4-7-86

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

005039

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Diuron Mutagenicity Data

FROM: Thomas Edwards, Pharmacologist
Hazard Evaluation Division (TS-769)

TO: Robert Taylor, Product Manager (25)
Registration Division (TS-767)

THRU: Clint Skinner, Section Chief
Review Section III

and

Theodore Farber, Chief
Toxicology Branch, HED (TS-769)

4 7 86

Chemical: Diuron

Caswell No.: 410

EPA Registration No.: 352-324

Accession Nos.: 258877

Requested Actions: Review four mutagenicity studies submitted in responce to the Diuron Registration Standard.

Comments:

The mutagenicity study in Salmonella was found to be unacceptable for the reasons stated in the DER.

The other three studies are acceptable. The CHO/HPRT Assay and the in Vitro Unscheduled DNA Synthesis Assay did not indicate mutagenicity. Diuron was shown to be weakly clastogenic.

The Salmonella study must be repeated. Also note other recommendations in the DERs.

751

EPA: 68-02-4225
DYNAMAC No. 1-059A-1
March 14, 1986

005039

DATA EVALUATION RECORD

DIURON

Mutagenicity--Salmonella typhimurium/Mammalian-Microsome Mutagenicity Test

STUDY IDENTIFICATION: Poet, L. B., Arce, G. T., and Sarrif, A. M. Mutagenicity evaluation in Salmonella typhimurium (Unpublished study No. HLR 471-84 prepared and submitted by E.I. du Pont de Nemours and Co., Inc., Newark, DE; dated July, 1985.) Accession No. 258877.

APPROVED BY:

I. Cecil Felkner, Ph.D.
Department Manager
Dynamac Corporation

Signature: Ira Cecil Felkner

Date: 3-14-86

1. CHEMICAL: Diuron; N'-(3,4-dichlorophenyl)-N,N-dimethyl-urea; 3-(3,4-chlorophenyl)-1,1-dimethylurea.

2. TEST MATERIAL: Diuron, IN-14,740-145, N.B. 5103-129, lot No. T50906, batch No. 04, sample No. H-15.580, was described as a tan solid with a purity of 98.19%; the impurities were not identified.

3. STUDY/ACTION TYPE: Mutagenicity--*Salmonella typhimurium*/mammalian-microsome mutagenicity test.

4. STUDY IDENTIFICATION: Poet, L. B., Arce, G. T., and Sarrif, A. M. Mutagenicity evaluation in *Salmonella typhimurium* (Unpublished study No. HLR 471-84 prepared and submitted by E.I. du Pont de Nemours and Co., Inc., Newark, DE; dated July, 1985.) Accession No. 258877.

5. REVIEWED BY:

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

Signature: Nancy E. McCarroll
Date: 3-14-86

Brenda Worthy, M.T.
Independent Reviewer
Dynamac Corporation

Signature: Brenda Worthy
Date: 3-14-86

6. APPROVED BY:

I. Cecil Felkner, Ph.D.
Genetic Toxicology
Technical Quality Control
Dynamac Corporation

Signature: Ira Cecil Felkner
Date: 3-14-86

W. Thomas Edwards
EPA Reviewer

Signature: ______________
Date: ______________

Clint Skinner, Ph.D.
EPA Section Head

Signature: ______________
Date: ______________

7. CONCLUSIONS:

A. Under the conditions of this assay, five doses of diuron without S9 activation (0.5, 1, 2.5, 5, and 10 µg/plate) and five doses of diuron with S9 activation (10, 25, 50, 100, and 250 µg/plate) elicited neither a cytotoxic nor a mutagenic effect in two independent Salmonella/mammalian-microsome mutagenicity assays. Since cytotoxicity was not achieved in the mutation assay and the preliminary range-finding study indicated that a wide range of nonprecipitating cytotoxic doses were available, we conclude that diuron was not adequately tested.

B. The study is unacceptable.

8. RECOMMENDATIONS: The assay should be repeated with a dose range for the test material that includes a cytotoxic level to ensure that conditions are optimal for the detection of genotoxicity. Additionally, a QA/GLP statement is required.

Items 9 and 10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):

A. Materials and Methods: (See Appendix A for details.)

1. Test Material: Diuron, IN-14,740-145, N.B. 5103-129, lot No. T50906, batch No. 04, sample No. H-15,580, was described as a tan solid with a reported purity of 98.19%; the impurities were not reported. Stability, storage conditions, or other characteristics that define the test material were not reported. The authors stated, however, that the test material was assumed stable during the conduct of the assay. Based on solubility information furnished with the test material, dimethylsulfoxide (DMSO) was chosen as the solvent; the source of this information was not specified.

2. Bacterial Strains: S. typhimurium strains TA1535, TA97, TA98, and TA100 were obtained from B. Ames, Berkeley, CA. Strain maintenance and culture preparation for the assay were not reported. It was assumed, however, that the strains were maintained and prepared for the assay as described by Ames et al.[2] since the authors reported that the assay was performed in a manner similar to this cited reference.

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[1] Only items appropriate to this DER have been included.

[2] Ames, B.N., J. McCann, and E. Yamasaki. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat. Res. 31 (1975): 347-364.

3. Metabolic Activation: The S9 fraction used for metabolic activation was derived from the livers of 8- to 9-week-old male Charles River CD rats induced with Aroclor 1254. Preparation and storage were assumed by the reviewers to comply with the method of Ames et al.[3] The S9 mix contained 1.6 mg of protein/mL; based upon the reviewers' assumption that the protein concentration of the S9 was approximately 40 mg/mL,[4] we calculated that the S9 mix contained approximately 4% S9 protein.

4. Cytotoxicity Assay: A cytotoxicity assay was conducted in the presence and absence of S9 activation with strain TA1535. To tubes containing 0.1 mL of an overnight broth culture of *S. typhimurium* TA1535, adjusted to contain 10^3 bacteria in 2 mL of top agar (0.6% agar, 0.6% NaCl, 0.05 mM biotin, and 0.15 mM L-histidine), were added 0.1 mL of the solvent or an unspecified number of dilutions of the test material. For the S9-activated cytotoxicity test, 0.5 mL of the S9 mix was added to the reaction mixture. The contents of each tube were mixed and poured over Davis minimal agar plates. Plates were incubated at 37°C for 48 hours, and revertant colonies were counted. Based on the findings of the cytotoxicity assay, the authors stated, "Concentrations of the test sample that were nontoxic and, if possible, slightly toxic were selected for the mutagenesis assay."

5. Mutagenicity Assay: The mutagenicity assay was conducted as described for the cytotoxicity assay with the following exceptions:

   a. The four tester strains were exposed to the test material at a cell density of 10^8 bacteria per plate.

   b. The amount of histidine in the top agar was reduced to trace levels (0.05 mM L-histidine).

   c. The appropriate direct-acting or promutagenic positive controls for each strain were included.

6. Statistical Analysis: The data were analyzed for significance by the method of Snee and Irr[5] using the power transformation $y=x^{0.2}$.

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[3] Ibid.

[4] Maron, D. M. and B. Ames. Revised methods for the *Salmonella* mutagenicity test. *Mutat. Res.* 113(1983): 173-215.

[5] Snee, R. D. and J. D. Irr. Design of a statistical method for the analysis of mutagenesis at the hypoxanthine guanine phosphoribosyl transferase locus of cultured Chinese hamster ovary cells. *Mutat. Res.* 85 (1981): 77-93.

7. Evaluation Criteria: The assay was considered positive if a) the probability was < 0.01 that the number o' revertants at each of the test sample concentrations was not greater than the number of revertants in the solvent control and b) the probability was < 0.01 that there was not a positive correlation between the number of revertants and increasing concentrations of the test sample.

