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

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FEB 12 1993

OFFICE OF
PESTICIDES AND TOXIC
SUBSTANCES

MEMORANDUM

SUBJECT: **BROMACIL - Review of Mutagenicity Studies Submitted in Response to 9/91 DCI**

TO: Mario F. Fiol
PM Team Reviewer (73)
Reregistration Branch, SRRD (H7508C)

FROM: Linda L. Taylor, Ph.D. Linda Taylor 2/2/93
Toxicology Branch II, Section II,
Health Effects Division (H7509C)

THRU: K. Clark Swentzel K. Clark Swentzel 2/2/93
Section II Head, Toxicology Branch II
Health Effects Division (H7509C)

and

Marcia van Gemert, Ph.D. Marcia van Gemert 2/3/93
Chief, Toxicology Branch II/HFAS/HED (H7509C)

Registrant:	DuPont
Chemical:	5-Bromo-3-sec-butyl-6-methyluracil
Synonyms:	Bromacil; INN-976
Caswell No.:	111
Shaughnessy No.	012301
DP Barcode:	D182796
Submission:	S425703
ID #:	012301
Case:	818592
Action Requested:	None specified.

Comment: In response to the Agency's September, 1991 Data Call-In for Bromacil, the Registrant has submitted four mutagenicity studies, which have been reviewed. The DER for each study is appended. Three of the four studies are classified Acceptable, and all three mutagenicity guideline requirements (Categories I, II, and III) have been satisfied.

1). Mutagenicity Evaluation of Bromacil in the CHO/HPRT Assay, dated 12/9/88 [MRID # 424656-01]. Under the conditions of S-9 activation, adequate dose levels were clearly not mutagenic and cytotoxicity was achieved. Due to the inconclusive results in the nonactivated phase of the study, this study is classified Unacceptable, and it does not satisfy the guideline requirement

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(84-2) for genetic effects, Category I, Gene Mutations.

2). Mutagenicity Testing of Bromacil in the Salmonella Typhimurium Plate Incorporation Assay, dated 9/9/88 [MRID # 424657-01]. Under the conditions of two independently-performed assays, six doses of Bromacil (ranging from 50 to 5000 µg/plate) with and without S-9 activation were not mutagenic in S. typhimurium tester strains TA1535, TA97, TA98, and TA100. Under nonactivated conditions, the highest dose was cytotoxic in all tester strains. Based on these findings, it was concluded that Bromacil was tested over an appropriate range of concentrations and was not genotoxic in this mutation assay. This study is classified Acceptable, and it satisfies the guideline requirement (84-2a) for genetic effects, Category I, Gene Mutations.

3). Mutagenicity: In Vivo Micronucleus Assay in Mice, dated 12/5/88 [MRID # 424658-01]. Oral administration of 5, 75, or 500 mg/kg Bromacil to mice of both sexes did not cause a significant increase in the frequency of micronucleated polychromatic erythrocytes in bone marrow cells harvested 24, 48, or 72 hours postexposure. In the high-dose mice, signs of toxicity (including mortality) and cytotoxicity (evidenced by reductions in the ratio of polychromatic erythrocytes to normochromatic erythrocytes in males) was observed. Based on these findings, it was concluded that Bromacil was tested over an appropriate range of doses and found to be nongenotoxic in the mouse micronucleus assay. This study is classified Acceptable, and it satisfies the guideline requirement (84-2) for genetic effects, Category II, Structural Chromosome Aberrations.

4). Assessment of Bromacil in the In Vitro Unscheduled DNA Synthesis Assay in Rat Primary Hepatocytes, dated 9/9/88 [MRID # 424659-01]. Under the conditions of two independently-performed assays, Bromacil at concentrations ranging from 0.1 to 500 µg/mL did not increase the frequency of UDS in primary rat hepatocytes. Higher levels (≥ 1000 µg/mL) were cytotoxic. Based on these findings, it was concluded that Bromacil was tested over an appropriate range of concentrations and failed to induce a genotoxic response in this in vitro assay. This study is classified Acceptable, and it satisfies the guideline requirement (84-4) for genetic effects, Category III, Other Mutagenic Mechanisms.

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DATA EVALUATION REPORT

BROMACIL

Study Type: Mutagenicity: In Vivo Micronucleus Assay in Mice

Prepared for:

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

Prepared by:

Clement International Corporation
9300 Lee Highway
Fairfax, VA 22031-1207

Principal Reviewer	Kristin Jacobson, MSPH	Date 1/26/93
Independent Reviewer	Nancy E. McCarroll, B.S.	Date 1/26/93
QA/QC Manager	Sharon Segal, Ph.D.	Date 1/26/93

Contract Number: 68D10075
Work Assignment Number: 2-25
Clement Number: 85
Project Officer: Caroline Gordon

GUIDELINE SERIES 84: MUTAGENICITY MICRONUCLEUS

MUTAGENICITY STUDIES 010032

EPA Reviewer: Byron T. Backus, Ph.D. Signature: Byron T. Backus
Review Section II, Toxicology Branch II Date: 2/1/93
Health Effects Division (H-7509C)
EPA Reviewer: Linda Taylor, Ph.D. Signature: Linda Taylor
Review Section II, Toxicology Branch II Date: 2-2-93
Health Effects Division (H-7509C)
EPA Section Head: K. Clark Swentzel Signature: K. Clark Swentzel
Review Section II, Toxicology Branch II Date: 2/2/93
Health Effects Division (H-7509C)

DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity: In vivo micronucleus assay in mice

EPA IDENTIFICATION Numbers:

Tox. Chem. Number: 012301

MRID Number: 424658-01

TEST MATERIAL: Bromacil

SYNONYMS/CAS NUMBER: 2,4(1H,3H)-Pyrimidinedione, 5-bromo-6-methyl-3-(1-methylpropyl)-uracil, 5-bromo-3-sec-butyl-6-methyluracil, 314-40-9

SPONSOR: E.I. du Pont de Nemours and Co., Inc., Wilmington, DE

STUDY NUMBERS: Medical Research Number 8452-001; Haskell Laboratory Report Number 783-88

TESTING FACILITY: Haskell Laboratory, E.I. du Pont de Nemours and Co., Inc., Newark, DE

TITLE OF REPORTS: Mouse Bone Marrow Micronucleus Assay of Bromacil (IN N976)

AUTHOR: D.A. Vlachos

REPORTS ISSUED: December 5, 1988

CONCLUSIONS--EXECUTIVE SUMMARY: The oral gavage administration of 5, 75, or 500 mg/kg of bromacil to male and female mice did not cause a significant increase in the frequency of micronucleated polychromatic erythrocytes (MPEs) in bone marrow cells harvested 24, 48, or 72 hours postexposure. In the high-dose animals, signs of toxicity (including mortality) and cytotoxicity (evidenced by reductions in the ratio of polychromatic erythrocytes (PCEs) to normochromatic erythrocytes (NCEs) in males) were observed. Based on these findings, we conclude that bromacil was tested over an appropriate range of doses and found to be nongenotoxic in the mouse micronucleus assay.

STUDY CLASSIFICATION: Acceptable. The study satisfies Guideline requirements (§84-2) for genetic effects Category II, Structural Chromosome Aberrations.

A. **MATERIALS:**

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1. **Test Material:** Bromacil

Description: Tan solid
Identification numbers: IN N976; Haskell number 16,473; Lot number 180-806 3T Batch 31
Purity: Approximately 95%
Receipt date: Not reported
Stability: Assumed to be stable under the conditions of the assay
Contaminants: None listed
Vehicle used: Corn oil
Other provided information: Storage conditions were not reported. Dosing solutions were prepared immediately prior to testing, and mixed constantly during dosing with a homogenizer. Analytical determinations were not performed to verify test concentrations of test material stability.

