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

008115

OCT 3 1990

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Carbaryl (1-naphthyl N-methylcarbamate)

TOX Chem No. 160
Project No. 0-0762

FROM: Ray Landolt 9/27/90
Review Section I, Toxicology Branch II
Herbicide, Fungicide, and Antimicrobial Support
Health Effects Division (H7509C)

TO: Dennis Edwards, Jr., PM 12
Insecticide - Rodenticide Branch
Registration Division (H7504C)

THRU: Mike Ioannou, Section Head
Review Section I, Toxicology Branch II
Herbicide, Fungicide, and Antimicrobical Support
Health Effects Division (H7509C) J M Ioannou 9/27/90
and
Marcia van Gemert, Branch Chief Marcia van Gemert 10/1/90
Toxicology Branch II
Herbicide, Fungicide, and Antimicrobical Support
Health Effects Division (7509C)

Registrant: Rhone-Poulenc AG Company letter of January 26, 1990

Action Requested: Review four mutagenicity studies conducted with carbaryl as a requirement of CDFA under their Birth Defects Prevention Act.

Conclusion: The following studies were cited as data gaps in the California Department of Food and Agriculture memorandum of October 25, 1988. The Agency (February 2, 1989) did not concur with CDFA that there were data gaps for mutagenicity.

1. Unscheduled DNA Synthesis Assay in Primary Rat Hepatocytes.

   Carbaryl is not genotoxic in this test syetem at 5.0 to 25.0 ug/mL.

   Not acceptable- MRID No. 413703-01
   May be upgraded if either the raw data or calculated means and standard deviations for net nuclear grain counts are provided.

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2. Mammalian Cells in Culture Gene Mutation Assay in Chinese Hamster Ovary (CHO) Cells.

   The data does not support the conclusion that carbaryl is negative in this test system under both the S9 metabolic activation and nonactivation conditions of this assay.

   Not acceptable- MRID No. 413703-02 and MRID No. 414202-01
   The data are insufficient to characterize the mutagenic activity of carbaryl in this test system.

3. Salmonella typhimurium/Mammalian Microsome Mutagenicity Assay (84-2a).

   Acceptable-MRID No. 413703-03
   Carbaryl is not mutagenic in this test system. The test material was assayed to a level (2000 ug/plate +/-S9) that was cytotoxic to all strains but failed to induce a mutagenic response over a nonactivated and S9-activated concentration range of 5 to 2000 ug/plate.

4. Mammalian Cells in Culture Cytogenetic Assay in Chinese Hamster Ovary (CHO) Cells (84-2b).

   Acceptable-MRID No. 413703-04
   Carbaryl was clastogenic in the cultured CHO cells with metabolic activation.

EPA No.: 68D80056
DYNAMAC No.: 305-A
TASK No.: 3-05A
September 19, 1990

DATA EVALUATION RECORD

CARBARYL

Mutagenicity--Unscheduled DNA Synthesis Assay
in Primary Rat Hepatocytes

APPROVED BY:

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

Signature: Robert J. Weir

Date: 9/19/90

Guideline Series 84: **Mutagenicity**

EPA No.: 68D80056
DYNAMAC No.: 305-A
TASK No.: 3-05A
September 19, 1990

DATA EVALUATION RECORD

CARBARYL

Mutagenicity--Unscheduled DNA Synthesis Assay
in Primary Rat Hepatocytes

REVIEWED BY:

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

Signature: Nancy E. McCarroll
Date: 9-19-90

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

Signature: for I. Cecil Felkner
Date: 9/19/90

APPROVED BY:

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

Signature: Nicolas P. Hajjar
Date: September 19, 1990

Raymond Landolt, Ph.D.
EPA Reviewer, Section I
Toxicology Branch II
(H-7509C)

Signature: Raymond Landolt
Date: September 25, 1990

Mike Ioannou, Ph.D.
EPA Section Head, Section I
Toxicology Branch II
(H-7509C)

Signature: M. Ioannou
Date: 9-26-90

DATA EVALUATION RECORD

TOX. Chem. No.:
EPA File Symbol:

CHEMICAL: Carbaryl.

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

MRID NUMBER: 413703-01.

TEST MATERIAL: Carbaryl technical.

SYNONYM(S): None listed.

STUDY NUMBER: 10862-0-447.

SPONSOR: Rhone-Poulenc AG Co., Research Triangle Park, NC.

TESTING FACILITY: Hazleton Laboratories America, Inc., Kensington, MD.

TITLE OF REPORT: Mutagenicity Test on Carbaryl Technical in the *in vitro* Rat Primary Hepatocyte Unscheduled DNA Synthesis Assay.

AUTHOR: M. A. Cifone.

REPORT ISSUED: November 22, 1989.

CONCLUSIONS - Executive Summary:

Under the conditions of two independent assays, six doses of carbaryl technical ranging from 0.5 to 25.0 µg/mL in the first assay and six doses ranging from 5.0 to 25.0 µg/mL in the repeat assay did not induce an appreciable increase in the net nuclear grain counts of treated rat hepatocytes. Doses >25.0 µg/mL were severely cytotoxic; reduced cell survival (≈25%) was observed at 25.0 µg/mL in both assays. Although an increase in the percentage of cells with ≥6 grains per nucleus was seen in the initial test, the increase was confined to a single dose (10 µg/mL) and was not dose related or reproducible. The data suggest, therefore, that carbaryl technical was assayed to an appropriate level with no reproducible evidence of a genotoxic effect. However, the study was compromised for the following reasons:

1. Presentation of average values without the actual grain counts or some indication of variability (standard deviations) is not an acceptable practice.

2. No information on test material stability, storage conditions, or the analytical data from the chemical analysis of frozen samples of the test material dilutions was reported.

We assess, therefore, that the study does not fully satisfy Guideline requirements for genetic effects, Category III, Other Mutagenic Mechanisms.

Study Classification: The study is unacceptable, but it can be upgraded if either the raw data or calculated means and standard deviations for net nuclear grain counts and the missing test material information are provided.

A. MATERIALS:

1. Test Material:

Name:	Carbaryl (technical)
Description:	White powder.
Batch/Lot No.:	87191.
Purity:	99.3%
Contaminants:	Not listed.
Solvent used:	Dimethylsulfoxide (DMSO).
Other comments:	Storage conditions were not reported. The test material formed a clear, pale yellow solution in DMSO at a concentration of 75 mg/mL. Solutions of the test material were prepared for each trial, and samples of the dilutions were frozen and retained for chemical analysis.

2. Indicator Cells: Primary rat hepatocytes were obtained by the in situ perfusion of the livers of adult male Fischer 344 rats purchased from Harlan Sprague-Dawley, Inc. (Trial 1, 221.9 g; Trial 2, 247.9 g).

3. Control Substances: DMSO at a final concentration of 1% was used as the solvent control, and 2-acetylaminofluorene (2-AAF) at 0.10 µg/mL was used as the positive control.

B. STUDY DESIGN:

1. Cell Preparation:

a. Perfusion Technique: The livers were perfused with Hanks' balanced salts containing 0.5 mM EGTA and HEPES buffer, pH 7.2, for 4 minutes and with incomplete William Medium E (WMEI) containing 50 to 100 units/mL collagenase for 10 minutes. Livers were excised, removed to culture dishes containing WMEI and collagenase, and mechanically dispersed to release the hepatocytes.

b. Hepatocyte Harvest/Culture Preparation: Recovered cells were centrifuged, resuspended in WME containing 10% fetal calf serum, L-glutamine, and antibiotics (WME+), counted, and aliquoted (0.5×10^6 cells/3 mL WME+) onto plastic coverslips. The cultures were placed in a humidified, 37°C, 5% CO_2 incubator for a 1.7- or 1.9-hour attachment period. Unattached cells were removed; viable cells were refed and established as monolayer cultures.

2. Dose Selection: Initially, 15 concentrations of the test material (0.025 to 750 µg/mL) were assayed. When the viability estimate was obtained (18.1 hours after treatment initiation), at least six of these doses were chosen for analysis of nuclear labeling, starting with the highest dose that resulted in a sufficient number of survivors with intact morphologies and proceeding to successively lower doses.

3. UDS Assay:

a. Treatment: Five replicate monolayer cultures were exposed to the selected doses of the test material, negative (DMSO) control, or positive control (2-AAF, 0.1 µg/mL) for 18.1 to 19.0 hours in WMEI containing 5 µCi/mL [^{3}H]thymidine. Treated monolayers were washed twice with WMEI; two of the five replicates for each treatment group were used to determine cytotoxicity.

These cultures were refed, reincubated, and monitored for cytotoxicity at 21.1 to 22.8 hours posttreatment by trypan blue exclusion.

b. UDS Slide Preparation: The remaining cultures were washed with media containing 1 mM thymidine. Treated hepatocytes, attached to coverslips, were exposed to 1% sodium citrate for ≈10 minutes, fixed in acetic acid/ethanol (1:3), dried, and mounted.

c. Preparation of Autoradiographs/Grain Development: Slides were coated with Kodak NTB2 emulsion, dried for 7 to 10 days at 4°C in light-tight desiccated boxes, developed in Kodak D-19, fixed, stained with Williams' modified hematoxylin and eosin, coded, and counted.

d. Grain Counting: The nuclear grains of morphologically normal cells (50/coverslip) for each test dose and negative and positive controls were counted microscopically. Net nuclear grain counts were determined by subtracting the average cytoplasmic grain count of three nuclear-sized areas adjacent to each nucleus from the nuclear grain counts of each cell.

