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

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JUL 30 1987

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

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MEMORANDUM

SUBJECT: Antifoulant C-9211M
EPA File Symbol 707-RTL

FROM: Carlos A. Rodriguez Carlos A. Rodriguez 7/27/87
Review Section VI
Toxicology Branch
Hazard Evaluation Division (TS-769C)

TO: John H. Lee, PM 31
Disinfectants Branch
Registration Division (TS-767C)

THRU: Judith W. Hauswirth, Ph.D. Judith W. Hauswirth 7/27/87
Section Head, Review Section VI
Toxicology Branch
Hazard Evaluation Division (TS-769C)

APPLICANT: Rohm & Haas Company
Independence Mall West
Philadelphia, PA 19105

Present Action

Review and evaluate the data submitted on CHO/HGPRT, Gene Mutation Assay, In Vitro Cytogenetic Assay in Chinese Hamster Ovary (CHO) Cells and Teratology Study in Rabbits to support the registration of the Antifoulant C-9211M product.

Evaluation

The mutagenicity studies, in vitro cytogenic assay in CHO cells, and CHO/HGPRT gene mutation assay with Antifoulant C-9211M, were found to be acceptable and warrant the conclusion that the test material is not mutagenic in these assay systems.

In the rabbit teratology study the NOEL for maternal toxicity could not be established due to weight loss and decreased urination and defecation at all dose levels tested (5, 25, and 70 mg/kg).

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This study demonstrated no embryotoxic or developmental toxicity effects in rabbit fetuses at dose levels of 5 and 25 mg/kg. Due to compound-related abortions, insufficient litters were available at 70 mg/kg/day (HDT), to assess developmental toxicity at this dose level. A tentative NOEL* of 25 mg/kg is established for developmental toxicity.

This study is classified "Supplementary" pending submission of the chemical analytical data from assays conducted for this study in order to evaluate the adequacy of the dosing suspension.

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* Note: If the registrant wishes to determine a NOEL for development in the rabbit, other than the 25 mg/kg tentatively determined, Toxicology Branch recommends that the study be repeated with the high dose set between 25 and 70 mg/kg.

CONFIDENTIAL BUSINESS INFORMATION
DOES NOT CONTAIN
NATIONAL SECURITY INFORMATION (EO 12065)

EPA: 68-02-4225
DYNAMAC No. 290-C
July 15, 1987

DATA EVALUATION RECORD

C-9211M

Teratogenicity Study in Rabbits

STUDY IDENTIFICATION: Rodwell, D. E. A teratology study in rabbits with C-9211 (formulation). (Unpublished report No. 85RC-59 by WIL Research Laboratories, Inc., Ashland, OH, for Rohm and Haas Co., Spring House, PA; dated January 14, 1986.) Accession No. 262569.

APPROVED BY:

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

Signature: I. Cecil Felkner

Date: 7-15-87

006312

MANUFACTURING PROCESS INFORMATION IS NOT INCLUDED

1. CHEMICAL: C9211M; 4,5-dichloro-2-n-octyl-3(2H)isothiazolone.

2. TEST MATERIAL: C9211M, lot No. RCL 2030, was identified as an amber liquid containing [redacted] and 40% active ingredient.

3. STUDY/ACTION TYPE: Teratogenicity study in rabbits.

4. STUDY IDENTIFICATION: Rodwell, D. E. A teratology study in rabbits with C-9211 (formulation). (Unpublished report No. 85RC-59 by WIL Research Laboratories, Inc., Ashland, OH, for Rohm and Haas Co., Spring House, PA; dated January 14, 1986.) Accession No. 262569.

5. REVIEWED BY:

Michael Narotsky, B.A.
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Date: 7/24/87

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Acting EPA Section Head

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Date: 7/27/87

7. CONCLUSIONS:

A. The NOEL for maternal toxicity of C-9211 in rabbits could not be established due to weight loss and decreased urination and defecation at all dose levels tested (5, 25, and 70 mg/kg). The LOEL for this s' is 5 mg/kg.

The NOEL for . . . ntal toxicity in this study is 25 mg/kg. Due to compound-related abortions, combined with several maternal deaths, at 70 mg/kg, there were insufficient litters available to comprehensively assess developmental toxicity at this dose level.

B. This study is classified Core Supplementary pending submission of results from chemical analyses of the dosing suspensions.

8. RECOMMENDATIONS: We recommend that chemical analytical data from assays conducted for this study be submitted in order to evaluate the adequacy of the dosing suspensions.

Items 9 and 10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):

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

1. Test Material: C-9211, lot No. RCL 2030, was identified as an amber liquid containing [redacted] and 40% active ingredient. Appropriate amounts of the bulk material (adjusted for purity) were mixed in 0.5% aqueous methylcellulose to yield daily doses of 0 (vehicle control), 5, 25, and 75 mg/kg when administered at a volume of 10 mL/kg. Since the test material contained [redacted] an additional (xylene-control) group was tested; this group received a mixture of xylene in aqueous methylcellulose at a volume of 10 mL/kg body weight. The xylene concentration was equal to that of the high-dose preparation (10.50 g xylene/1000 mL mixture). Samples of the preparations were obtained on the first day of dosing, midway through dosing, and on the last day of preparation.