2. Control Materials:

Vehicle: Mice received corn oil via oral gavage at a dosing volume of 15 ml/kg.

Positive: Cyclophosphamide (CP), at a final dose of 40 mg/kg, was dissolved in distilled deionized water and administered by oral gavage at a dosing volume of 10 mL/kg.

3. Test Compound:

Route of administration: Oral gavage

Volume of test substance administered: 15 mL/kg

Dose levels used:

(a) Acute dose range-finding study: 50, 250, 500, 1000, 2500, and 4000 mg/kg

(b) Micronucleus assay: 5, 75, and 500 mg/kg

Note: Initially, 750 mg/kg was selected as the high dose; however, severe toxicity was evident within minutes of administration of 500 mg/kg. Accordingly, 500 mg/kg was substituted as the high dose and the low- and mid-dose were adjusted to 5 and 75 mg/kg, respectively.

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4. Test Animals:

(a) Species: Mouse Strain: CR1:CD®-1(ICR)BR Age: ≈7 weeks (at dosing) Weight Range: 29.5-34.6 g (males); 18.4-26.7 g (females) Sex: Males and females Source: Charles River Breeding Laboratories, Inc., Raleigh, NC

(b) Number of animals used per dose:
Acute study: 3-5 (males); 3-5 (females) /treatment group

Micronucleus assay (primary dose groups/sacrifice time):

- Treatment groups: 5 males 5 females
- Positive control: 5 males 5 females
- Vehicle control: 5 males 5 females

Note: To allow for possible mortality, an additional male and female were included in the high-dose group for each sacrifice time. These animals were sacrificed and their cells were scored along with the survivors in each group.

(c) Animals were properly maintained? Yes.

B. TEST PERFORMANCE:

1. Acute Dose Range-finding Study: Animals received single oral gavage administrations of the selected doses of bromacil and were observed for signs of toxicity and/or mortality at least daily for 3 days.

2. Micronucleus Assay:

Treatment and sampling times:

(a) Test compound:
Dosing: x once _____ twice (24 hours apart)
Sampling (after last dose): _____ 6 hours _____ 12 hours
x 24 hours x 48 hours x 72 hours

(b) Vehicle control:
Dosing: x once _____ twice (24 hours apart)
Sampling (after last dose): x 24 hours x 48 hours
x 72 hours

(c) Positive control:
Dosing: x once _____ twice (24 hours apart)
Sampling (after last dose): x 24 hours _____ 48 hours
_____ 72 hours

3. Tissues and Cells Examined:

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__x__ bone marrow _____ other (list):
Number of polychromatic erythrocytes (PCEs) examined per animal: 1000
Number of normochromatic erythrocytes (NCEs, more mature RBCs) examined per animal: Number observed while scoring 1000 erythrocytes (PCEs+NCEs)

4. Details of Cell Harvest and Slide Preparation: At 24, 48, and 72 hours after administration of the test material or vehicle control, the appropriate groups of animals were euthanized with CO_2. Animals in the positive control group were sacrificed 24 hours postexposure. Bone marrow cells from both femurs of each animal were aspirated into prewarmed fetal bovine serum, centrifuged, resuspended and spread onto slides using a Miniprep® automatic blood smearing instrument. Prepared slides were fixed in absolute methanol, stained with acridine orange solution (0.0125 mg/mL), coverslipped, coded and scored for PCEs, micronucleated PCEs (MPEs), and NCEs.

Note: When stained with acridine orange, MPEs appear as bright yellow-green staining bodies within the orange PCEs, and NCEs have a dark green appearance.

5. Statistical Methods: To determine whether the results from any dose or cell harvest time were significantly different ($p<0.05$) from vehicle controls, the data were evaluated using an analysis of variance on transformed data (arcsine square-root function) and Dunnett's test. Males and females were analyzed separately.

6. Evaluation Criteria: No criteria were provided to evaluate assay validity, a positive response, or the biological significance of the findings.

7. Protocol: Not provided

C. REPORTED RESULTS:

1. Acute Dose Range-finding Study: All mice exposed to 2500 and 4000 mg/kg died within 2 days of treatment. At 1000 mg/kg, 4 mice (1 male and 3 females) died within 3 days. No other deaths were reported. Clinical signs observed in animals surviving treatment included convulsions and absence of righting reflex in higher dose groups, and mild lethargy in the 50-mg/kg dose group. Based on these findings, doses of 75, 500, and 750 mg/kg were selected for the micronucleus assay.

2. Micronucleus Assay: Animals administered the selected doses of bromacil were observed for mortality and other signs of compound toxicity immediately after dosing, ≈4 hours postdosing, and daily thereafter. Based on clinical signs (i.e., partially closed eyes,

lethargy, low posture, tremors, and/or uncoordinated gait) observed within minutes of dosing in the 500-mg/kg group, the selected doses for the micronucleus assay were adjusted to 5, 75, and 500 mg/kg. Some of these clinical signs were still evident four hours postdosing; in addition, several animals were prostrate, or had labored breathing or wet fur. One high-dose male died within 24 hours postdosing. Most clinical signs subsided in the surviving animals by 48 hours postdosing. Significant ($p<0.01$) body weight loss was also observed in high-dose males sacrificed 24 and 48 hours posttreatment. In the mid-dose group (75 mg/kg), most mice displayed labored breathing, lethargy, and partially closed eyes following dosing; in general, these signs subsided by 4 hours postdosing. The low-dose (5 mg/kg) animals appeared normal throughout the study.

Representative results from the micronucleus assay are presented in Table 1. Significant ($p<0.01$) cytotoxicity (evidenced by reductions in PCE/NCE ratios) was noted in high-dose males sacrificed 48 hours posttreatment. There were no significant increases in the frequency of MPEs in male or female mice at any treatment level. Although the percentage of MPEs was elevated in high-dose males sacrificed 48 hours posttreatment, the increase was neither time or dose related. By contrast, the frequency of MPEs in male and female mice treated with the positive control (40 mg/kg CP) was significantly increased ($p<0.01$).

Based on the overall results, the study author concluded that bromacil was not genotoxic in this in vivo mouse micronucleus assay.

D. REVIEWERS' DISCUSSION/CONCLUSIONS: We assess that the study was properly conducted and that the study author's interpretation of the data was correct. Bromacil was evaluated to a level (500 mg/kg) that caused mortality and other clinical signs of toxicity but failed to induce a clastogenic response. In addition, the senstivity of the test system to detect genotoxicity was demonstrated by the significant increases ($p<0.01$) in MPEs in both male and female mice exposed to the positive control (40 mg/kg CP).

We conclude, therefore, that the study provided acceptable evidence that bromacil did not increase the frequency of micronuclei in PCEs in this in vivo micronucleus assay.

E. QUALITY ASSURANCE MEASURES: Was the test performed under GLPs? Yes. (A quality assurance statement documenting the performance of quarterly audits of representative studies for this type of assay was signed and dated November 30, 1988.)

F. CBI APPENDIX: Appendix A, Materials and Methods -- Micronucleus Assay, CBI pp. 7-10

CORE CLASSIFICATION: Acceptable. The study satisfies Guideline requirements (§84-2) for genetic effects Category II, Structural Chromosome Aberrations.