4. Evaluation Criteria:

a. Assay Validity: For the assay to be considered valid, the following criteria must be satisfied: (1) hepatocytes recovered from the perfusion step and monolayer cultures used for the assay must show ≥70% viability; (2) the negative control should have net nuclear grain counts of -5.0 to 1.0, and ≤10% of the cells should contain ≥6 grains/nucleus; (3) the positive control must demonstrate the sensitivity of the test system to detect UDS; (4) data must be obtained from at least two replicate cultures/dose; and (5) the highest dose must show cytotoxicity, the limit of solubility, or reach the maximum recommended dose for this assay (5 mg/mL).

b. Positive Response: The assay was considered positive if: (1) the increase in the mean net nuclear grain count was ≥6 grains/nucleus over the negative control value, and (2) the percentage of nuclei with ≥6 grains exceeded 10% of the negative control population.

C. REPORTED RESULTS:

1. Chemical Analysis: Although the report indicated that samples of the dilutions prepared for the assay were frozen

and retained for chemical analysis, the results from analytical determinations, if performed, were not reported.

2. UDS Assay: Two trials were conducted. For the initial test, 15 doses ranging from 0.0.5 to 750 µg/mL were assayed. The report indicated that doses >100 µg/mL were insoluble in culture medium and that exposure to test doses >25 µg/mL resulted in severe cytotoxicity; however, no data were presented to support either statement.

For the remaining doses, survival ranged from 76.9% at 25.0 µg/mL to 100.0% at doses ≤1.0 µg/mL. Based on these findings, the six doses selected for the evaluation of nuclear labeling were 0.5, 1.0, 2.5, 5.0, 10.0, and 25.0 µg/mL. Results presented in Table 1 indicate that there was no appreciable increase in net nuclear grains at the scored doses of the test material; however, the percent of cells with ≥6 grains per nucleus (20.7%) was increased at 10.0 µg/mL compared with the control (4.7%). This marked increase in the percentage of cells with ≥6 grains per nucleus was neither dose related nor observed at any other assayed level of the test material. Based on the observed increase at a single dose, the assay was repeated using 11 test concentrations ranging from 0.625 to 50.0 µg/mL. In agreement with the earlier results, doses >25 µg/mL were reported to be severely cytotoxic. Percent survival for the remaining levels ranged from 71.0% at 25 µg/mL to 97.7% at ≤5.0 µg/mL. The doses selected to evaluate UDS activity were 5.0, 7.51, 10.0, 15.0, 20.0, and 25.0 µg/mL. None of the scored levels induced an appreciable increase in net nuclear grains or the percentage of cells with ≥6 grains per nucleus (Table 2).

In both trials, the ability of the test system to detect genotoxicity was demonstrated by the results obtained with the positive control (0.1 µg/mL 2AAF).

Based on the overall results, the study author concluded: "The test material, carbaryl technical, did not induce significant changes in the nuclear labeling of rat primary hepatocytes in two independent trials for an applied concentration range of 25.0 µg/mL to 0.500 µg/mL. Carbaryl technical was therefore evaluated as inactive in the Rat Primary Hepatocyte UDS Assay."

D. REVIEWERS' DISCUSSION/INTERPRETATION OF STUDY RESULTS:

We assess that the study was properly conducted and that the study author's interpretation of the data was correct. The highest concentration of carbaryl technical (25 µg/mL) that was evaluated for UDS caused a reproducible reduction in cell

TABLE 1. Representative Results of the Initial Unscheduled DNA Synthesis Rat Hepatocyte Assays With Carbaryl Technical

Treatment	Dose/mL	Cells Scored	Percent Survival (18.1 hours post-exposure)	Average Cytoplasmic Grain Count[a]	Average Net Nuclear Grain Count[a]	Average Percent Nuclei with ≥6 Grains
Solvent Control						
Dimethyl-sulfoxide	1%	150	100.0	13.13	-0.78	4.7
Positive Control						
2-Acetylaminofluorene	0.1 µg	150	73.9	12.58	23.04[b]	99.3[b]
Test Material						
Carbaryl technical	5.0 µg[c]	150	96.4	13.71	-0.45	4.0
	10.0 µg	150	88.7	10.77	1.91	20.7[b]
	25.0 µg[d]	150	76.9	10.38	-0.18	4.0

[a]Average value from triplicate coverslips except for the high dose (25.0 µg/mL); counts for this group are from duplicate coverslips.

[b]Fulfills one or both of the reporting laboratory's criteria for a positive effect.

[c]Results for lower levels (2.5, 1.0, and 0.5 µg/mL) did not indicate a genotoxic effect.

[d]Concentrations >25.0 µg/mL were reported to be severely cytotoxic and, therefore, were not scored; concentrations >100 µg/mL were insoluble in culture medium.

TABLE 2. Representative Results of the Repeat Unscheduled DNA Synthesis Rat Hepatocyte Assays With Carbaryl Technical

Treatment	Dose/mL	Cells Scored	Percent Survival (19.0 hours post-exposure)	Average Cytoplasmic Grain Count[a]	Average Net Nuclear Grain Count[a]	Average Percent Nuclei with ≥6 Grains
Solvent Control						
Dimethyl-sulfoxide	1%	150	100.0	9.09	-1.85	0.7
Positive Control						
2-Acetylaminofluorene	0.1 µg	150	70.8	9.99	17.87[b]	97.3[b]
Test Material						
Carbaryl technical	10.0 µg[c]	150	98.6	10.54	-1.49	4.7
	15.0 µg	150	84.6	8.78	-0.73	2.7
	20.0 µg	150	86.6	9.63	-0.19	4.0
	25.0 µg[d]	150	71.0	9.57	-0.21	3.3

[a]Average value for triplicate coverslips except for 15.0- and 20.0-µg/mL dose groups; counts for these groups are from duplicate coverslips.

[b]Fulfills reporting laboratory's criteria for a positive effect.

[c]Results for lower levels (7.51 and 5.0 µg/mL) did not indicate a genotoxic effect.

[d]Concentrations >25.0 µg/mL were reported to be severely cytotoxic and, therefore, were not scored.

survival (≈23% in Trial 1 and 29% in Trial 2) but was not genotoxic. Although an increased percentage of cells with ≥6 grains per nucleus was scored for the 10-µg/mL treatment level in the first trial, this finding was not reproduced in the repeat assay, which evaluated a narrow range of test material doses between 10 and 25 µg/mL. In addition, there was no evidence of a subtle dose-related effect on net nuclear grain counts or the percentage of cells with ≥6 grains per nucleus in either assay. We conclude, therefore, that carbaryl technical was assayed to a cytotoxic level with no appreciable evidence of a genotoxic effect. However, the study is not acceptable because of the method used to report UDS assay results. Presentation of average values without some indication of the distribution of net grains per nucleus (e.g., standard deviations) is not an acceptable practice. Furthermore, there was no information on test material stability, storage conditions, or the results from the chemical analysis (if performed) of sample dilutions that were frozen at the time of preparation.

E. QUALITY ASSURANCE MEASURES: A quality assurance statement was signed and dated November 22, 1989.

F. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 12-18.

APPENDIX A

Materials and Methods

(CBI pp. 12-18)

Carbaryl toxicology Branch review

Page _____ is not included in this copy.

Pages 14 through 20 are not included in this copy.

The material not included contains the following type of information:

___ Identity of product inert ingredients

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___ Identity of the source of product ingredients

___ Sales or other commercial/financial information

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___ Information about a pending registration action

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EPA No.: 68D80056
DYNAMAC No.: 305-B
TASK No.: 3-05B
September 19, 1990

DATA EVALUATION RECORD

CARBARYL

Mutagenicity--Mammalian Cells in Culture Gene Mutation Assay in Chinese Hamster Ovary (CHO) Cells

APPROVED BY:

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

Signature: Robert J. Weir

Date: 9/19/90

Guideline Series 84: **MUTAGENICITY**

EPA No.: 68D80056
DYNAMAC No.: 305-B
TASK No.: 3-05B
September 19, 1990

DATA EVALUATION RECORD

CARBARYL

Mutagenicity--Mammalian Cells in Culture Gene Mutation Assay in Chinese Hamster Ovary (CHO) Cells

REVIEWED BY:

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

Signature: Nancy E. McCarroll
Date: 9-19-90

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

Signature: R. Cecil Felkner for I. Cecil Felkner
Date: 9/19/90

APPROVED BY:

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

Signature: Nicolas P. Hajjar
Date: September 19, 1990

Raymond Landolt, Ph.D.
EPA Reviewer, Section II
Toxicology Branch II
(H-7509C)

Signature: Raymond Landolt
Date: September 25, 1990

Mike Ioannou, Ph.D.
EPA Section Head, Section II
Toxicology Branch II
(H-7509C)

Signature: M. Ioannou
Date: 9/26/90

DATA EVALUATION RECORD

Tox. Chem. No.: 160

EPA File Symbol:

CHEMICAL: Carbaryl.

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

ACCESSION OR MRID NUMBER: 413703-02 (original submission).
414202-01 (revised submission).

SYNONYM/CAS NUMBER.: None listed.

SPONSOR: Rhone-Poulenc AG Co., Research Triangle Park, NC.

TESTING FACILITY: Hazleton Laboratories America, Inc., Kensington, MD.