B. Protocol: A protocol was not provided.

12. REPORTED RESULTS:

A. Cytotoxicity Assay:

The cytotoxicity assay was conducted with 10, 50, 100, 500, 1,000, and 5,000 μg/plate of the test material in the presence and absence of S9 activation using strain TA1535 as the indicator. Nonactivated results from duplicated plates indicated that the test material precipitated at 5,000 μg/plate, and no colonies were recovered at doses ranging from 5,000 down to 50 μg/plate. At the remaining dose, 10 μg/plate, an approximately 50% survival of the bacterial strain was reported.

In the presence of S9 activation, precipitation was observed at 5,000 μg/plate and both the 1,000- and 5,000-μg/plate levels were lethal. Approximately 40% of the cells survived treatment with 500 μg/plate; below this concentration, > 85% cell recovery was reported.

B. Mutagenesis Assay

Two independent experiments were performed with the test material in the presence and absence of S9 activation. Based on the cytotoxicity assay results, the nonactivated assays were conducted with 0.5, 1, 2.5, 5, and 10 μg/plate of diuron; under S9-activated conditions, 10, 25, 50, 100, and 250 μg/plate were assayed.

Results of the first experiment indicated that at the dose range selected for either the nonactivated or S9-activated assay, no definitive cytotoxicity or appreciable increase in histidine revertants of any strain occurred. Representative results from the initial assay are presented in Table 1.

Although a decrease in His^+ colonies of TA98 was noted at the 250-μg/plate S9-activated dose in the second assay, the overall results were similar to the first assay. As shown in Table 2, no definitive evidence of cytotoxicity or increase in His^+ colonies of any strain accompanied exposure to the five selected nonactivated or S9-activated doses.

TABLE I. Representative Results of the First Salmonella typhimurium Mutagenicity Assay with Diuron

Substance	S9 Activation	Dose (µg/plate)	Revertants per Plate of Bacterial Tester Strain[a] TA1535	TA97	TA98	TA100
Solvent Control						
Dimethylsulfoxide	-	-	19	120	26.5	119
	+	-	12	142	37	130.5
Positive Controls						
N-methyl-N'-nitro-N-nitrosoguanidine	-	4	3821	-	-	4017.5
9-aminoacridine	-	50	-	634.5	-	-
2-nitrofluorene	-	25	-	-	2602	-
2-aminoanthracene	+	1	-	1098	-	1320
	+	2	191.5	-	3091	-
Test Material						
Diuron	-	10[b]	15.5	125	25	109
	+	250[b]	12.5	134	29.5	116

[a] Average of duplicate plates; calculated by our reviewers.

[b] Highest dose tested with +/-S9; revertant counts for lower doses (5, 2.5, 1, and 0.5 µg/plate/-S9 and 100, 50, 25 and 10 µg/plate/+S9) were comparable to the solvent control and, therefore, were not selected as representative.

TABLE 2. Representative Results of the Second Salmonella typhimurium Mutagenicity Assay with Diuron

Substance	S9 Activation	Dose (µg/plate)	Revertants per Plate of Bacterial Tester Strain[a] TA1535	TA97	TA98	TA100
Solvent Control						
Dimethylsulfoxide	-	-	21.5	117.5	23.5	123
	+	-	12.0	148.5	44	130
Positive Controls						
N-methyl-N'-nitro-N-nitrosoguanidine	-	4	3000.5	-	-	3160
9-aminoacridine	-	50	-	492.5	-	-
2-nitrofluorene	-	25	-	-	2664.5	-
2-aminoanthracene	+	1	-	1028	-	1524
	+	2	215	-	2805	-
Test Material						
Diuron	-	10[b]	19.5	117	21.5	130.5
	+	250[b]	13.5	143	19.0	136

[a] Average of duplicate plates; calculated by our reviewers.

[b] Highest dose tested with +/-S9; revertant counts for lower doses (5, 2.5, 1, and 0.5 µg/plate/-S9 and 100, 50, 25 and 10 µg/plate/+S9) were comparable to the solvent control and, therefore, were not selected as representative.

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. The authors concluded, "Diuron was nonmutagenic when tested in *Salmonella typhimurium* strains TA1535, TA97, TA98 and TA100 by the protocol described in Methods."

B. A quality assurance statement was not provided.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

We assess that the study was executed in an appropriate manner and within the confines of this study, the data were interpreted correctly. It is commendable that the authors used *S. typhimurium* TA97 in these assays. This plasmid-bearing derivative of TA1537 is now recommended by Maron and Ames[6] as the replacement for TA1537. However, the study was flawed by the lack of a cytotoxic effect at the highest dose assayed in both the nonactivated and S9-activated test. Cytotoxicity was clearly revealed over a wide range of non-precipitating doses in the cytotoxicity assay; the authors, therefore, should have had no difficulties in selecting an appropriate starting concentration.

Since it is generally agreed that mutagenicity and cytotoxicity are closely related, we assess that the dose range of diuron evaluated in this study is inadequate to support the authors' conclusions. The study should be repeated at higher test concentrations to ensure that cytotoxicity is achieved, hence assuring that conditions are optimal for the detection of mutagenic events.

The revertant counts for TA1535 and TA100 exposed to 4 µg/plate N-methyl-N'-nitro-N-nitrosoguanidine appeared extremely high. We assume that the study authors used an electronic colony counting device; the upper accuracy limit for most conventional electronic colony counters generally does not exceed 3,000 colonies. It is possible that a correction factor was used; if so, it should have been reported. Nevertheless, the ability of these two strains to detect a mutagen that causes nonactivated base-pair substitution was demonstrated.

Item 15--see footnote 1.

16. CBI APPENDIX: Appendix A, Material and Methods (Protocol), CBI pp. 2-3.

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[6] Maron, D. M. and Ames, B. (1983): 173-215.

APPENDIX A

Materials and Methods

DIURON SCIENTIFIC REVIEWS

Page ______ is not included in this copy.

Pages __11__ through __12__ are not included in this copy.

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 product 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 (EO 12065)

EPA: 68-02-4225
DYNAMAC No. 1-059A-2
March 5, 1986

DATA EVALUATION RECORD

DIURON

Mutagenicity--CHO/HGPRT Forward Gene Mutation Assay

STUDY IDENTIFICATION: Rickard, L. B., Ullman, D. V., Choy, W. N., and Fisher, R. L. Mutagenicity evaluation of diuron in the CHO/HGPRT assay. (Unpublished study No. HLR 282-85 prepared and submitted by E. I. duPont de Nemours and Co., Inc., Newark, DE; dated June 28, 1985.) Accession No. 258877.

APPROVED BY:

I. Cecil Felkner, Ph.D.
Department Manager
Dynamac Corporation

Signature: I. Cecil Felkner

Date: 3-5-86

1. CHEMICAL: Diuron; N'-(3,4-dichlorophenyl)-N,N-dimethyl-urea; 3-(3,4-chlorophenyl)-1,1-dimethylurea.

2. TEST MATERIAL: Diuron, IN-14,740-145, N.B. 5103-129, lot No. T50906, batch No. 04, sample No. H-15,580, was described as a tan solid with a purity of 98.19%; the impurities were not identified.

3. STUDY/ACTION TYPE: Mutagenicity--CHO/HGPRT forward gene mutation assay.

4. STUDY IDENTIFICATION: Rickard, L. B., Ullman, D. V., Choy, W. N., and Fisher, R. L. Mutagenicity evaluation of diuron in the CHO/HGPRT assay. (Unpublished study No. HLR 282-85 prepared and submitted by E. I. duPont de Nemours and Co., Inc., Newark, DE; dated June 28, 1985.) Accession No. 258877.