Table 1. Representative Results of the Micronucleus Assay in Mice with Bromacil

Substance	Dose/kg	Exposure Time[a] (hours)	Sex	Number of Animals Analyzed per Group	Number of PCEs Analyzed per Group	Number of MPEs per Group	Mean Percent MPEs ± S.E.	Mean PCE/NCE Ratio ± S.E.
Vehicle Control								
Corn oil	15 mL	24	M	5	5000	11	0.22 ± 0.09	1.05 ± 0.14
		48	M	5	5000	13	0.26 ± 0.02	1.17 ± 0.21
		72	M	5	5000	10	0.20 ± 0.04	1.15 ± 0.23
	15 mL	24	F	5	5000	4	0.08 ± 0.05	1.22 ± 0.30
		48	F	5	5000	5	0.10 ± 0.03	1.35 ± 0.28
		72	F	5	5000	5	0.10 ± 0.03	1.49 ± 0.22
Positive Control								
Cyclophosphamide	40 mg	24	M	5	5000	98	1.96 ± 0.35*	0.83 ± 0.09
		24	F	5	5000	85	1.70 ± 0.16*	1.22 ± 0.09
Test Material								
Bromacil	500 mg[b]	24	M	5	5000	11	0.22 ± 0.11	0.91 ± 0.16
		48	M	6	6000	27	0.45 ± 0.15	0.57 ± 0.05*
		72	M	6	6000	11	0.18 ± 0.05	0.87 ± 0.13
	500 mg[b]	24	F	6	6000	14	0.23 ± 0.09	0.79 ± 0.12
		48	F	6	6000	11	0.18 ± 0.05	1.07 ± 0.15
		72	F	6	6000	6	0.10 ± 0.07	1.44 ± 0.49

[a]Time after compound administration by oral gavage

[b]One male at 500 mg/kg died prior to sacrifice at 24 hours. Other signs of toxicity in treated animals of the high-dose group included convulsions, partially closed eyes, labored breathing, and lethargy. No treatment-related clastogenic effects were seen in the low- (5 mg/kg) or mid- (75 mg/kg) dose groups.

*Significantly higher ($p<0.01$) than the corresponding vehicle control by ANOVA.

Abbreviations used: PCE = polychromatic erythocyte; MPE = micronucleated polychromatic erythrocyte; NCE = normochromatic erythrocyte

Note: Data were extracted from CBI pp. 14, and 16-19.

TABLE 1

GUIDELINE SERIES 84: MUTAGENICITY MICRONUCLEUS

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APPENDIX A

MATERIALS AND METHODS
(MICRONUCLEUS ASSAY)
CBI pp. 7-10

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Page_____ is not included in this copy.

Pages 11 through 14 are not included.

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FINAL

DATA EVALUATION REPORT

BROMACIL

Study Type: Mutagenicity: Salmonella typhimurium/Mammalian Microsome Mutagenicity Assay

Prepared for:

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

Prepared by:

Clement International Corporation
9300 Lee Highway
Fairfax, VA 22031-1207

Principal Reviewer	Kristin Jacobson Kristin Jacobson, MSPH	Date 1/26/93
Independent Reviewer	Nancy E. McCarroll Nancy E. McCarroll, B.S.	Date 1/26/93
QA/QC Manager	Sharon Segal Sharon Segal, Ph.D.	Date 1/26/93

Contract Number: 68D10075
Work Assignment Number: 2-25
Clement Number: 83
Project Officer: Caroline Gordon

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MUTAGENICITY STUDIES

EPA Reviewer: Byron T. Backus, Ph.D.
Review Section II, Toxicology Branch II
Health Effects Division (H-7509C)
EPA Reviewer: Linda Taylor, Ph.D.
Review Section II, Toxicology Branch II
Health Effects Division (H7509C)
Acting EPA Section Head: K. Clark Swentzel
Review Section II, Toxicology Branch II
Health Effects Division (H7509C)

Signature: Byron T. Backus
Date: 2/1/93
Signature: Linda Taylor
Date: 2-2-93
Signature: K. Clark Swentzel
Date: 2/2/93

DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity: Salmonella typhimurium/mammalian microsome mutagenicity assay

EPA IDENTIFICATION Numbers:

Tox. Chem. Number: 012301

MRID Number: 424657-01

TEST MATERIAL: Bromacil

SYNONYMS/CAS NUMBER: 2,4(1H,3H)-Pyrimidinedione,5-bromo-6-methyl-3-(1-methylpropyl)-uracil; 5-bromo-3-sec-butyl-6-methyluracil/314-40-9

SPONSOR: E.I. du Pont de Nemours and Co., Inc., Wilmington, DE

STUDY NUMBERS: Medical Research Number 8452-001; Haskell Laboratory Report Number 551-88

TESTING FACILITY: Haskell Laboratory, E.I. du Pont de Nemours and Co., Inc., Newark, DE

TITLE OF REPORT: Mutagenicity Testing of Bromacil in the Salmonella typhimurium Plate Incorporation Assay

AUTHOR: V.L. Reynolds

REPORT ISSUED: September 9, 1988

CONCLUSIONS--EXECUTIVE SUMMARY: Under the conditions of two independently performed Salmonella typhimurium/mammalian microsome plate incorporation assays, 6 doses of bromacil ranging from 50 to 5000 µg/plate +/- S9 were not mutagenic in S. typhimurium tester strains TA1535, TA97, TA98, or TA100. Under nonactivated conditions, the highest assayed dose (5000 µg/plate) was cytotoxic in all tester strains. Based on these findings, it was concluded

that bromacil was tested over an appropriate range of concentrations and was not genotoxic in this mutation assay.

STUDY CLASSIFICATION: Acceptable. While the Guidelines indicate that strain TA1537 should be used, we can accept substitution of strain TA97 (a strain derived from TA1537). The study satisfies Guideline requirements (§84-2a) for genetic effects Category I, Gene Mutations.

A. MATERIALS:

1. Test Material: Bromacil

Description: Tan solid
Identification Numbers: IN N976; Haskell number 16,473; Lot number 180-306 3T Batch 31
Purity: ≈95%
Receipt date: Not reported
Stability: Assumed to be stable under the conditions of this study
Contaminants: None listed
Solvent used: Dimethyl sulfoxide (DMSO)
Other provided information: Storage conditions for the test material were not reported. Analytical determinations were not performed to verify test concentrations or test material stability.

2. Control Materials:

Negative: None

Solvent/final concentration: DMSO, 0.1 mL/plate

Positive:

Nonactivation:	
Sodium azide	2 µg/plate TA1535, TA100
2-Nitrofluorene	25 µg/plate TA98
ICR-191	2 µg/plate TA97
Activation:	
2-Aminoanthracene	2 µg/plate TA1535, TA98
	1 µg/plate TA97, TA100

3. Activation: S9 derived from 8-9-week old male Crl:CD®BR

x Aroclor 1254	x induced	x rat	x liver
___ phenobarbital	___ noninduced	___ mouse	___ lung
___ none		___ hamster	___ other
___ other		___ other	

The rat liver S9 homogenate was obtained from Sitek Research Laboratories, Rockville, MD; it was identified as lot number 871215. The S9 mix was prepared as follows:

Component:	Amount/mL
Cofactor solution	0.7 mL
Glucose-6-phosphate (5 µmoles)	
KCl (33 µmoles)	
NADP (4 µmoles)	
$MgCl_2$ (8 µmoles)	
$NaPO_4$ (100 µmoles, pH 7.4)	
S9 (5.6 mg/mL in phosphate buffered saline)	0.3 mL

4. Test Organism Used: S. typhimurium strains
   x TA97 x TA98 x TA100 _____ TA102 _____ TA104
   x TA1535 _____ TA1537 _____ TA1538
   list any others:

   Test organisms were properly maintained? Not reported.
   Checked for appropriate genetic markers (rfa mutation, R factor)? Not reported; however, spontaneous revertant frequencies were within acceptable ranges.