TITLE OF REPORT: Mutagenicity Test on Carbaryl (Technical) in the CHO/HGPRT Forward Mutation Assay.

AUTHOR: R. R. Young.

STUDY NUMBER: 10862-0-435.

REPORT ISSUED: November 6, 1989.

CONCLUSIONS - Executive Summary: Carbaryl technical was evaluated in two nonactivated and three S9-activated Chinese hamster ovary (CHO) cell forward mutation assays; the results were as follows:

a. Nonactivated: The findings from both nonactivated assays were in good agreement and indicated that over a concentration range of 1 to 300 μg/mL, the test material did not induce a mutagenic response. Doses ≥200 μg/mL were severely cytotoxic (<10% cell survival), and <50% of the cells survived exposure to ≥50 μg/mL. A significantly increased mutation frequency (MF) relative to the concurrent negative control was observed at 150 μg/mL in the first nonactivated trial; however, the increased MF (7.5×10^{-6}) was within the generally accepted background range for CHO cells ($0\text{-}20 \times 10^{-6}$).[1]

b. S9-Activated: Carbaryl was less cytotoxic in the presence of S9 activation as indicated by increased survival at comparable levels in the preliminary cytotoxicity test (e.g., 29.5% survival at 62.5 μg/mL -S9 as compared with 95.7% survival at 62.5 μg/mL +S9) and the initial mutation assay (e.g., 18.1% survival at 100 μg/mL -S9 as compared with 46.8% at 100 μg/mL +S9). Significantly increased MFs occurred at 150 and 200 μg/mL in the initial trial (dose range: 10 to 200 μg/mL); however, 1.2% of the cells survived exposure at 200 μg/mL, and the increased MF at 150 μg/mL was well within normal background variation.

The second S9-activated trial was aborted because of excessive cytotoxicity at test material levels of ≥10 μg/mL. The study author attributed the increased cytotoxicity to the use of a new batch of S9.

Results from the third S9-activated trial (dose range: 1 to 80 μg/mL) showed severe cytotoxic effects at levels ≥60 μg/mL; no evidence of mutagenic effect was seen at the remaining doses.

We assess that the results of the assays provide no clear indication of a mutagenic response; however, the study does not fully support a negative conclusion for the following reasons:

- The conflicting cytotoxicity data for the S9-activated assays provide no assurance that the final S9-activated mutation assay was conducted over an appropriate dose range. It is noteworthy that doses selected for the in

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[1]Hsie, A. W.; Casciano, D. A.; Couch, D. B.; Krahn, D. F.; O'Neil, J. P.; and Whittefield, B. L. The use of Chinese hamster ovary cells to quantify specific locus mutation and to determine mutagenicity of chemicals. Mutat. Res. 86 (1981): 1193-214.

vitro cytogenetic studies conducted with carbaryl[2] were based on the finding that the nonactivated test material was more cytotoxic than S9-activated carbaryl; this conclusion agrees with the preliminary cytotoxicity and first mutation assay results. Since the study author concluded that the new S9 batch was responsible for the increased cytotoxicity and the inclusion of $CaCl_2$ into the S9-cofactor mix did not substantially lessen the effect, the batch should have been replaced with a fully characterized batch. Additionally, there is concern that the increased cytotoxicity was accompanied by a decrease in assay sensitivity (see Section 5, Reviewers' Discussion/Conclusions).

- Results from the in vitro cytogenetic assay revealed that carbaryl (+/-S9) severely suppressed cell cycle kinetics. For these studies, prolonged cell harvests (20 and 30 hours posttreatment) were used to demonstrate the dramatic clastogenic effects of S9-activated carbaryl. Based on this information, we assess that the 7-day period allowed for cells to recover from carbaryl treatment may have been too short to permit full expression of induced mutations. Normally, a 7 to 9 day expression time is adequate for CHO cells (doubling time: 12 to 16 hours). However, considering the adverse effects of carbaryl on cell cycling time, the expression period should have been increased to 9 days to ensure that conditions were optimum for detection of mutagenesis.

We conclude, therefore, that the study does not fully satisfy Guideline requirements for Category I, Gene Mutations.

Study Classification: The study is unacceptable.

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[2]Murli, H. Mutagenicity test on carbaryl technical in an in vitro cytogenetic assay measuring chromosome aberration frequencies. (Unpublished study No. 10862-447 prepared by Hazleton Laboratories America, Inc., for Rhone-Poulenc AG, Co.; dated August 31, 1989.) MRID No. 413703-04.

MAMMALIAN CELLS IN CULTURE GENE MUTATION

A. MATERIALS:

1. Test Material:

Name:	Carbaryl (technical).
Description:	White powder.
Batch/Lot No.:	87191.
Purity:	99.3%
Contaminants:	Not listed.
Solvent used:	Dimethylsulfoxide (DMSO).
Other comments:	The test material was stored at room temperature in the dark and was found to be soluble in DMSO at 100 mg/mL. The report indicated that concentrations ≥ 500 µg/mL formed a white precipitate when added to culture medium (Ham's F12), but none of the applied concentrations altered the pH of the culture medium outside the range of 7.0 to 7.8.

2. Control Materials:

Negative: Ham's F12 medium supplemented with L-glutamine, 8% fetal bovine serum, and antibiotics. (Note: The negative control was used only for the cytotoxicity assessment.)

Solvent/final concentration: DMSO/1%.

Positive: Nonactivation (concentrations, solvent): 5-Bromo-2'-deoxyuridine (BrdU) was prepared in DMSO to yield a final concentration of 50 µg/mL.

Activation (concentrations, solvent): 3-Methylcholanthrene (MCA) was prepared in DMSO to yield a final concentration of 5 µg/mL.

3. Activation: S9 derived from

X Aroclor 1254	X induced	X rat	X liver
_____ phenobarbital	_____ noninduced	_____ mouse	_____ lung
_____ none		_____ hamster	_____ other
_____ other		_____ other	

If other, describe below. Describe S9 composition (if purchased, give details).

The S9 fraction (Lot Nos. 0249 and 0282) was obtained from Molecular Toxicology, Inc., and was tested for enzymatic activity using either benzo(a)pyrene or MCA prior to use.

S9 mix composition:

Component	Final Concentration in Cultures
NADP (sodium salt)	1.0 mM
Glucose-6-phosphate	5.0 mM
Calcium chloride	2.0 mM
Potassium chloride	6.6 mM
Magnesium chloride	2.0 mM
Phosphate buffer	2.0 mM
S9 homogenate	20.0 µL/mL

4. 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? Yes.
Periodically "cleansed" against high spontaneous background? Yes.

5. Locus Examined:

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

__X__ hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)
Selection agent: _____ 8-azaguanine (8-AG)
(give concentration) 4 µg/mL (24 mM) 6-thioguanine (6-TG)

____ Na^+/K^+ATPase
Selection agent: _____ ouabain
(give concentration)

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

6. Test Compound Concentrations Used:

a. Preliminary cytotoxicity assay: Ten concentrations ranging from 1.95 to 1000 µg/mL were assayed with or without S9 activation.

b. Mutation assay:

1) Nonactivated conditions: Two trials were performed; the doses evaluated were as follows:

- Trial 1: 1, 10, 30, 50, 80, 100, 150, 200, and 300 µg/mL.
- Trial 2: 10, 50, 100, 150, 200, 250, and 300 µg/mL.

2) Activated conditions: Three trials were performed; the doses evaluated were as follows:

- Trial 1: 10, 50, 80, 100, 120, 150, 200, and 300 µg/mL.
- Trial 2: This assay was aborted because of excessive cytotoxicity at doses ≥10 µg/mL.
- Trial 3: 1, 5, 10, 20, 40, 60, 80, 100, and 130 µg/mL.

B. TEST PERFORMANCE:

1. Cell Treatments:

a. Cells exposed to test compound for:
4 hours (nonactivated) 4 hours (activated)

b. Cells exposed to positive controls for:
4 hours (nonactivated) 4 hours (activated)

c. Cells exposed to negative and/or solvent controls for:
4 hours (nonactivated) 4 hours (activated)

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

e. After expression, cells cultured for 7 to 10 days in selection medium to determine numbers of mutants and for 7 to 10 days without selection medium to determine cloning efficiency.

2. Protocol: See Appendix A, Materials and Methods.

3. Preliminary Cytotoxicity Assay: The concentration range of the 10 doses assayed in the preliminary cytotoxicity test was 1.95 to 1000 μg/mL +/-S9. Slight precipitation of the test material was observed at concentrations ≥500 μg/mL. No cells survived treatment with the three highest nonactivated and S9-activated doses (250, 500, or 1000 μg/mL). For the remaining nonactivated doses, relative percent survival (RPS) ranged from 24.4% at 125 μg/mL to ≥80.5% at <15.6 μg/mL (Table 1). In the presence of S9 activation, RPS survival ranged from 28.3% at 125 μg/mL to >95% at doses ≥62.5 μg/mL. Based on these results, nine levels without S9 activation (1 to 300 μg/mL) and eight levels, spanning the same concentration range, with S9 activation were tested in the mutation assay.