5. REVIEWED BY:

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

Signature: Nancy E. McCarroll
Date: 3-5-86

Brenda Worthy, M.T.
Independent Reviewer
Dynamac Corporation

Signature: Brenda Worthy
Date: 3-5-86

6. APPROVED BY:

I. Cecil Felkner, Ph.D.
Genetic Toxicology
Technical Quality Control
Dynamac Corporation

Signature: Ira Cecil Felkner
Date: 3-5-86

W. Thomas Edwards
EPA Reviewer

Signature: ____________
Date: ____________

Clint Skinner, Ph.D.
EPA Section Head

Signature: ____________
Date: ____________

7. CONCLUSIONS:

A. Under the conditions of the nonactivated assay, no significant increase in the mutation frequency (MF) of Chinese hamster ovary (CHO) cells at the HGPRT locus resulted from exposure to five doses of Diuron (0.01, 0.5, 1.0, 1.125, and 1.250 mM) which included a marginally acceptable cytotoxic upper dose. Additionally, the cloning efficiencies (CE) for the solvent control were barely acceptable.

In the presence of S9 activation, doses ranging from 0.05 to 0.75 mM diuron elicited a definitive cytotoxic response (0.5 and 0.75 mM), but did not induce a mutagenic effect.

B. The nonactivated assay is considered marginally acceptable and the S9 activated assay is acceptable.

8. RECOMMENDATIONS: It is recommended that future assays be performed under rigorously controlled conditions with a more suitable cell population to achieve higher cloning efficiencies for the control cultures and ensure results that can be unequivocally interpreted. A statement of compliance with QA/GLP should be included in all future reports.

Items 9 and 10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):

A. Materials and Methods: (See Appendix A for details.)

1. Test Material:

a. Description: Diuron, IN-14,740-145, N.B. 5103-129, lot No. T50906, batch No. 04, sample No. H-15,580, was described as a tan solid with a purity of 98.19%; tne impurities were not identified. Stability, storage conditions, or other characteristics that define the test material were not reported. The authors stated, however, that diuron was assumed to be stable under the conditions of administration.

b. Solvent Selection: Solubility of the test material was determined in phosphate-buffered saline, ethanol, dimethylsulfoxide (DMSO), and acetone. Although the specifics of the solubility determinations were not provided,

[1] Only items appropriate to this DER have been included.

the authors stated that, "DMSO was used as the solvent in this test. The highest concentration of chemical which did not lead to excessive precipitate formation in the treatment medium was the maximum concentration tested in the preliminary cytotoxicity experiments."

2. Indicator Cells: Chinese hamster ovary (CHO) cells, clone CHO-K1-BH3, were obtained from Dr. Abraham Hsie, Oak Ridge National Laboratory, and were maintained as monolayers in Ham's F12 containing 5% fetal bovine serum (F12) without hypoxanthine. Monolayers were incubated at 37±1.5°C in a humidified atmosphere containing 5±1% CO_2; cells were collected by trypsinization.

3. Metabolic Activation: The S9 microsomal fraction used for metabolic activation was derived from the livers of 8- to 9-week-old male CrL:CD(SD)BR rats injected ip with 500 mg/kg of Aroclor 1254. The concentration of protein and cytochrome P-450 was determined following preparation of the hepatic microsomal fraction. The concentration of S9 in treatments with activation was 1 mg protein/mL.

4. Preliminary Cytotoxicity Assay: Preliminary cytotoxicity studies were performed with and without S9 activation. However, neither the methods nor the concentrations of the test material used in the cytotoxicity assay were reported. Based on these results, concentrations were selected for the mutation studies. The authors stated, "Ideally, the highest concentration of test chemical used should give about 10% survival as compared to the control."

5. Mutagenicity Assay:

   a. Exposure: Seeded cultures (5×10^5 cells/25-cm^2 flasks) were incubated overnight, culture medium was removed, and flasks were randomly assigned to treatment groups. For the nonactivated assay, duplicate flasks of cells received 3 mL of F12 treatment medium containing 30 µL of the appropriate solution of the test material, solvent (DMSO), or positive control, 0.5 mM ethylmethanesulfonate (EMS). The nonactivated exposure lasted for 18-20 hours. In the presence of S9 activation, cells were exposed for 5 hours to the appropriate levels of the test material, solvent, or positive control, 0.015 mM 9,10-dimethyl-1,2-benzanthracene (DMBA).

   b. Posttreatment Cytotoxicity Assessment: At the conclusion of the exposure, cells were washed with F12; cells exposed in the absence of S9 activation were subcultured immediately and cells treated with S9 were reincubated for 21-25 hours prior to subculture. At the appropriate subculture interval, cytotoxicity was assessed by plating

200 cells/60-mm dishes (six dishes) for each treatment. Following 7 days of incubation, cells were stained and counted, and cell survival was determined.

c. Cell Recovery/Mutant Expression: Expression of 6-thioguanine-resistant mutants (6-TG^r) was accomplished by initially plating 1 x 10^6/100-mm dish (one dish) and allowing a 7-day recovery period. Recovered cells were plated for survival as described in the posttreatment cytotoxicity assessment. Mutant cells were selected by plating 2 x 10^5 cells/100-mm dishes (five dishes) in media containing 1 x 10^5 M 6-TG. Following a 7-day incubation, colonies were stained and counted. MF was expressed as the number of 6-TG^r colonies/10^6 surviving cells at the time of mutant selection.

6. Statistical Analysis: Data were analyzed for significance at p = 0.01 in accordance with the method of Snee and Irr.[2]

7. Evaluation Criteria:

a. Assay Validity: The assay was considered acceptable if a) the CE of the solvent control was between 42 and 93% and b) the average spontaneous MF was between 0 and 45 mutants/10^6 cells. The report indicated that these values were derived from the results of 20 experiments obtained over a 2-year period.

b. Positive Response: The assay was considered positive if a) the MF of one or more of the sample concentrations was significantly greater than that of the solvent control, where significance was judged at the 0.01 level, and b) the correlation between MF and the concentration of the test sample was significantly greater than 0, where significance was judged at the 0.01 level.

B. Protocol: A protocol was not provided.

12. REPORTED RESULTS:

A. Preliminary Cytotoxicity Assay: The details of the preliminary cytotoxicity assay were not reported. However, the authors indicated that 10% survival was achieved at 1.25 mM (-S9) and at 0.75 mM (+S9). Based on these results, 1.25 mM was selected as the maximum concentration for the nonactivated assays; 0.75 mM was used as the starting concentration for the S9-activated tests.

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[2] Snee, R. D. and J. D. Irr. Design of a statistical method for the analysis of mutagenesis at the hypoxanthine-guanine phosphoribosyl transferase locus of cultural Chinese hamster ovary cells. Mutat. Res. 85(1981): 77-93.

B. Mutation Assays:

1. Nonactivated Assays: Three assays were conducted with 0.01, 0.5, 1.0, 1.125, and 1.250 mM concentrations of diuron in the absence of S9 activation. As shown in Table 1, the results from trials 1 and 3 compare favorably; a moderate cytotoxic effect, as indicated by 23.6 and 39.3% cell survivals, respectively, was found after exposure to 1.25 mM diuron. At the remaining doses, little or no evidence of cytotoxicity was noted. No appreciable increase in MFs accompanied exposure of the CHO cells to the five selected doses of diuron in trials 1 and 3.