5. Test Compound Concentrations Used:

   (a) Preliminary cytotoxicity assay: Six levels (0, 10, 50, 100, 500, 1000, and 5000 µg/plate +/- S9) were assayed with strain TA98. Duplicate plates were prepared per dose per condition.

   (b) Mutation assay: Six doses (0, 50, 100, 500, 1000, 2500, and 5000 µg/plate +/- S9) were evaluated with the four tester strains. Duplicate plates per dose, per strain, per condition were prepared.

B. TEST PERFORMANCE:

1. Type of Salmonella Assay:
   x Standard plate test
   ___ Pre-incubation (_____) minutes
   ___ "Prival" modification
   ___ Spot test
   ___ Other (describe)

2. Preliminary Cytotoxicity Assay: A mixture containing 0.1 mL of the appropriate concentration of the test material or solvent and 0.1 mL of an overnight broth culture of tester strain TA98 (diluted to yield $\approx 10^3$ bacteria/plate) was added to 2 mL top agar containing excess histidine. For the S9-activated test, 0.5 mL of the S9 mix was added before the mixture was poured onto plates containing Davis minimal agar. Duplicate plates were used per dose, per condition. Plates were incubated at 37°C for 48 hours, and colonies were counted.

   Mutation Assays: In general, similar procedures were used for the preliminary cytotoxicity assay and the mutation assays. In the mutation assays, however, the four tester strains were used, with 0.1 mL inoculations of $\approx 10^8$ bacteria added to 0.1-mL volumes of the selected

test material concentration, solvent or positive controls. The mixtures were added to 2 mL top agar (supplemented with 0.05 mM histidine and 0.05 mM biotin) and poured onto plates containing Davis minimal agar. Duplicate plates were used per strain, per dose, per condition. Following incubation at 37°C for 48 hours, revertant colonies were counted; means and standard deviations combined for both trials were determined.

3. Statistical Analysis: The data were evaluated for statistical significance at $p<0.05$ using a multiple linear regression analysis.

4. Evaluation Criteria: The test material was considered positive if there was a ≥2-fold increase in the average number of induced revertants at one or more test concentrations, and there was a dose-related response.

5. Protocol: Not provided

C. REPORTED RESULTS:

Preliminary Cytotoxicity Assay: At 5000 µg/plate +/- S9, cytotoxicity was indicated by a 64-72% reduction in colonies of S. typhimurium TA98. Lower doses (≤1000 µg/plate +/- S9) were not cytotoxic. Based on these results, the six doses selected for the mutation assay ranged from 50 to 5000 µg/plate.

Mutation Assay: Representative data from the initial and confirmatory mutation assays are presented in Tables 1 and 2, respectively. Reduced revertant colony counts for the four tester strains were seen on plates containing the highest nonactivated dose (5000 µg/plate). There was no clear indication of a cytotoxic response at lower nonactivated doses or at any S9-activated doses. Similarly, there was no indication of a mutagenic response in any strain, at any dose, with or without S9 activation in either trial. In contrast, the positive control compounds induced marked increases in the number of revertant colonies of the corresponding tester strain.

Based on these findings, the study author concluded that bromacil was not genotoxic in this bacterial mutation assay.

D. REVIEWERS' DISCUSSION/CONCLUSIONS: We assess that the study author's interpretation of the data was correct. Dose levels of bromacil ranging from 50 µg/plate to 5000 µg/plate, both in the presence and absence of S9 activation, did not significantly increase the frequency of revertant colonies. The highest nonactivated dose (5000 µg/plate) was cytotoxic for all tester strains. In addition, the sensitivity of the test system to detect mutagenesis was adequately demonstrated by the responses induced by the positive controls in the appropriate tester strains. It was concluded, therefore, that bromacil was adequately tested and was negative in this bacterial assay system.

E. QUALITY ASSURANCE MEASURES: Was the test performed under GLP? Yes. (A quality assurance statement was signed and dated September 6, 1988.)

F. CBI APPENDICES: Appendix A, Materials and Methods, CBI pp. 7-9 010032

CORE CLASSIFICATION: Acceptable; the study satisfies the data Guideline requirement (§84-2a) for genetic effects Category I, Gene Mutations.

Table 1: Representative Results of the Initial Salmonella typhimurium/Mammalian Microsome Mutation Assay with Bromacil

Substance	Dose per Plate	S9 Activation	Revertants per Plate of Bacterial Tester Strain[a] TA1535	TA97	TA98	TA100
Solvent Control						
Dimethyl sulfoxide	0.1 mL	-	18	111	20	124
	0.1 mL	+	11	147	34	127
Positive Controls						
Sodium azide	2 µg	-	657	--	--	769
ICR-191	2 µg	-	--	2070	--	--
2-Nitrofluorene	25 µg	-	--	--	1371	--
2-Aminoanthracene	2 µg	+	281	--	2157	--
	1 µg	+	--	1171	--	1625
Test Material						
Bromacil	2500 µg[b]	-	16	126	16	97
	5000 µg	-	9	49	11	77
	2500 µg[b]	+	11	151	33	104
	5000 µg	+	12	117	29	91

[a]Average counts from duplicate plates; calculated by our reviewers
[b]Results for lower doses (50, 100, 500, or 1000 µg/plate +/- S9) did not suggest a cytotoxic or mutagenic effect.

Note: Data were extracted from the study report, CBI p. 13-20.

Table 2: Representative Results of the Confirmatory Salmonella typhimurium/Mammalian Microsome Mutation Assay with Bromacil

Substance	Dose per Plate	S9 Activation	Revertants per Plate of Bacterial Tester Strain[a] TA1535	TA97	TA98	TA100
Solvent Control						
Dimethyl sulfoxide	0.1 mL	-	15	114	15	96
	0.1 mL	+	14	121	26	100
Positive Controls						
Sodium azide	2 µg	-	456	--	--	657
ICR-191	2 µg	-	--	2040	--	--
2-Nitrofluorene	25 µg	-	--	--	1704	--
2-Aminoanthracene	2 µg	+	192	--	2072	--
	1 µg	+	--	1752	--	1456
Test Material						
Bromacil	2500 µg[b]	-	7	122	13	100
	5000 µg	-	5	32	15	55
	2500 µg[b]	+	10	166	29	101
	5000 µg	+	8	133	24	88

[a]Average counts from duplicate plates
[b]Results for lower doses (50, 100, 500, or 1000 µg/plate +/- S9) did not suggest a cytotoxic or mutagenic effect.

Note: Data were extracted from the study report, CBI p. 13-20.

APPENDIX A

MATERIALS AND METHODS
CBI pp. 7-9

BROMACIL TXR 010032

Page_____ is not included in this copy.

Pages 24 through 26 are not included.

The material not included contains the following type of information:

____ Identity of product inert ingredients.

____ Identity of product impurities.

____ Description of the product manufacturing process.

____ Description of quality control procedures.

____ Identity of the source of product ingredients.

____ Sales or other commercial/financial information.

____ A draft product label.

____ The product confidential statement of formula.

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X FIFRA registration data.