4. Mutagenicity Assay: Two nonactivated and three S9-activated mutation assays were performed. The results were as follows:

   a. Nonactivated assays: In the first trial, the highest dose (300 μg/mL) was severely cytotoxic; for the remaining doses, survival relative to the control was dose dependent and ranged from 6.0% at 200 μg/mL to 115.9% at the lowest assayed level (1 μg/mL). The mutation frequency (MF) for cultures exposed to 150 μg/mL was significantly higher ($p \leq 0.01$) than the concurrent solvent control; however, this value was well within the reporting laboratories' acceptable background MF range ($\geq 15 \times 10^{-6}$) and did not exceed the generally accepted background range for CHO cells in this test system (0 to 20×10^{-6}).[3]

   The MFs for the remaining levels were not significantly higher than the solvent control values. A second nonactivated assay was performed using a narrower range of test material concentrations (10, 50, 100, 150, 200, 250, and 300 μg/mL). In the repeat assay, cultures exposed to 250 and 300 μg/mL were not cloned because there was excessive cytotoxicity. The MF at 200 μg/mL was significantly ($p < 0.05$) higher than the solvent control but within the normal background range; similar results were obtained for the lowest assayed dose (10 μg/mL) in the repeat assay. Representative results from the nonactivated trials are presented in Table 2.

[3]Hsie, A. W. et al. Mutat. Res. 86 (1981): 193-214.

TABLE 1. Representative Results from the Preliminary Cytotoxicity Assay with Carbaryl Technical

Substance	Dose μg/mL	S9 Activation	Average No. of Surviving Colonies[a]	Relative Percent Survival
Negative Control				
F_{12} medium	--	-	132.0	92.1
	--	+	150.3	107.1
Solvent Control				
Dimethylsulfoxide	--	-	143.3	100.0
	--	+	140.3	100.0
Test Material				
Carbaryl technical	15.6[b]	-	117.7	82.1
	31.6	-	43.3	30.2
	62.5	-	42.3	29.5
	125.0[c]	-	35.0	24.4
	62.5[d]	+	134.3	95.7
	125.0[c]	+	39.7	28.3

[a]Average count from triplicate plates.

[b]Relative percent survival for the remaining doses (7.8, 3.9, and 1.95 μg/mL) was ≥80.5%.

[c]Higher levels (250, 500, and 1000 μg/mL +/-) were 100% cytotoxic.

[d]Relative percent survival for the remaining doses (31.3, 15.6, 7.8, 3.9, and 1.95 μg/mL) was ≥100%.

Note: Compound precipitation was reported at doses ≥500 μg/mL +/-S9.

TABLE 2. Representative Results of the Nonactivated CHO Forward Gene Mutation Assays with Carbaryl Technical

Substance	Dose/mL	Relative Percent Survival	Total Mutant Colonies/ 12 Dishes	Percent Cloning Efficiency	Mutation Frequency[a] x 10^{-6}
Solvent Control[b]					
Dimethylsulfoxide	1%[c]	100.0	4	98.3	1.7
	1%[d]	100.0	9	86.8	4.2
Positive Control					
5-Bromo-2'-deoxyuridine	50 µg[c]	39.9	227	84.7	111.7*
	50 µg[d]	63.9	243	93.7	108.1*
Test Material					
Carbaryl technical	100 µg[c,e]	18.1	8	97.2	3.4
	150 µg	15.3	15	83.7	7.5*[f]
	200 µg[g]	6.0	0	79.3	0.0
	10 µg[d]	138.0	19	90.5	8.7[f]
	50 µg	24.5	14	81.7	7.1
	100 µg	14.6	2	88.2	0.9
	150 µg	11.4	4	70.7	2.4
	200 µg[g]	2.1	8	52.5	6.3[f]

[a] Mutation Frequency = $\frac{\text{Total mutants}}{\text{No. of dishes} \times 2 \times 10^5 \times \text{cloning efficiency}}$.

[b] Results for individual solvent control cultures were averaged by our reviewers.

[c] Results from the initial trial.

[d] Results from the repeat trial.

[e] Results for lower doses (80, 50, 30, 10, and 1 µg/mL) did not indicate a mutagenic response.

[f] Significantly higher than the solvent control but within the acceptable background range of 0 to 20 x 10^{-6}.

[g] Higher doses (300 µg/mL in the initial trial and 250 and 300 µg/mL in the repeat trial) were excessively cytotoxic.

*Significantly higher ($p \leq 0.01$) than the solvent control as determined by the tables of Kastenbaum and Bowman.

b. S9-Activated assays: Severe cytotoxicity prevented the cloning of cells exposed to 300 µg/mL; test material cytotoxicity for the remaining dose resulted in 1.2% survival at 200 µg/mL to 87.3% survival at 10 µg/mL. As shown in Table 3, both the total number of mutant colonies and the MF ($p \leq 0.05$) were increased at 200 µg/mL; however, these increases do not provide reliable evidence of mutagenicity because of the severe cytotoxicity (1.2% survival) observed at this level. A significant, but within normal background range, MF was also noted at 150 µg/mL. For the remaining S9-activated levels, the MFs were not appreciably higher than the solvent control. A second S9-activated assay was attempted but was aborted because of excessive cytotoxicity at doses ≥10 µg/mL. The study author stated that the increase in cytotoxicity as compared with the first trial "was believed to be associated with the use of a new lot of S9." Accordingly, a third S9-activated trial was performed with nine concentrations (1, 5, 10, 20, 40, 60, 80, 100, and 130 µg/mL). The test material was severely cytotoxic at 100 and 130 µg/mL, and <10% survival was seen at 60 and 80 µg/mL. We noted, however, that there was an ≈55% reduction in total surviving colonies in the solvent control group of the third trial as compared with the first S9-activated trial. Below 60 µg/mL, survival ranged from 21.9% at 40 µg/mL to 72.5% at 1 µg/mL. There were no appreciable increases in total mutant colonies or MFs at any cloned dose of carbaryl.

In both the nonactivated and S9-activated trials, the positive controls (50 µg/mL BrdU -S9 and 5 µg/mL MCA +S9) induced marked increases in the total mutant population and significant ($p \leq 0.01$) increases in the MFs. However there was an ≈50% decline in the MF and an ≈45% decline in mutant colonies for MCA in the third assay as compared to the first assay.

Based on the overall results, the study author concluded: "The test material, carbaryl (technical), is considered negative for inducing forward mutations at the HGPRT locus in Chinese hamster ovary cells under both the S9 metabolic activation and nonactivation conditions of the assay."

TABLE 3. Representative Results of the S9-Activated CHO Forward Gene Mutation Assays with Carbaryl (Technical)

Substance	Dose/mL	No. of Surviving Colonies	Relative Percent Survival	Total Mutant Colonies/ 12 Dishes	Percent Cloning Efficiency	Mutation Frequency[a] x 10^{-6}
Solvent Control[b]						
Dimethylsulfoxide	1%[c]	206	100	7	90.9	3.0
	1%[d]	113	100	31	103.5	12.5
Positive Control						
3-Methylcholanthrene	5 µg[c]	187	61.0	866	76.5	471.7*
	5 µg[d]	112	43.9	492	92.0	222.8*
Test Material						
Carbaryl technical	80 µg[c,e]	172	51.0	4	95.0	1.8
	100 µg	141	46.8	11	88.4	5.2
	120 µg	121	37.2	9	104.7	3.6
	150 µg	54	12.6	14	80.9	7.2[f]
	200 µg[g]	15	1.2	37	62.6	24.8*
	20 µg[d,e]	128	57.3	4	90.7	1.8
	40 µg	70	21.9	19	90.5	8.7
	60 µg	34	6.8	2	91.9	0.9
	80 µg	9	2.0	3	90.2	1.4

[a]Mutation Frequency = $\frac{\text{Total mutants}}{\text{No. of dishes} \times 2 \times 10^{5} \times \text{cloning efficiency}}$.

[b]Results for individual solvent control cultures were averaged by our reviewers.

[c]Results from the initial trial.

[d]Results from the repeat trial.

[e]Results for lower doses (50 and 10 µg/mL in the initial trial and 10, 5, and 1 µg/mL in the repeat trial) did not indicate a mutagenic response.

[f]Significantly higher ($p \leq 0.05$) than the solvent control but within the acceptable background range of 0 to 20 x 10^{-6}.

[g]Higher doses (300 µg/mL in the initial trial and 130 and 100 µg/mL in the repeat trial) were excessively cytotoxic.

*Significantly higher ($p \leq 0.01$) than the solvent control as determined by the tables of Kastenbaum and Bowman.

5. Reviewers' Discussion/Conclusions:

We assess that the data are not sufficient to support the study author's conclusions for the following reasons:

a. Based on the findings from the preliminary cytotoxicity assay and the initial mutation assay with and without S9 activation, the nonactivated test material was more cytotoxic than the S9-activated test material. For example, as shown in Table 1, the RPS at 62.5 µg/mL -S9 was 29.5% as compared to 95.7% at this level in the presence of S9 activation. Similarly, in the initial mutation assay, the RPS at 100 µg/mL -S9 was 18.1% (Table 2) as compared with 46.8% in the presence of S9 activation (Table 3).