   Also presented in Table 1 are the results of trial 2. As shown, the CE for the solvent control was low (49.5), a pronounced cytotoxic effect was reported at 1.25 mM diuron, and a dose-related increase in MF was observed. However, it is our assessment that due to the low CE for the solvent control and the lack of agreement with the findings from Trials 1 and 3 these results were invalid. The authors reported, "Statistical analysis of all three trials indicated no significant increase in the mutant frequencies at any concentration tested and no positive dose-response."

2. S9-Activated Assays: Two independent assays were conducted with five doses of the test material in the presence of S9 activation. Concentrations used in trial 1 were 0.05, 0.1, 0.25, 0.5, and 0.75 mM. No cells survived treatment with 0.5 and 0.75 mM diuron; at 0.25 mM, a moderate cytotoxic effect was observed. The remaining doses were not cytotoxic, and no statistically significant increases were observed in the MFs at the doses used for mutant selection.

   Due to the extreme cytotoxicity at the two highest concentrations in trial 1, the starting dose for the second assay was lowered. Levels assayed in trial 2 were 0.05, 0.1, 0.2, 0.25, and 0.5 mM diuron. Marked cytotoxicity (15% cell survival) was observed at 0.5 mM, and a moderate cytotoxic effect was reported at 0.25 mM diuron. The remaining doses were not cytotoxic, and no significant increase in MFs resulted from exposure of CHO cells to the five S9-activated doses of the test material.

   Representative data from both S9-activated trials are presented in Table 2.

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. The authors concluded, "Diuron was negative in the CHO/HGPRT Gene Mutation Assay both with and without S9 activation."

B. A quality assurance statement was not present.

TABLE 1. Representative Results of the CHO Forward Mutation Assay with Diuron in the Absence of S9 Activation 005039

Substance	Trial	Dose (mM)	% Cell Survival[a,b] Post-exposure	% Cloning Efficiency[a,c]	No. of Mutants[a]	Mutation Frequency[a,d] $\times 10^{-6}$
Solvent Control						
DMSO	1	0	100	62.2	0	0
	2	0	100	49.5	3.5	7.0
	3	0	100	59.0	13.0	22.0
Positive Control						
Ethylmethane-sulfonate	1	0.5	58.3	53.2	81.0	152.0
	2		31.3	42.1	95.0	225.7
	3		17.1	53.8	90.0	167.3
Test Material						
Diuron[e]	1	0.5	103.0	66.3	0	0
	2		88.6	45.5	10.0	22.0
	3		60.7	66.6	3.5	5.3
	1	1.0	57.9	52.7	1.5	2.9
	2		72.8	50.0	12.5	25.0
	3		64.6	61.1	2.0	3.3
	1	1.125	70.1	63.9	3.0	4.7
	2		34.7	43.4	22.0	50.7
	3		79.0	64.6	6.5	10.1
	1	1.250[f]	23.6	52.5	0	0
	2		8.4	0.0	0	0
	3		39.3	61.1	9.0	14.7

[a]Average of duplicate values; calculated by our reviewers.

[b]$\% \text{ Cell Survival Postexposure} = \dfrac{\text{No. of colonies on nonselective plates/dose level}}{\text{No. of colonies on nonselective plates/solvent control}} \times 100$

[c]$\% \text{ Cloning Efficiency} = \dfrac{\text{No. of colonies on nonselective plates}}{\text{No. of cells plated}} \times 100$

[d]$\text{Mutation Frequency} = \dfrac{\text{No. of mutants}}{\text{Cloning efficiency}} \times 100$

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[e]Results for lowest dose tested (0.01 mM) were comparable to the control value in all trials.

[f]Highest dose assayed.

TABLE 2. Representative Results of the CHO Forward Mutation Assay with Diuron in the Presence of S9 Activation

Substance	Trial	Dose (mM)	% Cell Survival[a,b] Post-exposure	% Cloning Efficiency[a,c]	No. of Mutants[a]	Mutation Frequency[a,d] $\times 10^{-6}$
Solvent Control						
DMSO	1	0	100	81.1	9.5	11.7
	2	0	100	67.2	1.5	2.2
Positive Control						
9,10-dimethyl-1,2-benzanthracene	1	0.015	76.9	69.6	91.0	130.8
	2		102.0	68.8	57.5	83.6
Test Material						
Diuron	1	0.25[e]	25.1	75.5	1.0	1.3
	2		44.9	77.9	1.5	1.9

[a] Average of duplicate values; calculated by our reviewers.

[b] $$\text{\% Cell Survival Postexposure} = \frac{\text{No. of colonies on nonselective plates/dose level}}{\text{No. of colonies on nonselective plates/solvent control}} \times 100$$

[c] $$\text{\% Cloning Efficiency} = \frac{\text{No. of colonies on nonselective plates}}{\text{No. of cells plated}} \times 100$$

[d] $$\text{Mutation Frequency} = \frac{\text{No. of mutants}}{\text{Cloning efficiency}} \times 100$$

[e] Highest marginally cytotoxic dose; lower doses (0.1 and 0.05 mM in trial 1 and 0.2, 0.1, an 0.05 mM in trial 2) were comparable to control values and were, therefore, not selected a representative.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

The dose-related increases in MF and severe cytotoxicity observed in the second nonactivated assay were not reproducible; the data from this assay conflicted with the findings from the first and third trials. Hence, we conclude that the results were invalid and did not constitute evidence of mutagenicity. The highest dose tested in the two other nonactivated assays (first and third assays) may not have been sufficiently cytotoxic to ensure that the test material was adequately evaluated. However, there was no apparent increase in mutant colonies at 1.25 mM Diuron, hence we assess that this level was marginally acceptable as the starting concentration.

The CEs for the solvent control in trials 1 and 3 were also marginally acceptable; however, CE for trial 2 (49.5%) was low and unacceptable. Although we agree that the CEs for trials 1 and 3 (62.2 and 59.0%) were within the range considered acceptable both by the reporting laboratory and O'Neill et al.,[3] it is our assessment that the CE of healthy cells exposed to DMSO should approach approximately 70% recovery.

The S9-activated assays were well conducted; cytotoxicity was clearly demonstrated at 0.5 and 0.75 mM diuron, and test MFs were comparable to the solvent control.

The sensitivity of the test system to detect mutagenic activity within the concentration range of the test material was adequately shown with 0.5 mM EMS (-S9) and 0.015 mM DMBA (+S9).

Item 15--see footnote 1.

16. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 4-7.

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[3] O'Neill, J. P., P. A. Brimer, R. Machanoff, G. P. Kirsch, and A. W. Hsie. A quantitative assay of mutation induction at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells (CHO/HGPRT system): Development and definition of the system. Mutat. Res. 45(1977): 91-101.

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APPENDIX A

Materials and Methods

DIURON SCIENTIFIC REVIEWS

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Pages __23__ through __26__ are not included in this copy.

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___ Identity of the source of product ingredients

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NATIONAL SECURITY INFORMATION (EO 12065)

EPA: 68-02-4225
DYNAMAC No. 1-059A-3
March 14, 1986

DATA EVALUATION RECORD

DIURON

Mutagenicity--Unscheduled DNA Synthesis in Primary Rat Hepatocytes

STUDY IDENTIFICATION: Arce, G. T. and Sarrif, A. M. Assessment of diuron in the *in vitro* unscheduled DNA synthesis assay in primary rat hepatocytes. (Unpublished study No. HLR 349-85 prepared and submitted by E. I. duPont de Nemours and Co., Inc., Newark, DE; dated July 10, 1985.) Accession No. 258877.