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

010032

FINAL

DATA EVALUATION REPORT

BROMACIL

Study Type: Mutagenicity: Gene Mutation in Cultured Chinese Hamster Ovary Cells (CHO/HGPRT)

Prepared for:

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

Prepared by

Clement International Corporation
9300 Lee Highway
Fairfax, VA 22031-1207

Principal Reviewer	Kristin Jacobson, MSPH	Date 1/26/93
Independent Reviewer	Nancy E. McCarroll, B.S.	Date 1/26/93
QA/QC Manager	Sharon Segal, Ph.D.	Date 1/26/93

Contract Number: 68D10075
Work Assignment Number: 2-25
Clement Number: 84
Project Officer: Caroline Gordon

010032

GUIDELINE § 84: MUTAGENICITY
MAMMALIAN CELLS IN CULTURE GENE MUTATION

EPA Reviewer: Byron T. Backus, Ph.D. — Signature: Byron T. Backus — Date: 2/1/93
EPA Review Section II, Toxicology Branch II
Health Effects Division (H-7509C)

EPA Reviewer: Linda Taylor, Ph.D. — Signature: Linda Lee Taylor — Date: 2-2-93
EPA Review Section II, Toxicology Branch II
Health Effects Division (H-7509C)

EPA Section Head: K. Clark Swentzel — Signature: K. Clark Swentzel — Date: 2/2/93
EPA Review Section II, Toxicology Branch II
Health Effects Division (H-7509C)

DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity: Gene mutation in cultured Chinese hamster ovary cells (CHO/HGPRT)

EPA IDENTIFICATION NUMBERS:

Tox Chem. Number: 012301

MRID Number: 424656-01

TEST MATERIAL: Bromacil

SYNONYMS/CAS NUMBER: 2,4(1H,3H)-Pyrimidinedione, 5-bromo-6-methyl-3-(1-methylpropyl)-uracil; 5-bromo-3-sec-butyl-6-methyluracil/314-40-9

SPONSOR: E.I. du Pont de Nemours and Co., Inc., Wilmington, DE

STUDY NUMBERS: Haskell Laboratory Report Number 739-88; Medical Research Number 8452-001

TESTING FACILITY: Haskell Laboratory, E.I. du Pont de Nemours and Co., Inc., Newark, DE

TITLE OF REPORT: Mutagenicity Evaluation of Bromacil in the CHO/HPRT Assay

AUTHOR: K.S. Bentley

REPORT ISSUED: December 9, 1988

CONCLUSIONS--EXECUTIVE SUMMARY: No conclusions can be reached from the series of Chinese hamster ovary (CHO) cell HGPRT assays conducted with nonactivated doses of bromacil ranging from 99 to 990 µg/mL. The inability to reproduce comparable results among replicate cultures for individual treatment groups or among the three trials precludes a full assessment of the biological significance of the increased mutations frequencies obtained in Trial 1 and

Trial 3 (see Section C Reported Results and Section D Reviewers' Discussion). By contrast to the lack of uniform data under nonactivated conditions, S9-activated doses of bromacil ranging from 248 to 1980 µg/mL were clearly not mutagenic and cytotoxicity was achieved at ≥990 µg/mL in both trials. We conclude, however, that the inconclusive results from the nonactivated phase of testing renders the study unacceptable.

STUDY CLASSIFICATION: Unacceptable. The study does not satisfy Guideline requirements (§84-2) for genetic effects, Category I, Gene Mutations.

A. MATERIALS:

1. Test Material: Bromacil

Description: Tan solid
Identification number: IN N976; Haskell number 16,473; lot number 180-806 3T Batch 31
Purity: 95.1%
Receipt date: Not reported
Stability: Assumed to be stable under the conditions of the study
Contaminants: None listed
Solvent used: Dimethyl sulfoxide (DMSO)
Other provided information: Neither the storage conditions for the test material nor the frequency of dose solution preparation were reported. Analytical determinations were not performed to verify test concentrations or test material stability.

2. Culture Medium: Ham's F12 medium without hypoxanthine, containing L-glutamine, 25 mM HEPES buffer, 5% dialyzed heat-inactivated fetal bovine serum (DHIFBS), and antibiotics. Culture medium for the S9-activated treatment did not contain DHIFBS.

3. Control Materials:

Negative: None

Solvent: DMSO, at a final concentration of 1%

Positive:

Nonactivation: Ethyl methanesulfonate (EMS) was prepared in phosphate buffered saline (pH 7.0) to yield a final concentration of 62.1 µg/mL.

Activation: 9,10-Dimethyl-1,2-benzanthracene (DMBA) was prepared in DMSO to yield a final concentration of 3.8 µg/mL.

MAMMALIAN CELLS IN CULTURE GENE MUTATION

4. Activation: S9 derived from 8-9-week old male Crl:CD®BR

x Aroclor 1254	x induced	x rat	x liver
____ phenobarbital	____ noninduced	____ mouse	____ lung
____ none		____ hamster	____ other
____ other		____ other	

The S9 homogenate was purchased from Sitek Research Laboratories, Rockville, MD, and the protein content was determined by the performing laboratory; these data were not provided. The S9 mix was prepared as follows:

Component	Final Concentration
Glucose 6-phosphate	5 mM
NADP	1.5 mM
$MgCl_2$	5.6 mM
S9 fraction (1 mg protein/mL)	1 mg/mL

5. Test Cells: Mammalian cells in culture

____ mouse lymphoma L5178Y cells
x Chinese hamster ovary (CHO) cells
____ V79 cells (Chinese hamster lung fibroblasts)
____ other (list):

Properly maintained? Yes.
Periodically checked for mycoplasma contamination? Yes.
Periodically checked for karyotype stability? Not reported.
Periodically "cleansed" against high spontaneous background? Not reported.

6. Locus Examined:

____ thymidine kinase (TK)
selection agent: _____ bromodeoxyuridine (BrdU)
_____ fluorodeoxyuridine (FdU)

x hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)
selection agent: _____ 8-azaguanine (8-AG)
0.01 µmole/mL 6-thioguanine (6-TG)

____ Na^+/K^+ATPase
selection agent: _____ ouabain

____ other (locus and/or selection agent; give details)

7. Test Compound Concentrations Used:

(a) Preliminary range-finding study: The number of doses used in range-finding assay were not specified. Information was

presented for doses of 990 and 2475 µg/mL.

(b) Mutation assay: Three nonactivated and two S9-activated trials were conducted; doses used were as follows:

Nonactivated conditions: 99, 297, 594, 792, and 990 µg/mL (all trials)

S9-activated conditions:
Trial 1: 248, 495, 990, 1485 and 1980 µg/mL
Trial 2: 248, 495, 743, 990 and 1180 µg/mL

B. TEST PERFORMANCE:

1. Cell Treatments:

(a) Cells exposed to the test compound, negative controls and positive controls for:
18-19 hours (nonactivated) 5 hours (activated)

(b) After washing, cells were cultured for at least 7 days (expression period) before cell selection.

(c) After expression, $2x10^5$ cells/dish (5 dishes/flask) were cultured for 6-8 days in selection medium to determine numbers of mutants; 200 cells/dish (6 dishes/flask) were cultured for 6-8 days in nonselection medium to determine cloning efficiency.

2. Statistical Methods: The mutation frequency (MF) data were transformed (using Snee and Irr's (1981)[1] formula, Y=(mutant frequency + 1)$^{0.15}$) and analyzed using Student's t-test and Dunnett's test. Dose-response relationships were analyzed by a two-way analysis of variance (ANOVA). A combined statistical analysis was presented for the results of the three nonactivated trials; results of the statistical analysis of the data from each S9-activated trial were performed and presented separately.