The finding that S9-activated carbaryl was less cytotoxic than the test material without S9 activation is in agreement with the preliminary cytotoxicity assessment of carbaryl in the CHO cytogenetic assay. Although the cell lines that were used were not identical and the length of exposure differed (4 hours for the mutation assay; 2 hours for the preliminary phase of the cytogenetic testing), the general trend of increased cytotoxicity without S9 activation was consistent.

b. With the above consideration in mind, we assess that the increased cytotoxicity reported for the aborted second S9-activated trial and the completed third trial appears to be related to the "new batch" of S9 used in both tests. Hence, the findings do not constitute valid evidence of test material cytotoxicity. Additionally, the inclusion of $CaCl_2$ in the S9 mix did not appear to lessen the cytotoxicity of the "new batch" of S9 as indicated by the ≈55% reduction in total surviving colonies in the solvent control and the reduced MF for MCA in the third trial as compared with the first trial. These data suggest that not only was cytotoxicity increased, but assay sensitivity was decreased. We conclude, therefore, that the questionable batch of S9 should have been discarded and the assay repeated with a fully verified new S9 batch.

c. The length of time for expression of mutations (7 days) may have been too short. Since the study author did not furnish the raw data for daily cell counts, we have based this assessment on the evidence of severe cell

cycle delay observed both with and without S9 activation in the in vitro cytogenetic assay conducted with carbaryl.[4] Under normal conditions, an expression period of 7 to 9 days is considered adequate for a population of CHO cells[5] having the normal doubling time of 12 to 16 hours.[5] However, the data clearly established that carbaryl interfered with cell-cycle kinetics. Since both assays were conducted by the same laboratory, prudence should have dictated that the mutation assay be performed with a 9-day expression time to allow optimum conditions for detection of induced mutations.

We conclude, therefore, that the data are insufficient to classify carbaryl as not mutagenic in this test system.

6. Was the test performed under GLP? YES. (A quality assurance statement was signed and dated November 6, 1989.)

7. CBI Appendix: Appendix A, Materials and Methods, CBI pp. 11-20.

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[4]Murli, H. DER 305-B. MRID No. 413703-04; dated August 31, 1989.

[5]Li, A. P.; Carver, J. H.; Choy, W. N.; Hsie, A. W.; Grupa, R. S.; Loveday, K. S.; O'Neill, J. P.; Riddle, J. C.; Stankowski Jr., L. F.; and Yang, L. L. (1987). A guide for the performance of the Chinese hamster ovary cell/hypoxanthine-guanine phosphoribosyl transferase gene mutation assay. Mutat Res. 189: 135-141.

APPENDIX A

Materials and Methods
(CBI pp. 11-20)

Carbaryl toxicology Branch review

Page _____ is not included in this copy.

Pages 37 through 46 are not included in this copy.

The material not included contains the following type of information:

___ Identity of product inert ingredients

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___ Sales or other commercial/financial information

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008115

EPA No.: 68D80056
DYNAMAC No.: 305-C
TASK No.: 3-05C
September 19, 1990

DATA EVALUATION RECORD

CARBARYL

Mutagenicity--*Salmonella typhimurium*/Mammalian Microsome Mutagenicity Assay

APPROVED BY:

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

Signature: Robert J. Weir

Date: 9/19/90

EPA No.: 68D80056
DYNAMAC No.: 305-C
TASK No.: 3-05C
September 19, 1990

DATA EVALUATION RECORD

CARBARYL

Mutagenicity--Salmonella typhimurium/Mammalian Microsome Mutagenicity Assay

REVIEWED BY:

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

Signature: Nancy E. McCarroll
Date: 9-19-90

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

Signature: R Klein for I. Cecil Felkner
Date: 9/19/90

APPROVED BY:

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

Signature: Nicolas P. Hajjar
Date: September 19, 1990

Raymond Landolt, Ph.D.
EPA Reviewer, Section I
Toxicology Branch II
(H-7509C)

Signature: Raymond Landolt
Date: September 26, 1990

Mike Ioannou, Ph.D.
EPA Section Head, Section I
Toxicology Branch II
(H-7509C)

Signature: M Ioannou
Date: 9/26/90

DATA EVALUATION RECORD

TOX. Chem. No.:
EPA File Symbol:

CHEMICAL: Carbaryl.

STUDY TYPE: Salmonella/mammalian activation gene mutation assay.

ACCESSION OR MRID NUMBER: 413703-03.

SYNONYMS/CAS NUMBER: None listed.

SPONSOR: Rhone-Poulenc AG Co., Research Triangle Park, NC.

TESTING FACILITY: Hazleton Laboratories America, Inc., Kensington, MD.

TITLE OF REPORT: Mutagenicity Test on Carbaryl (Technical) in the Ames Salmonella/Microsome Reverse Mutation Assay.

AUTHOR: T. E. Lawlor.

STUDY NUMBER: 10862-0-401.

REPORT ISSUED: September 6, 1989.

CONCLUSIONS - Executive Summary:

Carbaryl (technical) was initially evaluated in the Salmonella typhimurium/microsome mutagenicity assay over a concentration range of 5 to 1000 µg/plate. The test material was not mutagenic; however, the highest assayed dose was cytotoxic in S. typhimurium strains TA98 and TA100, but not in strains TA1535, TA1537, or TA1538. Accordingly, the assay was repeated with six concentrations (10 to 2000 µg/plate +/-S9). Results from the repeat assay indicated that 2000 µg/plate +/-S9 was cytotoxic in all strains; 1000 µg/plate +/-S9 induced a cytotoxic effect in strains TA98 and TA100, and the remaining doses were not mutagenic. We assess, therefore, that carbaryl (technical) was assayed to an appropriately high concentration with no evidence of mutagenicity in a well-controlled study. The study satisfies Guideline requirements for genetic effects, Category I, Gene Mutations.

Study Classification: The study is acceptable.

A. MATERIALS:

1. Test Material:

Name:	Carbaryl (technical)
Description:	White powder.
Batch/Lot No.:	87191.
Purity:	99.3%
Contaminants:	Not listed.
Solvent used:	Dimethylsulfoxide (DMSO).
Other comments:	The test material was stored at room temperature and formed a solution in DMSO at 100 mg/mL.

2. Control Materials:
Negative: DMSO
Solvent/final concentration: 50 µL/plate
Positive: Nonactivation:
Sodium azide 2.0 µg/plate TA100, TA1535
2-Nitrofluorene 1.0 µg/plate TA98, TA1538
ICR-191 2.0 µg/plate TA1537
Other:

Activation: 2-Aminoanthracene (2-anthramine)
2.5 µg/plate all strains.

3. Activation: S9 derived from

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

If other, describe below. Describe S9 composition (if purchased, give details).

The S9 fraction was obtained from an unspecified commercial source and was characterized, using the Salmonella/mammalian microsome mutation assay, for its ability to metabolize known promutagens.

One mL of the S9 mix contained the following components:

H_2O	0.70 mL
1.00 M NaH_2PO_4/Na_2HPO_4 (pH 7.4)	0.10 mL
0.25 M Glucose-6-phosphate	0.02 mL
0.10 M NADP	0.04 mL
0.20 M $MgCl_2$/0.825 M KCl	0.04 mL
S-9	0.10 mL
TOTAL	1.00 mL

4. Test Organism Used: S. typhimurium strains

_____	TA97	__x__	TA98	__x__	TA100	_____	TA102	_____	TA104
__x__	TA1535	__x__	TA1537	__x__	TA1538; list any others:				

Test organisms were properly maintained: YES.
Checked for appropriate genetic markers (rfa mutation, R factor): YES.

5. Test Compound Concentrations Used:

a. Preliminary Cytotoxicity Assay: Ten doses (6.67, 10.0, 33.3, 66.7, 100, 333, 667, 1000, 3330, and 5000 µg/plate were evaluated with or without S9 activation in S. typhimurium strain TA100. Single plates were used per dose per condition.

b. Initial Mutation Assay: The first assay was conducted with six doses (5, 10, 50, 100, 500, and 1000 µg/plate) in the presence or absence of S9 activation.

c. Repeat Mutation Assay: Because of the lack of cytotoxicity in strains TA1535, TA1537, and TA1538, a repeat assay was performed with all five tester strains. Doses used in the repeat assay were 10, 50, 100, 500, 1000, and 2000 µg/plate +/-S9.

B. TEST PERFORMANCE:

1. Type of Salmonella Assay:

__x__ Standard plate test
_____ Pre-incubation (__) minutes
_____ "Prival" modification
_____ Spot test
_____ Other (describe)

a. Protocol:

1) Plating Procedures:
In general, similar procedures were used for the preliminary cytotoxicity and the mutation assays.

To tubes containing 2.5-mL volumes of molten top agar containing 0.5 mM biotin and 0.5 mM histidine, 100 µL of an overnight broth culture of the appropriate tester strain and 50 µL of the appropriate test material dose, solvent, or positive controls were added. For the S9-activated test, 0.5 mL of the S9 cofactor mix was added to tubes containing 2.0 mL of top agar; tester strains and test and control solutions were

added as described. The contents of the tubes were mixed, poured over Vogel-Bonner minimal medium E, and incubated at 37°C for 48 hours. At the end of incubation, plates either were immediately scored or were refrigerated and subsequently counted with an automatic colony counter. Means and standard deviations were determined for the mutation assay.

2) Sterility Controls:
A sterility test was performed on the highest dose of the test material and 0.5 mL of the S9 mix as described for the mutation assay.

3) Evaluation Criteria:

a) Assay Validity: The assay was considered valid if the following criteria were met: (1) the presence of the appropriate genetic markers was verified for each strain; (2) the spontaneous revertant rate of each strain fell within the reporting laboratory's acceptable ranges; (3) cell densities were $\geq 5 \times 10^8$ cells/mL; and (4) all positive controls caused at least a 3-fold increase in revertants per plate compared with the respective solvent control.

b) Positive Response: The test material was considered positive if it caused a ≥2-fold increase in the number of mean revertant colonies for strains TA98 or TA100 or if it induced a ≥3-fold increase in the rate in strains TA1535, TA1537, or TA1538, and the increase was accompanied by a dose response to increasing concentrations of the test material.