APPROVED BY:

I. Cecil Felkner, Ph.D.
Department Manager
Dynamac Corporation

Signature: Ira Cecil Felkner

Date: 3-14-86

1. CHEMICAL: Diuron; N'-(3,4-dichlorophenyl)-N,N-dimethyl-urea; 3-(3,4-chlorophenyl)-1,1-dimethylurea.

2. TEST MATERIAL: Diuron, IN-14,740-145, N.B. 5103-129, lot No. T50906, batch No. 04, sample No. H-15,580, was described as a tan solid with a purity of 98.19%; the impurities were not identified.

3. STUDY/ACTION TYPE: Mutagenicity--Unscheduled DNA synthesis in primary rat hepatocytes.

4. STUDY IDENTIFICATION: Arce, G. T. and Sarrif, A. M. Assessment of diuron in the *in vitro* unscheduled DNA synthesis assay in primary rat hepatocytes. (Unpublished study No. HLR 349-85 prepared and submitted by E. I. duPont de Nemours and Co., Inc., Newark, DE; dated July 10, 1985.) Accession No. 258877.

5. REVIEWED BY:

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

Signature: Nancy E McCarroll
Date: 3-14-86

Brenda Worthy, M.T.
Independent Reviewer
Dynamac Corporation

Signature: Brenda Worthy
Date: 3-14-86

6. APPROVED BY:

I. Cecil Felkner, Ph.D.
Genetic Toxicology
Technical Quality Control
Dynamac Corporation

Signature: Ira Cecil Felkner
Date: 3-14-86

W. Thomas Edwards
EPA Reviewer

Signature: ______
Date: ______

Clint Skinner, Ph.D.
EPA Section Head

Signature: ______
Date: ______

7. CONCLUSIONS:

A. Under the conditions of two independent assays for unscheduled DNA synthesis (UDS) in primary rat hepatocytes, 0.001, 0.01, 0.1, 0.33, 1.0, and 20.0 mM diuron did not induce a significant increase in UDS. Although statistically significant increases in net nuclear grain counts were demonstrated in both assays at 0.33, 1.0, and 20.0 mM diuron, these increases occurred at cytotoxic levels. Similarly, depressed average nuclear grain counts and cytoplasmic grain counts were observed at these levels. We conclude that the positive effect observed at these doses was artifactual and was caused by cytotoxicity rather than genotoxicity.

B. The study is acceptable.

8. RECOMMENDATIONS: It is recommended that future UDS assays be conducted with a dose range that includes as the highest dose a concentration that exhibits a cytotoxic response. The remaining doses, covering at least a 2-log range, should be noncytotoxic. It is further recommended that a QA/GLP statement be included in future reports.

Items 9 and 10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):

A. Materials and Methods: (See Appendix A for details.)

1. Test Material:

a. Description: Diuron, IN-14,740-145, N.B. 5103-129, lot No. T50906, batch No. 04, sample No. H-15,580, was described as a tan solid with a reported purity of 98.19%; the impurities were not reported. Test material stability, storage conditions, or other characteristics were not reported. The authors stated that they assumed diuron was stable under the conditions of administration.

b. Solvent Selection: Solubility of the test material was determined in phosphate-buffered saline, ethanol, dimethylsulfoxide (DMSO), and acetone. Although the specifics of the solubility determinations were not provided, the authors stated that, "DMSO was found to be the most appropriate solvent and the highest concentration of diuron that dissolved in DMSO (1 M) was used in the assay."

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[1] Only items appropriate to this DER have been included.

2. Indicator Cells: Hepatocytes were harvested from an unspecified number of male CrL:CD(SD)BR rats obtained from Charles River, Kingston, NY. No further animal information was provided.

3. Cell Preparation:

   a. Hepatocyte Isolation: Rats were anesthetized with sodium pentobarbital (65 mg/kg, i.p.) and livers were perfused with Hanks' buffered salt solution, pH 7.35, and William's Medium E (WME) containing L-glutamine (292 mg/L), gentamicin (50 μg/mL), and collagenase (100 units/mL, type IV), buffered to pH 7.35. The length of time each liver was perfused with both solutions was not reported. Livers were excised, cleaned of extraneous tissue, placed in a sterile dish containing the collagenase perfusion solution, and combed to release the hepatocytes.

   b. Hepatocyte Harvest/Primary Culture Preparation: Recovered cells were collected by centrifugation, resuspended in complete WME minus collagenase, and filtered. Cell viability and density were determined by trypan blue exclusion. Multi-well culture plates, containing 2 mL of complete WME and a coverslip in each well, were inoculated with 5×10^5 viable hepatocytes/well. The cultures were placed in a humidified, 37±1.5 °C, 5±1% CO_2 incubator for a 2-hour attachment period. Cultures were washed with WME and refed with 2.0 mL treatment medium (WME containing L-glutamine, gentamicin, and 5 μCi/mL [^{3}H]thymidine).

4. UDS Assay:

   a. Treatment/Slide Preparation: Cytotoxicity was assessed in conjunction with the UDS assay. The UDS assay was conducted with an unspecified number of doses of the test material; the dilution factor(s) separating each dose level was similarly not indicated in the section on materials and methods in the report. Four to six prepared hepatocyte cultures were exposed for 18 hours to stock solutions and dilutions of the test material or positive control, dimethylbenzanthracene (DMBA), and the solvent control (DMSO). Cytotoxicity was assessed by measuring the levels of lactate dehydrogenase (LDH) activity in the treatment medium.[2] Coverslips were rinsed with WME, and the washed hepatocytes that were attached to the coverslips were treated with 1% sodium citrate for 15 minutes. Cells were fixed using three changes of ethanol: glacial acetic acid (3:1), rinsed, dried, and mounted.

[2] Technical Bulletin No. 226-UV, 8/82, Sigma Chemical Co.

b. Preparation of Autoradiographs/Grain Development: Slides were dipped into Kodak NT82 emulsion, dried for 2 hours, and stored at 4°C in desiccated slide boxes for 3 days. Slides were developed (developer not reported), stained with methyl-green pyronin Y (concentration and methodology not reported), coded, and counted.

c. Grain Counting: A maximum of 100 morphologically normal cells (25/slide) for each test dose, negative, and positive control group were scored for incorporation of tritiated thymidine into DNA. Net nuclear grain counts were determined by subtracting the nuclear grain count of each cell from the highest cytoplasmic grain count of the nuclear-sized areas adjacent to each nucleus. The net grains for each slide were averaged; slides with <25 cells were scored, but were not included in the data analysis.

5. Statistical Analysis: The difference between each concentration of the test material and the DMSO control, dose-response relationships, and differences in response between trials were analyzed in a two-variable analysis of variance. Linear and higher-order effects were evaluated by an F-test. If a dose-trend interaction was significant at $p < 0.01$, each trial was analyzed separately and further assessed in an analysis of covariance. Reported statistical references were Snedecor and Cochran,[3] Snee and Irr,[4] and Steel and Torrie.[5]

6. Evaluation Criteria: The assay was considered positive if a) an average increase of $\geq$ 5 net grains was seen at one or more test doses and the increases was statistically significant at $p < 0.01$ and b) the probability was <0.01 that there was not a positive correlation between average net grains and increasing test doses.

B. Protocol: A protocol was not presented.

---

[3] Snedecor, G. W. and W. G. Cochran, in: Statistical Methods, 7th Edition, Iowa State University Press, Ames, Iowa, 1980.

[4] Snee, R. D. and J. D. Irr, Design of a statistical method for the analysis of mutagenesis at the hypoxanthine-guanine phosphoribosyl transferase locus of cultural Chinese hamster ovary cells. Mutat. Res. 85(1981): 77-93.