3. Evaluation Criteria:

(a) Assay Validity: The study was considered valid if the average initial cell survival and cloning efficiency (CE) of controls were within the range of 50-115% and the average spontaneous mutant frequency was ≤20 in 10^6 surviving cells

Positive Response: The response was considered positive if there was a significant increase [illegible] the MF at one or more

[illegible]

concentrations, and there was a significant dose-response ($p<0.01$).

4. Protocol: Not provided.

C. REPORTED RESULTS:

1. Range-finding Study: The solubility limit of the test material in DMSO was reported to be 250 mg/mL, therefore, the highest final concentration tested in the range-finding study was near 2500 µg/mL. Nonactivated 990 µg/mL produced 80% cytotoxicity; the high dose (2450 µg/mL +S9) was reported to have produced "sufficient toxicity to preclude it from mutagenicity testing." No further information was presented in the study report. Based on these findings, the concentrations selected for the mutagenicity tests ranged from 99 to 990 µg/mL -S9 and from 248 to 1980 µg/mL +S9.

2. Mutation Assays:

(a) Nonactivated conditions: Representative results from the three nonactivated trials conducted with bromacil are presented in Table 1. In Trial 1, the high dose (990 µg/mL) was cytotoxic; relative percent CE was 22.9%. Our reviewers noted that the MFs and total mutant clones for one of the two replicate cultures treated with 297, 594, 792, and 990 µg/mL and both cultures exposed to 99 µg/mL were markedly increased compared to the solvent cultures. Average results combined by our reviewers for both cultures in each treatment group indicated a dose-reversal response (i.e., higher MFs as the dose decreased). The assay was, therefore, repeated with comparable doses. In agreement with Trial 1, 990 µg/mL was cytotoxic; there was, however, no indication of a mutagenic response at any concentration of bromacil (99-990 µg/mL). A third trial was conducted, presumably to confirm the results from Trial 2. However, in contrast to the previous findings, the high dose (990 µg/mL) was not cytotoxic. The relative percent CE was 78.8% at 990 µg/mL as compared to 22.9 and 11.5% at an equivalent level in Trials 1 and 2, respectively. Conflicting results were also obtained with the high dose group. One of the two replicate cultures exposed to 990 µg/mL had a marked increase in total mutant colonies and the MF, while the data from the parallel culture were clearly negative. MFs for each replicate treated with 990 µg/mL were 2.7 and 56.9×10^{-6}. Although the study author claimed that statistical analysis was performed on each trial separately and no significant increase in the MF was observed at any of the test concentrations in any trial, only statistical analysis of the data combined for the three trials was presented (see CBI p. 18). Of additional concern was the lack of comment on the biological significance, if any, of the marked increases in MFs for single

replicate cultures for the majority of treatment groups in Trial 1 and a similar finding for the high dose cultures in Trial 3. Owing to the inability to reproduce comparable results among replicates in individual treatment groups or among the three trials, our reviewers consider the data from the nonactivated trials to be inconclusive.

(b) S9-Activated Conditions: Results from the two S9-activated trials were in general agreement and indicated that levels ≥990 µg/mL were cytotoxic (Table 2). No appreciable increase in the number of mutant colonies or the MF was observed at any dose in either trial. It was noteworthy that the statistical analyses were performed and presented separately from the individual trials. The positive control (3.8 µg/mL DMBA) induced significant ($p<0.01$) increases in mutations at the HGPRT locus in both trials.

Based on the overall results, the study author concluded that bromacil was not mutagenic in the CHO/HGPRT forward gene mutation assay.

D. REVIEWERS' DISCUSSION AND INTERPRETATION OF RESULTS: We assess that the data are insufficient to support the study author's claim that bromacil was not mutagenic in this test system. The lack of concordance of the data from the three nonactivated trials precludes a full evaluation of the biological significance of the increased MFs obtained for single replicates at the majority of doses in Trial 1 and at the high dose in Trial 2. Although variability in MFs among replicate culture does often occur, it is generally associated with the random killing of the mutant fraction in one culture and the cytotoxicity experienced by chance in the wild-type cells in the parallel culture. In this study, however, the marked variations in MFs occurred at either slightly cytotoxic or noncytotoxic doses, and therefore cannot be considered an effect of compound cytotoxicity.

While the findings from the S9-activated trials provided convincing evidence that S9-activated bromacil was not mutagenic in this in vitro mammalian cell gene mutation assay, the inconclusive results from the nonactivated trials render the study unacceptable.

E. QUALITY ASSURANCE MEASURES: Was the test performed under GLP? Yes. (A quality assurance statement was signed and dated December 5, 1988.)

F. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 7-11.

CORE CLASSIFICATION: Unacceptable. The study does not satisfy Guideline requirements (§84-2) for genetic effects, Category I, Gene Mutations.

Table 1. Representative Results of the Nonactivated Chinese Hamster Ovary (CHO) Cell Forward Gene Mutation Assay with Bromacil

Substance	Dose/mL	S9 Activation	Relative Percent Cloning Efficiency (After Treatment)[a]	Average Number of Mutant Colonies[a]	Average Absolute Percent Cloning Efficiency (at Selection)	Average Mutation Frequency x10^{-6}[b]
Solvent Control						
Dimethyl sulfoxide	10 µL	-	100.0[c]	1	0.68	1.5
		-	100.0[d]	0	0.91	0.0
		-	100.0[e]	14	0.73	19.2
Positive Control						
Ethyl methanesulfonate	62.1 µg	-	72.2[c]	167	0.54	309.3*
		-	83.4[d]	172	0.80	215.0*
		-	95.3[e]	138	0.61	226.2*
Test Material						
Bromacil	99 µg	-	>100[c]	27	0.65	41.5
	297 µg	-	96.3	19[f]	0.69	27.5[f]
	594 µg	-	83.6	14[f]	0.53	26.4[f]
	792 µg	-	71.3	9[f]	0.63	14.3[f]
	990 µg	-	22.9	4[f]	0.59	6.8[f]
	792 µg[g]	-	28.0[d]	3	0.73	4.1
	990 µg	-	11.5	2	0.90	2.2
	792 µg[g]	-	100.8[e]	13	0.63	20.6
	990 µg	-	78.8	2/38[h]	0.73/0.67[h]	2.7/56.7[h]

[a]Based on the results of duplicate plates from two flasks per treatment, vehicle or positive control group; calculated by our reviewers. Minor differences between reported and recalculated values presumably resulted from the rounding-off of fewer significant digits by our reviewers.

[b]Mutation Frequency (MF) = $\frac{\text{Average number of total mutant colonies}}{\text{No. of Cells Plated } (1\times10^6) \times \text{Cloning Efficiency}}$; calculated by our reviewers.

[c]Results from Trial 1
[d]Results from Trial 2
[e]Results from Trial 3
[f]One of the duplicate cultures in this treatment group had increased total mutant colonies and MF; results for the remaining cultures in this group were negative.
[g]Findings for lower doses (99, 297, and 594 µg/mL in Trials 2 and 3) did not suggest a mutagenic response.
[h]Values for each replicate culture are presented separately.

*Significantly higher than control ($p \leq 0.01$) by Student's t-test

Note: Data were extracted from the study report; see CBI pp. 15-18.