2. Preliminary Assay: Ten doses ranging from 6.67 to 5000 µg/plate +/-S9 were assayed for cytotoxic effects on strain TA100. Slight precipitation of the test material and excessive cytotoxicity were observed at the two highest concentrations (i.e., 3330 and 5000 µg/plate) both with and without S9 activation. Colony counts were ≈50% less than the solvent control, at 1000 and 667 µg/plate +/-S9 and at 333 and 100 µg/plate +S9. Below these levels, TA100 revertants were generally lower than the solvent control, but the reductions were not clearly indicative of a cytotoxic effect.

3. Mutation Assay: Based on the above results, the mutation assay was conducted with six test concentrations ranging from 5 to 1000 µg/plate. The highest nonactivated and S9-activated dose induced a cytotoxic effect only in

strains TA98 and TA100 (Table 1). For the remaining levels either with or without S9 activation, there was no clear evidence of cytotoxicity or mutagenicity. The lack of a cytotoxic effect in strains TA1535, TA1537, and TA1538 prompted the performance of a repeat assay with six doses ranging from 10 to 2000 µg/plate. As shown in Table 2, 2000 µg/plate +/-S9 was cytotoxic in all strains; in agreement with the earlier findings, 1000 µg/plate was cytotoxic in strains TA98 and TA100 but not in the remaining strains. Also noted was an ≈50% decrease in TA100 revertant colonies at the 500-µg/plate nonactivated level. No appreciable increases in histidine revertants of any strains were observed at any dose either with or without S9 activation.

By contrast, all strains responded to the mutagenic action of the appropriate nonactivated or S9-activated positive control in both the initial and the repeat assays.

Based on the overall findings, the study authors concluded that carbaryl (technical) was not mutagenic in this test system.

4. Reviewers' Discussion/Conclusions: We assess that the mutation assays were well conducted and that the study author's interpretation of the results was correct. The test material was assayed to a level (2000 µg/plate +/-S9) that was cytotoxic to all strains but failed to induce a mutagenic response over a nonactivated and S9-activated concentration range of 5 to 2000 µg/plate. The ability of the test system to detect mutagenicity was adequately demonstrated in both the initial and repeat tests. We conclude, therefore, that carbaryl (technical) is not mutagenic in this test system.

5. Quality Assurance: The test was performed under good laboratory practices (a quality assurance statement was signed and dated September 6, 1989).

6. CBI Appendix: Appendix A, Materials and Methods, CBI pp. 14-21.

TABLE 1. Representative Results of the Initial Salmonella typhimurium Mutagenicity Assays with Carbaryl (Technical)

Substance	S9 Activation	Dose (µg/plate)	Revertants per Plate of Bacterial Tester Strain[a] TA1535	TA1537	TA1538	TA98	TA100
Solvent Control							
Dimethylsulfoxide	-	--	7 ± 1	3 ± 1	6 ± 1	15 ± 5	79 ± 16
	+	--	5 ± 1	7 ± 2	11 ± 5	21 ± 3	90 ± 13
Positive Control							
Sodium azide	-	2.0	500 ± 30	--	--	--	477 ± 47
2-Nitrofluorene	-	1.0	--	--	170 ± 18	122 ± 5	--
ICR-191	-	2.0	--	329 ± 41	--	--	--
2-Anthramine	+	2.5	155 ± 6	154 ± 5	1077 ± 184	858 ± 126	1088 ± 71
Test Material							
Carbaryl (technical)	-	500[b]	6 ± 2	6 ± 1	4 ± 2	8 ± 1	60 ± 4
	-	1000	4 ± 3	4 ± 1	7 ± 3	3 ± 1	19 ± 6
	+	500[b]	11 ± 2	6 ± 2	12 ± 4	16 ± 8	71 ± 7
	+	1000	5 ± 1	6 ± 3	13 ± 7	11 ± 4	43 ± 8

[a]Mean and standard deviation of counts from triplicate plates.

[b]Results for lower doses (5, 10, 50, and 100 µg/plate +/-S9) did not indicate a mutagenic response.

TABLE 2. Representative Results of the Repeat Salmonella typhimurium Mutagenicity Assays with Carbaryl (Technical)

Substance	S9 Activation	Dose (µg/plate)	Revertants per Plate of Bacterial Tester Strain[a] TA1535	TA1537	TA1538	TA98	TA100
Solvent Control							
Dimethylsulfoxide	-	--	10 ± 2	5 ± 3	4 ± 1	19 ± 1	93 ± 5
	+	--	12 ± 3	4 ± 0	8 ± 3	21 ± 2	112 ± 11
Positive Control							
Sodium azide	-	2.0	465 ± 14	--	--	--	665 ± 18
2-Nitrofluorene	-	1.0	--	--	187 ± 25	137 ± 25	--
ICR-191	-	2.0	--	328 ± 22	--	--	--
2-Anthramine	+	2.5	168 ± 44	143 ± 18	859 ± 2	985 ± 148	1133 ± 90
Test Material							
Carbaryl (technical)	-	500	11 ± 2	5 ± 0	4 ± 2	16 ± 5	49 ± 12
	-	1000	8 ± 3	5 ± 2	4 ± 3	10 ± 2	22 ± 6
	-	2000	7 ± 1	1 ± 2	1 ± 2	3 ± 2	1 ± 1
	+	500	10 ± 1	5 ± 2	8 ± 4	13 ± 3	95 ± 9
	+	1000	8 ± 2	7 ± 4	7 ± 1	7 ± 2	59 ± 12
	+	2000	4 ± 3	1 ± 1	2 ± 1	3 ± 1	6 ± 3

[a]Mean and standard deviation of counts from triplicate plates.

[b]Results for lower doses (10, 50, and 100 µg/plate +/-S9) did not indicate a mutagenic response.

APPENDIX A
Materials and Methods
(CBI pp. 14-21)

Carbaryl toxicology Branch review.

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Pages 58 through 65 are not included in this copy.

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___ Description of the product manufacturing process

___ Description of product quality control procedures

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___ Sales or other commercial/financial information

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___ Information about a pending registration action

X FIFRA registration data

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EPA No.: 68D80056
DYNAMAC No.: 305-D
TASK No.: 3-05D
September 19, 1990

DATA EVALUATION RECORD

CARBARYL

Mutagenicity--Mammalian Cells in Culture Cytogenetic Assay in Chinese Hamster Ovary (CHO) Cells

APPROVED BY:

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

Signature: Robert J. Weir

Date: 9/19/90

Guideline Series 84: **Mutagenicity**

EPA No.: 68D80056
DYNAMAC No.: 305-D
TASK No.: 3-05D
September 19, 1990

DATA EVALUATION RECORD

CARBARYL

Mutagenicity--Mammalian Cells in Culture Cytogenetic Assay in Chinese Hamster Ovary (CHO) Cells

REVIEWED BY:

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

Signature: Nancy E. McCarroll
Date: 9-19-90

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

Signature: Kathleen for I. Cecil Felkner
Date: 9/19/90

APPROVED BY:

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

Signature: Nicolas P. Hajjar
Date: September 19, 1990

Raymond Landolt, Ph.D.
EPA Reviewer, Section I
Toxicology Branch II
(H-7509C)

Signature: Raymond E. Landolt
Date: September 26, 1990

Mike Ioannou, Ph.D.
EPA Section Head, Section I
Toxicology Branch II
(H-7509C)

Signature: M Ioannou
Date: 9/26/90

DATA EVALUATION RECORD

TOX. Chem. No.:
EPA File Symbol:

CHEMICAL: Carbaryl.

STUDY TYPE: Mammalian cells in culture cytogenetic assay in Chinese hamster ovary (CHO) cells.

MRID NUMBER: 413703-04.

SYNONYM(S)/CAS NUMBER: None listed.

SPONSOR: Rhone-Poulenc AG Co., Research Triangle Park, NC.

TESTING FACILITY: Hazleton Laboratories America, Inc., Kensington, MD.

TITLE OF REPORT: Mutagenicity Test on Carbaryl Technical in an *in vitro* Cytogenetic Assay Measuring Chromosomal Aberration Frequencies in Chinese Hamster Ovary (CHO) Cells.

AUTHOR: H. Murli.

STUDY NUMBER: 10862-0-437.

REPORT ISSUED: August 31, 1989.

CONCLUSIONS - Executive Summary: Carbaryl (technical) was assayed for clastogenic effects in both the presence and absence of S9 activation using Chinese hamster ovary (CHO) cells. Because of severe cell cycle delay, which was more pronounced without S9 activation, a 20-hour cell harvest was selected to evaluate seven nonactivated doses ranging from 5 to 100 μg/mL. In the presence of S9 activation, cells exposed to carbaryl doses of 25, 50, 75, 100, 150, 200, 250, and 300 μg/mL were harvested at 20 hours, and cells exposed to 100, 150, 200, 250, and 300 μg/mL were harvested 30 hours posttreatment. Results indicated that the nonactivated test material was more cytotoxic than the S9-activated test material (i.e., few metaphases were recovered at 75 and 100 μg/mL, and moderate to slight cytotoxic effects were seen at doses ≥10.0 μg/mL). With the exception of a single rare complex aberration (quadriradial) scored at the 50.0-μg/mL dose level, there was no evidence of a clastogenic effect. By contrast, in the S9-activated assays, all scored doses (150, 200, 250, and 300 μg/mL) at both harvest times induced significant ($p \leq 0.01$) increases in the percentage of cells with aberrations, and the majority of doses induced significant ($p \leq 0.01$) increases in the percentage of cells with >1 aberration. The wide variety of induced aberrations (both simple and complex) was consistent with the findings from earlier in vitro cytogenetic studies conducted with carbaryl (see section 5, Reviewers' Dicsussion/Conclusions). Other investigators, however, found that carbaryl was clastogenic in the absence of S9 activation. Similarly, there was no indication in the currently reviewed study that carbaryl increased the frequency of numerical aberrations, which has been observed in other Chinese hamster cell lines. Additionally, the evaluation of slides prepared from lower doses would have provided important information since the data show little or no dose responsiveness and the lowest reactive level of carbaryl was not determined. We conclude, however, that the study was technically sound and, therefore, satisfies the Guideline requirements for Category 2, Structural Chromosome Aberrations.