[5] Steel, R. G. D. and J. H. Torrie, in: Principles and Procedures of Statistics, McGraw-Hill, 1980.

12. REPORTED RESULTS:

A. Cytotoxicity Assessment Results: Two independent UDS assays were conducted with 10 doses of the test material (0.001, 0.0033, 0.01, 0.033, 0.1, 0.33, 1.0, 3.33, 10.0, and 20.0 mM), three concentrations of DMBA (0.1, 0.5, and 1.0 mM), and the solvent control (DMSO). The cytotoxicity assessment was performed concurrent with the UDS assay.

Precipitation of the test material was reported for concentrations between 1 and 20 mM of the test material in both trials. The LDH activity of quadruplicate treatment medium samples from each test dose for both assays was measured as the cytotoxicity endpoint. Elevated LDH activity, indicative of cytotoxicity, was observed in a dose-related manner at test levels ranging from 0.033 to 1.0 mM in both tests. Peak enzymatic activity occurred at 1.0 mM in Trial 1 and at 0.33 in Trial 2; above this concentration, enzyme activity decreased, but remained higher than the DMSO control values for both studies.

A dose-related increase in LDH activity accompanied exposure to the increasing doses of DMBA.

Six test doses (0.001, 0.01, 0.1, 0.33, 1.0, and 20 mM) were selected for slide analysis. The rationale for dose selection was not reported; two doses (1.0 and 20 mM) exceeded the solubility limit and four doses (0.1, 0.33, 1.0, and 20 mM) were cytotoxic. Slides containing cells exposed to 0.1 mM DMBA and the solvent control were also analyzed.

B. UDS Assay Results:

Trial 1: A statistically significant and dose-dependent increase in net nuclear grain counts was observed at 0.33, 1, and 20 mM of the test material. However, the average nuclear grain counts and average cytoplasmic grain counts for all test doses were lower than the control values. Average nuclear and cytoplasmic grain counts were severely depressed at the three doses for which significant increases in net nuclear grain counts were reported. At these levels marked increases in LDH activity, indicative of severe cytotoxicity, were also observed. The authors reported that significant decreases and a significant negative dose response for cytoplasmic grain counts occurred at 0.33, 1, and 20 mM of the test material when compared to the control. At the remaining marginally cytotoxic or noncytotoxic doses (0.1, 0.01, and 0.001 mM), no significant increase in net nuclear grain counts or significant decrease in cytoplasmic grain counts were noted. Representative results are presented in Table 1.

TABLE 1. Representative Results of the Unscheduled DNA Synthesis Rat Hepatocyte Assay with Diuron (Trial 1)

		Cytotoxicity		UDS Activity			
Treatment	Dose (mM)	Lactate[a] dehydrogenase Activity (Units/L)	Increase[b] over Control	No. Cells Scored	Average[a] Nuclear Grain Count	Average[a] Cytoplasmic Grain Count	Mean[a] Net Nuclear Grain Count ± SD
<u>Solvent Control</u>							
Dimethylsulfoxide	0.0	97.9	—	100	15.3	17.8	-2.5±1.1
<u>Positive Control</u>							
Dimethylbenzanthracene	0.1	126.9	1.3	100	53.3	11.9	41.4±8.7**
<u>Test Material</u>							
Diuron	~~0.001~~	101.3	1.0	100	10.0	11.0	-1.0±2.2
	0.010	90.9	<1	100	13.7	15.0	-1.3±0.7
	0.100	126.9	1.3[d]	100	12.4	12.3	0.1±2.0
	0.330	179.4	1.8[d]	100	11.2	9.2**[f]	2.0±1.1**
	1.000[c]	299.4	3.1[d]	100	9.9	8.3**[f]	1.7±0.5**
	20.000[c]	197.4	2.0[d]	100	10.3	8.6**[f]	1.7±1.6**

[a]Average of four samples; calculated by our reviewers.

[b] $$\text{Increase over control} = \frac{\text{Test value}}{\text{Control value}}$$

[c]Compound precipitation occurred.

[d]Elevated enzyme activity, indicative of cytotoxicity.

**Significantly different from control value (p <0.01), by ANOVA.

**[f]Significantly decreased from control value (p <0.01), by ANOVA; indicative of cytotoxicity.

Note: An analysis of covariance on net nuclear grain counts using cytoplasmic grain counts as the covariant and dose and trial as factors indicated that the increased net nuclear grain counts resulted from decreased cytoplasmic grain counts and not from increased nuclear grain counts.

Trial 2: Trial 2 was conducted with the same concentrations of the test material. The results of Trial 2 compared favorably with Trial 1 and indicated that doses of diuron ranging from 0.33 to 20 mM induced a cytotoxic effect as shown in Table 2 by the elevated LDH values. In agreement with Trial 1, statistically significant and dose-related increases in net nuclear grain counts occurred at the three cytotoxic levels. At these same levels, decreased average nuclear grain counts and cytoplasmic grain counts were again observed. These decreases were significant and showed a significant negative trend. Below 0.33 mM (0.1, 0.01, and 0.001 mM), no appreciable cytotoxicity, appreciable decrease in cytoplasmic grain counts, nor significant increase in net nuclear grain counts was reported. Average nuclear grain counts for these doses were comparable to the solvent control. Representative data are presented in Table 2.

C. Additional Statistical Evaluations: Due to the reproducible and significantly increasing net nuclear grain counts accompanied by significantly decreasing cytoplasmic grain counts at 0.33, 1.0, and 20.0 mM diuron, the authors performed additional statistical evaluations. An analysis of covariance on nuclear grain counts, with the covariant being the cytoplasmic grain counts using dose and trial as factors, was performed on the combined data.

No significant increases in net nuclear grain counts were observed when the data were corrected for the decreases in cytoplasmic grain counts. The authors concluded, "the increase in net grain counts with dose was the result of a decrease in the cytoplasmic grain counts and not due to an increase in the nuclear grain counts. The statistically significant increases in the net nuclear counts, therefore, do not reflect UDS, but rather a cytoplasmic response, probably cytotoxicity."

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. The authors stated, "Diuron (H-15,580) was tested for its ability to induce unscheduled DNA synthesis (UDS) in primary rat hepatocyte cultures. The test sample was judged to be negative."

B. A quality assurance statement was not presented.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

We assess that the study was well conducted and that the authors correctly interpreted the data. The authors selected a dose range that included precipitating and cytotoxic levels of diuron. The authors assessed cytotoxicity by a highly sensitive method (LDH activity); because of the marked elevations in LDH activity at 0.33,

TABLE 2. Representative Results of the Unscheduled DNA Synthesis Rat Hepatocyte Assay with Diuron (Trial 2)

		Cytotoxicity		UDS Activity			
Treatment	Dose (mM)	Lactate[a] dehydrogenase Activity (Units/liter)	Increase[b] over Control	No. Cells Scored	Average[a] Nuclear Grain Count	Average[a] Cytoplasmic Grain Count	Mean[a] Net Nuclear Grain Count ± SD
Solvent Control							
Dimethylsulfoxide	0.0	228.9	—	100	15.0	17.4	-2.4±1.6
Positive Control							
Dimethylbenzanthracene	0.1	384.0	1.7	100	55.9	11.9	44.0±6.8**
Test Material							
Diuron	0.001	230.4	1.0	100	14.0	16.1	-2.1±2.2
	0.010	270.6	1.2	100	16.2	16.2	0.0±4.0
	0.100	324.7	1.4[d]	100	15.4	17.4	-2.0±2.2
	0.330	526.8	2.3[d]	100	13.9	13.9**f	0.0±2.4**
	1.000[c]	517.7	2.3[d]	75[e]	12.4	10.9**f	1.5±0.6**
	20.000[c]	526.4	2.3[d]	100	13.1	11.6**f	1.6±1.2**

[a]Average of four samples; calculated by our reviewers.