Table 2. Representative Results of the S9-Activated Chinese Hamster Ovary (CHO) Cell Forward Gene Mutation Assay with Bromacil

Substance	Dose/mL	S9 Activation	Relative Percent Cloning Efficiency (After Treatment)[a]	Average Number of Mutant Colonies[a]	Average Absolute Percent Cloning Efficiency (at Selection)	Average Mutation Frequency x10^{-6}[b]
Solvent Control						
Dimethyl sulfoxide	10 µL	+	100.0[c]	7	0.72	9.7
		+	100.0[d]	2	0.57	3.5
Positive Control						
9,10-Dimethyl-1,2-benz-anthracene	3.8 µg	+	95.2[c]	107	0.69	155.1*
		+	85.9[d]	164	0.67	244.8*
Test Material						
Bromacil	495 µg[e]	+	88.1[c]	9	0.69	13.0
	990 µg[f]	+	1.3	5	0.71	7.0
	743 µg[e]	+	77.7[d]	0	0.66	0.0
	990 µg	+	19.9	0	0.56	0.0
	1188 µg	+	7.3	2	0.59	3.4

[a]Based on the results of duplicate plates from two flasks per treatment, vehicle or positive control group; calculated by our reviewers. Minor differences between reported and recalculated values presumably resulted from the rounding-off of fewer significant digits by our reviewers.

[b]Mutation Frequency (MF) = $\frac{\text{Average number of total mutant colonies}}{\text{No. of Cells Plated } (1\text{x}10^6) \text{ x Cloning Efficiency}}$; calculated by our reviewers.

[c]Results from Trial 1

[d]Results from Trial 2

[e]Findings for the lower doses (248 µg/mL in Trial 1 and 248 and 495 µg/mL in Trial 2) did not suggest a mutagenic response.

[f]Higher doses (1485 and 1980 µg/mL) were severely cytotoxic (i.e., <1% of the cells survived treatment).

*Significantly higher than control ($p \leq 0.01$) by Student's t-test

Note: Data were extracted from the study report; see CBI pp. 19-22.

APPENDIX A

MATERIALS AND METHODS
CBI pp. 7-11

BROMACIL TXR 010032

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Pages 37 through 41 are not included.

The material not included contains the following type of information:

____ Identity of product inert ingredients.

____ Identity of product impurities.

____ Description of the product manufacturing process.

____ Description of quality control procedures.

____ Identity of the source of product ingredients.

____ Sales or other commercial/financial information.

____ A draft product label.

____ The product confidential statement of formula.

____ Information about a pending registration action.

X FIFRA registration data.

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

010032

FINAL

DATA EVALUATION REPORT

BROMACIL

Study Type: Mutagenicity: Unscheduled DNA Synthesis Assay in Primary Rat Hepatocytes

Prepared for:

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

Prepared by:

Clement International Corporation
9300 Lee Highway
Fairfax, VA 22031-1207

Principal Reviewer	Kristin Jacobson Kristin Jacobson, MSPH	Date 1/26/93
Independent Reviewer	Nancy L. McCarroll Nancy E. McCarroll, B.S.	Date 1/26/93
QA/QC Manager	Sharon A. Segal Sharon Segal, Ph.D.	Date 1/26/93

Contract Number: 68D10075
Work Assignment Number: 2-25
Clement Number: 86
Project Officer: Caroline Gordon

GUIDELINE §84: MUTAGENICITY UDS

EPA Reviewer: Byron T. Backus, Ph.D.
EPA Review Section II, Toxicology Branch II
Health Effects Division (H-7509C)
Signature: Byron T. Backus
Date: 2/1/93

EPA Reviewer: Linda Taylor, Ph.D.
EPA Review Section II, Toxicology Branch II
Health Effects Division (H-7509C)
Signature: Linda Taylor
Date: 2-2-93

EPA Section Head: K. Clark Swentzel
EPA Review Section II, Toxicology Branch II
Health Effects Division (H-7509C)
Signature: K. Clark Swentzel
Date: 2/2/93

DATA EVALUATION REPORT 010032

STUDY TYPE: Mutagenicity: In vitro unscheduled DNA synthesis assay in primary rat hepatocytes.

EPA IDENTIFICATION NUMBERS.:

Tox Chem. Number: 012301

MRID Number: 424659-01

TEST MATERIAL: Bromacil

SYNONYMS/CAS NUMBER: 2,4(1H,3H)-Pyrimidinedione, 5-bromo-6-methyl-3-(1-methylpropyl)-uracil, 5-bromo-3-sec-butyl-6-methyluracil/314-40-9

SPONSOR: E.I. du Pont de Nemours and Co., Inc., Wilmington, DE

STUDY NUMBERS: Haskell Laboratory Report Number 518-88; Medical Research Number 8452-001

TESTING FACILITY: Haskell Laboratory, E.I. du Pont de Nemours and Co., Inc., Newark, DE

TITLE OF REPORT: Assessment of Bromacil in the In Vitro Unscheduled DNA Synthesis Assay in Rat Primary Hepatocytes

AUTHOR: K.S. Bentley

REPORT ISSUED: September 9, 1988

CONCLUSIONS-EXECUTIVE SUMMARY: Under the conditions of two independently performed unscheduled DNA synthesis (UDS) assays, bromacil at concentrations ranging from 0.1 to 500 µg/mL did not increase the frequency of UDS in primary rat hepatocytes. Higher levels (≥1000 µg/mL) were cytotoxic. Based on these findings, it was concluded that bromacil was tested over an appropriate range of concentrations and failed to induce a genotoxic response in this in vitro assay.

GUIDELINE §84: MUTAGENICITY UDS

STUDY CLASSIFICATION: Acceptable. The study satisfies Guideline requirements (§84-4) for genetic effects, Category III, Other Mutagenic Mechanisms.

A. MATERIALS:

1. Test Material: Bromacil

   Description: Tan solid
   Identification number: IN N976; Haskell number 16,473; Lot number 180-806 3T Batch 31
   Purity: 95.1%
   Receipt date: Not reported
   Stability: Assumed to be stable for the duration of the study
   Contaminants: None listed
   Solvent used: Dimethyl sulfoxide (DMSO)
   Other provided information: Storage conditions were not reported. Test solutions were prepared immediately prior to use. Analytical determinations were not performed to verify test concentrations or test material stability.

2. Indicator Cells: Primary rat hepatocytes were obtained by in situ perfusion of the livers of adult male Crl:CD®BR rats obtained from Charles River Laboratories, Kingston, NY.

3. Control Substances:

   (a) Negative: None

   (b) Solvent: DMSO, at a final concentration of 1%

   (c) Positive: 2-Acetylaminofluorene (2-AAF) was prepared in DMSO and administered at concentrations of 0.022 and 0.22 µg/mL

4. Test Compound Concentrations Used:

   (a) Initial assay: 1, 10, 50, 100, 500, 1000, 1500, 2000, and 2500 µg/mL

   (b) Confirmatory assay: 0.1, 1, 10, 50, 100, 500, 1000, 1500, and 2000 µg/mL

B. STUDY DESIGN:

1. Cell Preparation:

   (a) Perfusion techniques: Rats were anesthetized with sodium pentobarbital. The livers were perfused with serum-free Williams' Medium E (WME) containing 10 mM HEPES buffer and

100 units/mL collagenase. Livers were excised, placed in the perfusion solution and combed to release the hepatocytes.

(b) Hepatocyte harvest/culture preparation: Recovered cells were centrifuged, resuspended in cold Hank's balanced salt solution (HBSS), and filtered to remove clumps of cells and debris. The cells were mixed with Percoll® working solution (90% Percoll®, 10% 10x-HBSS), centrifuged, washed twice and resuspended in Hepatocyte Plating Medium (HPM, consisting of complete WME with 10 mM HEPES buffer).