Study Classification: The study is acceptable; S9-activated carbaryl is clastogenic in CHO cells.

A. MATERIALS:

1. Test Material:

Name:	Carbaryl (technical).
Description:	White powder.
Batch/Lot No.:	87191.
Purity:	99.3%.
Contaminants:	Not listed.
Solvent used:	Dimethylsulfoxide (DMSO).
Other comments:	Storage conditions were not reported. The test material formed a clear, colorless solution in DMSO at concentrations ≤489 mg/mL. A fine white precipitate and pH of 8.0 to 8.5 were observed at a final concentration of 978 µg/mL of McCoy's 5a culture medium. The pH was adjusted to 7.5 with Hepes buffer to allow performance of the range-finding study with a high dose of 1000 µg/mL.

2. Control Materials:

Negative: McCoy's 5a medium supplemented with 10% fetal calf serum, glutamine, and antibiotics.

Solvent/final concentration: DMSO/10 µL/mL.

Positive: Nonactivation (concentrations, solvent): The solvent for Mitomycin C (MMC) was not reported but was assumed to be culture medium since MMC is water-soluble. Two doses of MMC were assayed in the preliminary cytotoxicity study (0.25 and 0.5 µg/mL), and two doses were tested in the cytogenetic assay (0.04 and 0.08 µg/mL). Only one dose from each phase of testing was analyzed.

Activation (concentrations, solvent): The solvent for Cyclophosphamide (CP) was not reported but was also assumed to be culture medium. Two doses of CP were assayed in the preliminary cytotoxicity study (12.5 and 20.0 µg/mL), and two doses were tested in the cytogenetic assay (10.0 and 12.5 µg/mL). Only one dose from each phase of testing was analyzed.

3. Activation: S9 derived from

X Arclor 1254	X induced	X rat	X liver
_____ phenobarbital	_____ noninduced	_____ mouse	_____ lung
_____ none		_____ hamster	_____ other
_____ other		_____ other	

The report did not indicate whether the S9 fraction was prepared in-house or purchased commercially. The S9 mix contained the following components:

Component	Concentration
NADP	1.5 mg/mL
Isocitric acid	2.7 mg/mL
S9	15.0 µl/mL

4. Test Compound Concentrations Used:

   a. Preliminary cytotoxicity assay. Nine half-log dilutions of 1000 µg/mL (dose range: 0.0334 to 1000 µg/mL) with and without S9 activation.

   b. Cytogenetic assay:

      1. Nonactivation: Seven doses (5.0, 7.5, 10.0, 25.0, 50.0, 75.0, and 100.0 µg/mL) were assayed with a delayed 20-hour cell harvest.

      2. S9 Activation: Two assays were performed; doses and harvest times were as follows:

         Trial 1: 25.0, 50.0, 75.0, 100.0, 150.0, 200.0, 250.0, and 300.0 µg/mL with a 20-hour cell harvest.

         Trial 2: 100.0, 150.0, 200.0, 250.0, and 300.0 µg/mL with a 30-hour cell harvest.

NOTE: Initially, a 10-hour cell harvest was planned for the S9-activated phase of testing; it was not performed.

5. Test Cells: The Chinese hamster ovary cells (CHO-WBL) used in this assay were originally obtained from Dr. Sheldon Wolff, University of California, San Francisco, CA.

   Properly maintained? YES.

   Cell line or strain periodically checked for Mycoplasma contamination? Not reported.

   Cell line or strain periodically checked for karyotype stability? YES.

B. TEST PERFORMANCE:

1. Cell Treatments:

a. Cells exposed to test compound for:
≈17.25 hours (nonactivated) 2 hours (activated)

b. Cells exposed to positive controls for:
≈17.25 hours (nonactivated) 2 hours (activated)

c. Cells exposed to negative and/or solvent controls for:
≈17.25 hours (nonactivated) 2 hours (activated)

2. Protocol:

a. Preliminary cytotoxicity assay: Cell cultures, seeded at 0.3×10^6 cells/flask, were exposed to half-log dilutions of the test material with or without S9 activation at doses ranging from 0.0334 to 1000 µg/mL, the negative control (McCoy's 5a medium), the solvent (DMSO), and the positive controls (MMC -S9 and CP +S9).

In the nonactivated system, cells were exposed for 2 hours to the test material; 5-bromo-2'-deoxyuridine (BrdUrd 10 µM) was added to the cultures, and incubation was continued for a further 23 hours. Cell monolayers were washed, refed with fresh complete medium containing BrdUrd, and reincubated in the presence of 0.1 µg/mL colcemid for 2.75 hours. In the S9-activated system, cultures were exposed for 2 hours without fetal calf serum. After exposure, cells were washed twice, refed with complete medium containing BrdUrd (10 µM), and reincubated for 23 hours. Colcemid was added, and cultures were incubated for an additional 2.5 hours. After incubation, monolayers were visually evaluated for confluency and dead cells. Metaphase cells were collected by mitotic shake-off, swollen in a hypotonic 0.075 M solution of KCl, fixed with methanol:acetic acid (3:1), and stained using a modified fluorescent-plus Giemsa technique. One hundred metaphase cells per culture were examined for the percentage of first (M_1), between first and second (M_{1+}), and second or more ($\geq M_2$) division metaphases. Based on the findings, doses and harvest times were selected for the cytogenetic assay.

b. Cytogenetic assays:

Treatment: Prepared cultures (in duplicate), seeded at 1.2×10^6 cells, were exposed to the selected test

material doses, the solvent, or the positive controls. In the nonactivated system, cells were dosed for 17.25 hours. Cultures were washed, refed medium containing colcemid, and reincubated for approximately 2.5 hours (≈20-hour cell harvest).

Under S9-activated conditions, 1.2×10^6 cells/flask (20-hour cell harvest) and 1×10^6 cells/flask (30-hour cell harvest) were exposed for 2 hours, washed, refed culture medium, and incubated for either 17.75 or 27.75 hours, respectively. Colcemid was added 2.5 hours before the cultures were harvested.

Metaphase cells were collected by mitotic shake-off and fixed in methanol:glacial acetic acid (3:1). Slides were stained with 5% Giemsa and coded to avoid bias.

Metaphase analysis: One hundred morphologically normal cells (containing 19 to 23 centromeres) per culture from four dose levels of the test material and the solvent control were scored for chromosome aberrations. Twenty-five cells were scored from one dose level only for each positive-control culture.

Statistical methods: The data were evaluated for statistical significance at $p < 0.01$ by Fisher's exact test. The negative (culture medium) and solvent controls were pooled if no statistical differences were found.

Evaluation criteria: The biological significance of the results was evaluated relative to the overall chromosome aberration frequencies, percentage of cells with aberrations, percentage of cells with >1 aberration, dose-response, and the types of aberrations observed. Gaps were tabulated but not considered in the evaluation.

3. Preliminary Cytotoxicity Assay: The cytotoxicity assay was conducted with test doses ranging from 0.0334 to 1000 µg/mL, each dose separated by half-log dilutions, in the presence or absence of S9 activation. The author reported that the highest concentration, i.e., 1000 µg/mL (+/-S9), formed a precipitate when added to the culture medium; below this level, the test material was soluble. Results with and without S9 activation were as follows:

   a. Nonactivation: No viable cells were recovered from cultures exposed to concentrations ≥100.0 µg/mL. Severe cell-cycle delay was observed at 33.4 µg/mL; moderate delay was also seen at 10.0 and 3.34 µg/mL (Table 1).

TABLE 1. Results from the Preliminary Test for Delay of Cell-Cycle Progression with Carbaryl (Technical)

Substance	Dose/mL	S9 Activation	% Cells[a] M_1	% Cells[a] M_{1+}	% Cells[a] $\geq M_2$	Monolayer Confluency (%)
Negative Control						
Culture Medium	--	-	0	6	94	100
	--	+	0	12	88	100
Solvent Control						
Dimethylsulfoxide	10 µL	-	2	25	73	100
	10 µL	+	0	7	93	100
Positive Control						
Mitomycin C	0.25 µg	-	39	61	0	100
Cyclophosphamide	12.5 µg	+	12	88	0	100
Test Material						
Carbaryl (technical)	3.3 µg[b]	-	6	81	13	88
	10.0 µg	-	2	79	19	88
	33.4 µg[c]	-	86	14	0	75
	10.0 µg[b]	+	0	23	77	100
	33.4 µg	+	0	39	61	100
	100.0 µg	+	3	71	26	100

[a]Percent cells in first (M_1), between first and second (M_{1+}), and in second or greater ($\geq M_2$) divisions.