[b]
$$\text{Increase over control} = \frac{\text{Test value}}{\text{Control value}}$$

[c]Compound precipitation occurred.

[d]Elevated enzyme activity, indicative of cytotoxicity.

[e]No cells on one of four replicate slides.

**Significantly different from control value (p <0.01), by ANOVA.

**[f]Significantly decreased from control value (p <0.01), by ANOVA; indicative of cytotoxicity.

Note: An analysis of covariance on net nuclear grain counts using cytoplasmic grain counts as the covariant and dose and trial as factors indicated that the increased net nuclear grain counts resulted from decreased cytoplasmic grain counts and not from increased nuclear grain counts.

1.0, and 20 mM diuron, the investigators probably should not have evaluated the hepatocytes from these doses. Dr. Charlene McQueen[6] has found that, in general, increased LDH activity compares favorably with overt signs of cytotoxic effects in rat hepatocytes. However, since LDH activity is a more sensitive measurement of cytotoxicity than gross appearance, Dr. McQueen recommends that hepatocytes exposed to test doses that yield $\geq$ 2-fold increases in LDH in the absence of cell morphology alterations should not be evaluated for nuclear grain formation. She further indicated that results from "subtoxic" doses frequently cause artifactual positive results. Using this information for our interpretations we, therefore, concur with the authors' conclusions that the statistically significant increase in net nuclear grain counts at 0.33, 1.0, and 20.0 mM diuron was caused by cytotoxicity rather than by DNA repair synthesis.

The ability of the test system to detect UDS was clearly demonstrated by the marked increase in the average nuclear grain count and the statistically significant increase in the net nuclear grain count of rat hepatocytes exposed to the positive control (DMBA, 0.1 mM).

Item 15--see footnote 1.

16. <u>CBI APPENDIX</u>: Appendix A, Materials and Methods, CBI pp. 5-8.

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[6] McQueen, C., Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, NY, personal communications.

APPENDIX A

Materials and Methods

DIURON SCIENTIFIC REVIEWS

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EPA: 68-02-4225
DYNAMAC No. 1-059A-4
March 14, 1986

005039

DATA EVALUATION RECORD

DIURON

Mutagencity--*In vivo* Cytogenetic Study in Rats

STUDY IDENTIFICATION: Sarrif, A. Assessment of diuron in the *in vivo* cytogenetic study in rats. (Unpublished study No. 36685 prepared and submitted by E. I. duPont de Nemours and Company, Inc., Haskell Laboratory, Newark, DE; dated June 20, 1985.) Accession No. 258877.

APPROVED BY:

I. Cecil Felkner, Ph.D.
Department Manager
Dynamac Corporation

Signature: I. Cecil Felkner

Date: 3-14-86

1. CHEMICAL: Diuron; 3-(3,4-dichlorophenyl)-1,1-dimethylurea. 005039

2. TEST MATERIAL: Diuron, IN-14,740-145, N.B. 5103-129, lot No. T50906, batch No. 04, was described as a tan solid with a purity of 98.19%.

3. STUDY/ACTION TYPE: Mutagenicity--*In vivo* cytogenetic study in rats.

4. STUDY IDENTIFICATION: Sarrif, A. Assessment of diuron in the *in vivo* cytogenetic study in rats. (Unpublished study No. 36685 prepared and submitted by E. I. duPont de Nemours and Company, Inc., Haskell Laboratory, Newark, DE; dated June 20, 1985.) Accession No. 258877.

5. REVIEWED BY:

Brenda Worthy, M.T.
Principal Reviewer
Dynamac Corporation

Signature: Brenda Worthy
Date: 3-14-86

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

Signature: Nancy E. McCarroll
Date: 3-14-86

6. APPROVED BY:

I. Cecil Felkner, Ph.D.
Genetic Toxicology
Technical Quality Control
Dynamac Corporation

Signature: Ira Cecil Felkner
Date: 3-14-86

W. Thomas Edwards
EPA Reviewer

Signature: ____________
Date: ____________

Clint Skinner, Ph.D.
EPA Section Head

Signature: ____________
Date: ____________

7. CONCLUSIONS:

A. Under the conditions of this assay, diuron administered orally at 50, 500, and 5000 mg/kg to male and female rats over the entire mitotic cycle induced a toxic response in the animals and a cytotoxic response in the target cell. Combined data from both sexes for the 48-hour cell harvest indicated that diuron at 5000 mg/kg caused a significant increase in percent abnormal cells/group and average aberrations/group. Although significant increases were not calculated for the lower doses at the 48-hour sacrifice interval, a significant dose-related effect was uncovered. We conclude, therefore, that diuron is clastogenic and that the effect, although not significant, was apparent in males.

B. The study is acceptable.

Items 8 through 10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):

A. Materials and Methods: (See Appendix A for details.)

1. Test Material: Diuron, lot No. T50906, batch No. 04, was described as a tan solid with a purity of 98.19%. The test material was suspended in corn oil, the solvent control.

2. Test Animals: An unreported number of 50-day-old male and female Sprague-Dawley rats were obtained from Charles River Breeding Laboratories, Inc., Kingston, NY.

3. Animal Maintenance: Prior to initiation of the study, the animals were quarantined and acclimated to laboratory conditions for 6 days. Animals were individually housed in an environment controlled for temperature (22-24°C), relative humidity (34-51%), and light (12-hour light/dark cycle). Purina Certified Rodent Chow and water were available *ad libitum*.

4. Group Assignment: One hundred and thirty animals were assigned to treatment groups by computer-generated random numbers. The mean pretreatment weight was 240 g (range, 215-275 g) for males and 188 g (range, 164-216 g) for females. All animals were identified by individual cage card numbers.

5. Dose Selection: A preliminary range-finding study was performed with groups of five male and five female Sprague-Dawley rats. The animals were dosed orally by gavage with

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[1] Only items appropriate to this DER have been included.

1000, 2500, 3500, or 5000 mg/kg of the test material suspended in corn oil at a volume of 12 mL/kg. No deaths occurred and the animals were sacrificed 4 days posttreatment. Two animals per sex in the highest dose group were sacrificed 48 hours after treatment, and bone marrow preparations were made to ensure that suitable metaphase spreads could be prepared. Based on this data, the highest dose selected for the cytogenetic study was 5000 mg/kg.

6. Compound Preparation and Administration: Three concentrations, 50, 500, and 5000 mg/kg of the test material, were prepared in corn oil and administered by oral intubation in a single dose (volume of 12 mL/kg). The positive control, cyclophosphamide (20 mg/kg), was prepared in distilled water at 2.5 mg/mL and administered orally at a volume of 8 mL/kg. The solvent control (corn oil) was administered as described at a volume of 12 mL/kg.

7. Animal Sacrifice: Five animals per sex per solvent and test groups were sacrificed at 6-, 24-, or 48-hour intervals. The positive control groups were sacrificed at 24 hours. Each animal was observed for clinical signs of toxicity. Two hours prior to the appropriate sacrifice interval, the animals were injected with a single dose of colchicine (1 mg/kg, ip) at which time terminal body weights were taken for all groups except the 6-hour sacrifice. Animals were killed by CO_2 asphyxiation.