Cell viability and density were assessed by trypan blue exclusion. Pooled hepatocyte suspensions were prepared from two livers for each trial, and chamber slides were inoculated with $5x10^5$ viable hepatocytes/chamber. Cells were allowed to attach to the surface of slides for an unstated length of time in a 37°C incubator. Four cultures were prepared per treatment level.

2. UDS Assay:

(a) Treatment/slide preparation: Prepared hepatocyte cultures were exposed for 18 hours to the selected doses of the test material, solvent or positive control. The treatment medium contained 5 µCi/mL [methyl-^{3}H]-thymidine. To evaluate cytotoxicity, the treatment medium was removed, centrifuged, and assayed for lactate dehydrogenase (LDH) activity. Cells attached to the slides were rinsed in WME, swollen in 1% sodium citrate for 8 minutes, fixed in ethanol:glacial acetic acid (3:1), rinsed in distilled deionized water, and air dried.

(b) Preparation of autoradiographs/grain development: Slides were dipped in Kodak NTB-2 emulsion, dried and stored in slide boxes containing a dessicant at -70°C for 4-5 days. Slides were developed, stained with methyl-green pyronin Y, coded and scored.

(c) Grain counting: Cells with normal nuclear morphology, apparent cytoplasm, one nucleus, and free of debris or staining artifacts were scored using a colony counter. A total of 25 cells per slide were evaluated by measuring the area of silver grains over the nucleus and the area of grains in two or more nucleus-sized regions in the cytoplasm immediately adjacent to the nucleus; the highest cytoplasm value was recorded.

Areas of grains were converted to numbers of grains using a conversion factor calculated for each slide. The conversion factor was defined as "the mean ratio of manually-counted grains to areas of the same grains from three nucleus-sized patches of the slide. Nuclear cytoplasmic areas were then multiplied by the factor to derive nuclear or cytoplasmic grains." Net nuclear grain (NNG) counts were determined by subtracting cytoplasmic grains from nuclear grains. Mean NNG and standard error, mean nuclear grains, mean cytoplasmic grains, median net nuclear

grains, and the percentage of cells in repair (i.e., cells with ≥5 NNG) were calculated for each slide. The average of individual mean NNGs at each dose level was calculated to determine the UDS response at that dose for each trial.

3. Statistical Analysis: Within-trial and between-trial means and respective standard errors calculated for each treatment level were first transformed by the equation Y=(NNG+20) (to obtain positive values for all data) and compared using a two-way analysis of variance (ANOVA). Dose-response patterns were evaluated using an F-test. If the dose-trial interaction was significant ($p<0.01$), the results of the two trials were analyzed separately.

4. Evaluation Criteria:

   (a) Assay validity: For the assay to be considered valid, the following were required: (1) hepatocyte viability ≥75%, with ≥25 scorable cells per culture and ≥50% scorable cultures per test concentration; (2) mean NNG <5 in solvent controls; and (3) mean NNG ≥5 in positive control cultures. Concentrations were not scored if variability across scored cultures was excessive when there was clear evidence of cytotoxicity.

   (b) Positive response: The test material was considered positive if the average UDS response for any concentration from both trials was ≥5 NNG and ≥3 standard deviations above the control response, and there was a dose-response pattern observed for average UDS responses in the absence of a negative correlation between test concentration and average cytoplasmic grains ($p<0.01$).

C. REPORTED RESULTS:

The limit of solubility of the test material in DMSO was reported to be 250 mg/mL. Accordingly, the nine doses selected for the UDS assay ranged from 1 to 2500 µg/mL. Precipitate, which cleared after incubation, was observed at doses ≥500 µg/mL. Representative data for the UDS assays are presented in Table 1. Cytotoxicity, as indicated by the increased release of LDH into the culture medium, was observed at doses ≥1000 µg/mL in both trials. Owing to severe cytotoxicity, cultures ≥1000 µg/mL could not be scored in the first trial; therefore, the concentrations were adjusted to 0.1-2000 µg/mL for the second trial. For all treatment levels, the mean net nuclear grains were negative values. By contrast, in cultures exposed to 0.022 or 0.22 µg/mL 2-AAF, there were significant ($p<0.01$) increases in UDS activity.

Based on these results, the study author concluded that bromacil was negative in the primary rat hepatocyte UDS assay.

D. REVIEWERS' DISCUSSION/CONCLUSIONS: We assess that the study was well-conducted and the study author correctly interpreted the data. In the first trial, the test material was assayed up to the solubility limit

(final culture concentration 2500 µg/mL). Concentrations ≥1000 µg/mL were cytotoxic in both trials. There was, however, no indication of UDS activity at doses ranging from 1-500 µg/mL (first trial) and 0.1-500 µg/mL (second trial). In addition, the sensitivity of the test system to detect genotoxicity was clearly demonstrated by the response obtained in cultures exposed to the positive control (0.022 or 0.22 µg/mL 2-AAF). It was concluded, therefore, that bromacil was tested over an appropriate range of concentrations and was found to be negative in this in vitro genotoxicity assay.

E. QUALITY ASSURANCE MEASURES: Was the test performed under GLPs? Yes. (A quality assurance statement was signed and dated September 6, 1988.)

F. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 7-13

CORE CLASSIFICATION: Acceptable. The study fully satisfies Guideline requirements (§84-4) for genetic effects Category III, Other Mutagenic Mechanisms.

Table 1. Representative Results of the Unscheduled DNA Synthesis Rat Hepatocyte Assays with Bromacil

Treatment	Dose/mL	Initial Trial LDH Activity (units/L)	Initial Trial Mean Net Nuclear Grains[a] ±S.E.	Confirmatory Trial LDH Activity (units/L)	Confirmatory Trial Mean Net Nuclear Grains[a] ±S.E.
Solvent Control					
Dimethyl sulfoxide	10 µL	108	-15.7±1.1	273	-3.8±1.8
Positive Control					
2-Acetylaminofluorene	0.022 µg[b]	64	21.6±5.9*	320	35.4±8.9*
Test Material					
Bromacil	500 µg[c]	132	-6.7±2.5	420	-5.6±0.1
	1000 µg[d]	288	e	601	e

[a]Means and standard errors of the counts of 100 cells; 25 cells from four slides were analyzed for each treatment.
[b]Positive results were also obtained with 0.22 µg/mL of the positive control; the data from the lower dose were selected as representative
[c]Lower doses (1, 10, 50, and 100 µg/mL in the initial assay, and 0.1, 1, 10, 50, and 100 in the confirmatory assay) did not show evidence of genotoxicity.
[d]Higher doses (1500, 2000 and 2500 µg/mL in the initial assay, and 1500 and 2000 µg/mL in the confirmatory assay) were also cytotoxic.
[e]Too cytotoxic to score

*Significantly higher ($p<0.01$) than the solvent control by Student's t-test

Note: Data were extracted from the study report; see CBI pp. 14-19.

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APPENDIX A

MATERIALS AND METHODS
CBI pp. 7-13

BROMACIL TXR 010032

Page_____ is not included in this copy.

Pages 50 through 56 are not included.

The material not included contains the following type of information:

____ Identity of product inert ingredients.

____ Identity of product impurities.

____ Description of the product manufacturing process.

____ Description of quality control procedures.

____ Identity of the source of product ingredients.

____ Sales or other commercial/financial information.

____ A draft product label.

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