[b]Results for lower doses (half-log dilutions to 0.0334) were not reported.

[c]Higher concentrations (100, 333, and 1000 µg/mL -S9 and 333 and 1000 µg/mL +S9) were completely cytotoxic. Compound precipitation was noted at the highest concentration (1000 µg/mL +/-S9).

Based on these findings; a 20-hour cell harvest was selected to evaluate seven test doses ranging from 5.0 to 100.0 µg/mL.

b. S9-Activated. In the presence of S9 activation, cytotoxicity and mitotic suppression were less severe. No viable cells were recovered from the two highest dose groups (334.0 and 1000.0 µg/mL); however, only slight to moderate cell cycle delay was observed at 10.0, 33.4, or 100.0 µg/mL.

Based on these observations, a 20-hour cell harvest was selected to assay a dose range of 25 to 300 µg/mL, and a 30-hour cell harvest was selected to evaluate doses of 100 to 300 µg/mL. Although the study author provided no explanation for the inclusion of a 30-hour cell harvest, we assume that the author was aware of the findings of Onfelt and Klastuska (1983)[1] showing that highly significant increases in chromosome aberrations were observed in V79 cells harvested 50 hours postexposure to carbaryl.

4. Cytogenetic Assay: Results from the nonactivated cytogenetic assay are presented in Table 2. As shown the two highest doses were cytotoxic. An analysis of metaphases scored from the 7.5-, 10.0-, 25.0-, and 50.0-µg/mL dose groups did not reveal a significant clastogenic effect. However, as shown in Table 3, in the presence of S9 activation, significant ($p \leq 0.01$) increases in the percentage of cells with aberrations were observed at all scored dose levels following both the 20- and 30-hour cell harvests. Significant ($p \leq 0.01$) increases in the percentage of cells with >1 aberration was also scored in the majority of dose groups from both harvest intervals. The findings indicated that at both harvest times, carbaryl induced marked increases in both simple and complex aberrations; peak clastogenic activity occurred 20 hours posttreatment (declining, but still significant at 30 hours).

Based on these findings, the study author concluded that carbaryl was clastogenic in the presence of S9 activation.

5. Reviewer's Discussion/Conclusions: We assess in agreement with the study author that carbaryl was clastogenic in the presence of S9 activation. Under both nonactivated and S9-activated conditions the test material induced cell cycle delay. Since cell

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[1]Onfelt, A., and Klasterska, I. Spindle disturbance in mammalian cells. II. Induction of viable aneuploid/polyploid cells and multiple chromatid exchanges after treatment of V79 Chinese hamster cells with carbaryl. Mutat. Res. (1983) 119: 319-330.

TABLE 2. Representative Results of the Nonactivated in vitro CHO Cell Cytogenetic Assays with Carbaryl (Technical)

Substance	Dose (μg/mL)	No. of Cells Scored	No. of Aberrations per Cell	% Cells with Aberrations	% Cells with >1 Aberration	Biologically Significant Aberrations[a] (No./Type)
Pooled Negative Control						
Medium and dimethylsulfoxide	--	200	0.00	0.0	0.0	--
Positive Control						
Mitomycin	0.08	25	0.28	24.0*	4.0	2TB; 2SB; 2TR; 1QR
Test Material						
Carbaryl (technical)	7.5	200	0.01	0.5	0.0	1D
	10.0[b]	200	0.01	1.0	0.0	1TB; 1CI
	25.0[b]	200	0.03	2.0	0.5	3TB; 2SB
	50.0[b,c]	200	0.02	1.5	0.5	2TB; 1SB; 1QR

[a]Abbreviations used:
TB - Chromatid break
SB - Chromosome break
D - Dicentric
TR - Triradial
QR - Quadriradial
CI - Chromosome intrachange

[b]Cytotoxic signs, which included a reduction in monolayer confluency, dead cells, and/or a reduction in mitotic cells, were observed at these levels.

[c]Few metaphases were available for analysis at higher doses (75 and 100 μg/mL).

*Significantly higher ($p \leq 0.01$) than the pooled negative control values as determined by Fisher's exact test.

TABLE 3. Representative Results of the S9-Activated in vitro CHO Cell Cytogenetic Assays with Carbaryl (Technical)

Substance	Dose (µg/mL)	Harvest Time (hours)	No. of Cells Scored	No. of Aberrations per Cell	% Cells with Aberrations	% Cells with >1 Aberration	Biologically Significant Aberrations[a] (No./Type)
Pooled Negative Control							
Medium and dimethylsulfoxide	--	20.0	200	0.02	1.5	0.5	4SB
		30.0	200	0.01	1.0	0.0	1≤3; 1D
Positive Control							
Cyclophosphamide	10.0	20.0	25	0.80	36.0*	24.0*	3TB; 5SB; 4TR; 4QR; 3CR; 1CI
	10.0	30.0	25	0.64	40.0*	12.0*	1TB; 6SB; 2TR; 1QR; 1CR; 2D; 1CI; 1DF; 1AB
Test Material							
Carbaryl (technical)	150.0	20.0	125[c]	0.67	44.8*	16.0*	24TB; 16SB; 4ID; 11TR; 23QR; 2CR; 1D; 2R; 1CI
	200.0[b]	20.0	100[c]	0.59	35.0*	17.0*	12TB; 8SB; 7ID; 6TR; 17QR; 5CR; 4CI
	250.0[b]	20.0	100[c]	0.95	57.0*	26.0*	22TB; 35SB; 5ID; 10TR; 18QR; 3CR; 2CI
	300.0[b]	20.0	125[c]	0.70	48.8*	17.6*	16TB; 38SB; 5ID; 9TR; 12QR; 4CR; 1R; 3CI
	150.0	30.0	200	>0.29	14.0*	5.5*	4TB; 18SB; 1ID; 5TR; 2QR; 3CR; 4D; 1DF; 2GT
	200.0[b]	30.0	200	>0.15	8.5*	1.5	1TB; 11SB; 1TR; 2QR; 1CR; 3D; 1CI; 1GT
	250.0[b]	30.0	200	>0.46	25.5*	9.5*	15TB; 26SB; 4ID; 15TR; 8QR; 12CR; 8D; 3CI; 1DF; 1GT
	300.0[b]	30.0	150[c]	>0.81	30.0*	16.0*	14TB; 30SB; 3ID; 11TR; 8QR; 7CR; 3D; 4CI; 1DF; 4GT

[a]Abbreviations used:

TB - Chromatid break
SB - Chromosome break
D - Dicentric
R - Ring
CR - Complex rearrangement
TR - Triradial
QR - Quadriradial
ID - Interstitial deletion
GT - >10 aberrations
CI - Chromosome intrachange
PU - Pulverized cell
DF - Dicentric with fragment
AB - Abnormal monocentric chromosome

[b]Cytotoxic signs, which included a reduction in monolayer confluency, dead cells, and/or a reduction in mitotic cells, were observed.

[c]Because of the high percentage of cells with aberrations, <200 cells/treatment group were scored.

*Significantly higher ($p \leq 0.01$) than the pooled negative control values as determined by Fisher's exact test.

cycle delay was more severe and also occurred at lower doses in the absence of S9 activation, it was not clear why only a 20-hour cell harvest was performed for the nonactivated phase of testing. However, the clastogenic effects in the presence of S9 activation were dramatic, and the wide variety of carbaryl-induced structural aberrations was consistent with those reported by other investigators using Chinese hamster fibroblasts (Ishidate and Odashima, 1977)[2] and Chinese hamster V79 cells (Onfelt and Klasterska, 1983).[3] It was noteworthy that these earlier studies found carbaryl to be clastogenic in the absence of exogenous metabolic activation. In the currently reviewed study, the only suggestive evidence of a nonactivated clastogenic response was the occurrence of a rare complex aberration (one quadriradial) in the high-dose group (50 µg/mL). It was also of interest to our reviewers that there was no indication of an increased incidence of aneuploid/polyploid cells since a collective body of evidence[3,4] exists suggesting that carbaryl induces numerical aberrations. Although the study was not specifically designed to detect aneuploidy (i.e., examination of cells in second or subsequent divisions), our reviewers expected to see an increase in numerical aberrations at the 30-hour cell harvest. Additionally, it would have been useful if several lower doses had been analyzed, since the data show little or no dose responsiveness and the lowest reactive level of carbaryl was not determined. Nevertheless, we conclude, that although the results of the current study are not in full agreement with earlier findings and questions remain regarding the effective clastogenic range of carbaryl, the study was technically sound and fulfills the Guideline requirements for Category 2, structural chromosome aberrations.

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[2]Ishidate, M., Odashima, S. 1977. Chromosome tests with 134 compounds on Chinese hamster cells in vitro: A screening for chemical carcinogens. Mutat. Res. 48: 337-354.

[3]Onfelt, A., and Klasterska, I. (1983) from p. 10 Mutat. Res. 119: 319-330.

[4]Sabharawal, P.S., and Lockard, J.M. 1979. Induction of sister chromatid exchange and polyploidy by carbaryl in V79 cells. In vitro. 15(3): 172-173.

6. Quality Assurance Measures: The test was performed under GLPs (a quality assurance statement was signed and dated August 31, 1989).

7. CBI Appendix: Appendix A, Materials and Methods, CBI pp. 12-17.

APPENDIX A

Materials and Methods
(CBI pp. 12-17)

Carbaryl toxicology Branch review

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