/mL caused cell rounding without lethality, and 166.7 µg/mL gave no toxicity. It is not clear to our reviewers whether the 555.7 µg/mL dose was actually used or if this was intended to indicate a different concentration (e.g., 255.7 or 55.57 µg/mL). Based on these results, a dose range of 5.0 to 500.0 µg/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 [illegible] and 166.7-µg/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 µg/mL). Cytoplasmic grain counts were high in all test groups,

---

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

[2]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. 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/mL	Cells Scored	Average Cytoplasmic Grain Count[a]	Mean Net Nuclear Grain Count[a] ± S.D.	Average Percent Nuclei with ≥5 Grains[a]
Solvent Control					
Culture medium	--[b]	100	37.6	-13.[illegible] ± 6.6	--
	--[c]	100	38.0	-14.5 ± 7.2	--
Positive Control					
2-Acetyl-aminofluorene	2.283 µg[b]	50	50.5	49.5 ± 7.3[d]	--
	2.335 µg[c]	50	51.0	55.8 ± 8.8[d]	--
Test Material					
2,4-D IPA	500.0 µg[b]	100	38.5	-11.6 ± 7.2	--
	500.0 µg[c]	100	38.7	-11.7 ± 6.7	--
	166.7 µg[b,e]	100	41.2	-13.1 ± 7.4	--
	166.7 µg[c,e]	100	42.3	-16.3 ± 7.5	--

[a] Calculated from 100 cells/slide in the solvent and test groups and 50 cells/slide in the positive control groups. [b] Owing to a technical problem, only one slide/group was scored.

[b] Data from assay No. 1.

[c] Data from assay No. 2.

[d] Cells reporting laboratory's criteria for a positive effect; nuclear grains were too high to be accurately counted; hence, nuclear grain counts were considered only good estimates.

[e] Data for the low dose (5.0 µg/mL) and intermediate doses (16.7 and 50.0 µg/mL) in both assays did not indicate a toxic effect.

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

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

APPENDIX A

Materials and Methods
(CBI pp. 9-13)

RD 2465-01

2,4-D DER

Page____ is not included in this copy.

Pages 53 through 61 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.

X Sales or other commercial/financial information.

____ A draft product label.

____ The product confidential statement of formula.

____ Information about a pending registration action.

X 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.