8. Cell Harvest and Slide Preparation and Staining:

   a. Cell Harvest: Bone marrow was collected from both femurs of each animal by aspiration into warm Hanks' balanced salt solution. Aspirates were centrifuged for 5 minutes and resuspended in warm 0.075 M KCl. After a 15-minute incubation period, cells were centrifuged and the resulting pellet was resuspended in cold fixative (methanol:acetic acid, 3:1).

   b. Slide Preparation and Staining: Three slides per animal were prepared. A drop of the cell suspension was placed onto a glass slide and flamed dried; the slides were stained with 10% Giemsa in Gurr phosphate buffer (pH 6.8), mounted, and coded.

9. Cytogenetic Analysis: Fifty metaphase cells per animal were scored for the presence of chromosomal abnormalities. Chromosomal aberrations were characterized as breaks, fragments, pulverized cells, translocations, or cells having 10 or more aberrations. The number of chromosomes present in each cell was counted. Gaps were recorded but not included in the final analysis. Each animal was considered an experimental unit and the sexes were combined for statistical analysis of the percent abnormal cells with more than one aberration and of aberrations per cell.

10. Statistical Analysis: The above parameters were evaluated for statistical significance by the Mann-Whitney U test or the Fisher exact test. Trend analysis was evaluated by the Jonckheere test.[2] Mitotic indices and body weight changes were tested using a two-way analysis of variance. Significance was at the 5% level.

B. Protocol: A protocol was not provided.

12. REPORTED RESULTS:

A. Clinical Observations: The following observations were noted at 24 and/or 48 hours after dosing. Red, orange, or yellow discharge from the mouth, nose, and/or eyes in eight males and three females; reduced activity or depression in five males and two females; wet, stained perineum in three males and two females; labored respiration in one male and two females; diarrhea in two females; salivation in one female; tremors in one male; and moribundity in one male and three females (one of the moribund females was found dead 48 hours after dosing) were observed in the 5000-mg/kg group. In the 500-mg/kg group, diarrhea was observed in one male and one female. In the 50-mg/kg group, red staining of the neck fur was noted in one female.

Significant body weight loss was observed at 24 hours in the male rats at the highest dose tested and in the combined analysis of males and females at this dose ($p < 0.001$). Significant ($p < 0.001$) weight loss was observed at 48 hours in both male and female animals in the highest dose groups and in the female rats in the mid-dose group. Combined analysis of weight data at 48 hours could not be performed because of significant interaction between treatment and sex.

B. Mitotic Index: Compared to the solvent control there was a significant depression in the mitotic index in male rats in the high-dose group at both the 24- ($p < 0.001$) and 48- ($p < 0.01$) hour sacrifices. The combined analyses indicated a significant reduction in the mitotic index at 24 ($p < 0.01$) and 48 hours ($p < 0.001$). No significant increase in bone marrow depression was observed for the remaining groups at any time interval. The positive control for both male and female groups also showed a significant depression ($p < 0.001$) in the mitotic index.

C. Chromosomal Aberrations: No significant differences between the solvent control and the test material groups were noted at the 6- or 24-hour sacrifices for aberrations per cell, percent

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[2] Jonckheere, A. R. A distribution-free K-sample test against ordered alternatives, Biometrika 41(1954): 133-145.

abnormal cells, or percent abnormal cells with more than one aberration. At the 48-hour sacrifice, slight but not significant increases in percent abnormal cells and the average number of aberrations/cell were recorded for both sexes following exposure to 500 and 5000 mg/kg diuron. Combined data for the percent abnormal cells and average number of aberrations/cell from the 5000-mg/kg group were significant ($p < 0.05$). A significant positive and linear dose trend ($p < 0.01$) was also observed for both parameters. Since there were no aberrations in the 48-hour control group, a comparison of the 48-hour data for the high-dose was made against the pooled negative controls from all three sacrifice intervals. The 48-hour high-dose group showed a small but significant effect ($p < 0.05$).

Representative results are presented in Table 1.

13. STUDY AUTHOR'S CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. The author concluded that "Under the conditions of this assay, diuron displayed weak clastogenic activity in bone marrow cells of rats sacrificed 48 hours after a 5000 mg/kg oral dose....The compound is considered weakly positive in this test."

B. A quality assurance statement was not submitted.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

We assess that the study was properly conducted and the author's interpretation of the data was correct. Diuron at the highest dose tested (5000 mg/kg) elicited a toxic response in the animals and induced a cytotoxic effect in the target cell. Therefore, the dose range selected for the *in vivo* bone marrow study was adequate. A calculation error was however uncovered. The percent cells/group with >1 aberration for 5000-mg/kg males at the 48-hour harvest was reported as 0.4; based on the furnished individual animal data, this value should be 0. Since this value was used to compute the group percent cells with >1 aberration, average number of aberrations/cell for males, and group average aberrations/cell, we recalculated all numbers from individual data; the correct results are presented in Table 1.

We also performed Fisher's exact test to determine if the erroneously reported values affected the statistical outcome. Based on our reevaluation of the statistical analysis, the increase in average number of aberrations per cell, although lower than the reported result, was still significant (p value = 0.0500). We conclude, therefore, that the reporting error did not alter the reported results and that diuron induced a clastogenic response. The findings further suggest that diuron is more active in males than females.

TABLE 1. Representative Results of the Cytogenicity Assay in Rats at 48 Hours with Diuron

Substance	Dose (mg/kg)	Sex	No. of Animals per Group	No. of Metaphases per Group	% Abnormal Cells per Group	% Cells per Group with >1 Aberration	Average No. of Aberrations per Group	Average No. of Mitoses per 500 Cells (S.E.)
Solvent Control								
Corn Oil		M	5	250	0.0	0.0	0.000	11.4(1.3)
		F	5	250	0.0	0.0	0.000	9.8(1.2)
		Combined	10	500	0.0	0.0	0.000	10.6(0.9)
Positive Control[a]								
Cyclophos-phamide	20	M	5	250	25.2	18.4	1.140	1.6(0.6)***
		F	4[b]	200	27.0	22.5	1.520	3.5(0.3)***
		Combined	9	450	26.0***	20.2***	1.309*	2.4(0.5)***
Test Material								
Diuron	50	M	5	250	0.0	0.0	0.000	8.4(0.9)
		F	5	250	0.4	0.0	0.004	9.2(1.0)
		Combined	10	500	0.2	0.0	0.002	8.8(0.6)
	500[c]	M	5	250	0.8	0.0	0.008	10.6(1.7)
		F	5	250	0.4	0.4	0.008	10.0(1.5)
		Combined	10	500	0.6	0.2	0.008	10.3(1.1)
	5000[c]	M	5	250	1.2	0.4[0.0][e]	0.016[0.012][e]	4.6(1.4)**
		F	4[d]	200	0.5	0.0	0.005	6.5(2.0)
		Combined	9	450	0.9*	0.2[0.0][e]	0.011*[0.0085]*	5.4(1.1)***

[a] Positive control results from 24-hour sacrifice.

[b] One animal yielded no scorable metaphases.

[c] Clinical signs of toxicity observed at 24 and 48 hours.

[d] One animal found dead prior to sacrifice.

[e] [ ]: Corrected results calculated by reviewers.

*Significantly different from control value (p <0.05), by the Fisher exact test.

**Significantly different from control value (p <0.01), by the Fisher exact test.

***Significantly different from control value (p <0.001), by the Fisher exact test.

NOTE: Results from the 6- and 24-hour harvests were comparable to the control and were therefore not presented.

The positive control, cyclophosphamide (20 mg/kg), administered via the same route as the test material, demonstrated the sensitivity of the assay to detect a clastogenic effect in animals of both sexes.

Item 15--see footnote 1.

16. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 3-9.

APPENDIX A

Materials and Methods

DIURON SCIENTIFIC REVIEWS

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