2. Animals and Experimental Design: Virgin female New Zealand White rabbits were obtained via the sponsor from Hazleton-Dutchland, Inc., Denver, PA, and individually housed. After 21 days of quarantine, 20 females, approximately 7 months old, were assigned to each of the five treatment groups using a weight-stratified randomization procedure. The females were artificially inseminated using semen collected from

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

MANUFACTURING PROCESS INFORMATION IS NOT INCLUDED

three stock males of the same source and strain; 100 U.S.P. units of human chorionic gonadotropin were then administered intravenously to each female. Insemination procedures were conducted on 4 days over a 9-day period. Each male was equally represented in each group on each day. The day of insemination was apparently regarded (but not explicitly stated) to be gestation day 0.

Females received their respective test mixtures by gavage on days 7 through 19. Doses were based on the most recently recorded individual body weights. On day 29, the females were killed and their litters were delivered by cesarean section.

3. Observations and Measurements: Clinical observations were recorded daily throughout gestation. The animals were examined both prior to and following dosing. Females that aborted or were moribund were killed and necropsied. Tissues were preserved in formalin for histological examination when the gross findings indicated a toxicological effect. Body weights were recorded on gestation days 0, 4, 7, 11, 15, 19, 25, and 29.

Females were killed on day 29 with an intravenous injection of T-61. Abdominal and thoracic viscera were examined, corpora lutea were counted in each ovary, and the numbers, types, and locations of uterine implantations were recorded. Implantation sites were classified as live fetuses, dead fetuses, late resorptions, and early resorptions. Uteri that appeared nongravid were stained with ammonium sulfide to detect sites of early resorption.

The crown-rump lengths of late resorptions were measured. Live fetuses were individually identified, weighed, sexed internally, and examined for visceral alterations using a modified Staples' technique. The heads of approximately one-half of the fetuses of each litter were removed, fixed in Bouin's solution, and examined by Wilson's method. The remaining fetal heads were not removed; the brains were examined via a mid-coronal slice. All eviscerated fetuses were stained with alizarin red S and examined for skeletal alterations.

Fetuses from dams that delivered on day 29 were examined as described for cesarean-delivered fetuses. Fetuses from dams that aborted before day 29 were examined externally and fixed in formalin.

4. Statistical Methods: Two-tailed tests were used to compare the dosed groups and the xylene-control group with the vehicle-control group. Maternal body weight, corpora lutea, implantation, and fetal weight data were evaluated using a one-way analysis of variance and Dunnett's test. The numbers

of postimplantation losses were compared by the Mann-Whitney U test. Fetal sex ratios were compared by the chi-square test with Yates' correction factor; incidences of fetal malformations and variations were analyzed by Fisher's exact test.

B. Protocol: A study protocol was not provided in the report.

12. REPORTED RESULTS:

Test Material Analyses: The study author stated that samples of the dosing suspensions were shipped to the sponsor for analysis; however, no assay results were reported.

Maternal Data: Deaths occurred in 1/20, 1/20, 0/20, 3/20, and 5/20 females in the vehicle and xylene controls and the low-, mid-, and high-dose groups, respectively. The death in the vehicle-control group and two deaths in each of the mid- and high-dose groups were associated with gavage trauma. An additional high-dose female died of cardiorespiratory arrest. The remaining two high-dose females that died had pale and soft livers, congested kidneys and lungs, and gastrointestinal hemorrhage; the causes of these deaths were undetermined. The death in the xylene-control group and the remaining mid-dose deaths were attributed to severe metrorrhagia; the xylene-control dam had aborted and the mid-dose dam had only late resorptions in utero.

Necropsy findings of surviving females were noted sporadically or with frequencies that were comparable to vehicle controls.

Decreased defecation and urination were observed with increasing, dose-related frequencies in all groups. Xylene-control incidences were slightly greater than in the control group, but were comparable to those in the low-dose group. Other clinical findings were observed with comparable frequencies in all groups.

Dose-related body weight losses during the dosing period occurred in all dosed groups and reached significance in the mid- and high-dose groups when compared to vehicle controls (Table 1). Xylene controls also had significant weight losses during the dosing period; their data were similar to the low-dose values.

Developmental Data: Pregnancy rates were slightly reduced in the xylene-control group and all dosed groups when compared to the vehicle controls. All values, however, were within expected ranges. Abortions occurred in zero, two, one, two, and six dams in the vehicle- and xylene-control groups and in the low-, mid-, and high-dose groups, respectively. As indicated above, one of the xylene controls subsequently died; metrorrhagia was diagnosed as the cause of death for this dam and for a mid-dose dam.

TABLE 1. Mean (±S.D.) Maternal Body Weights of Rabbits Gavaged with C-9211

Dose Level (mg/kg)	Body Weight (g) on Gestation Day 0	7	19	29
0[V]	3979±399	4036±414	4120±447	4181±328
0[X]	4009±333	4035±330	3985±407	3939±519
5	4071±397	4161±423	4117±419	4190±474
25	4013±379	4073±388	4005±432	4203±554
70	3955±341	4001±360	3551±372**	3804±529

Dose Level (mg/kg)	Body Weight Gain (g) on Gestation Days 0-7	7-19	19-25	25-29
0[V]	57± 77	84±127	38±172	-16±132
0[X]	26± 97	-52±149*	4±159	-49±159
5	90±120	-44±145	27±165	17± 87
25	60± 88	-115±157**	114±159	19±138
70	47± 94	-478±216**	37±217	99±131

[V]Vehicle control.

[X]Xylene control.

*Significantly different from vehicle-control value ($p \leq 0.05$).

**Significantly different from vehicle-control value ($p \leq 0.01$).

The numbers of corpora lutea were comparable for all groups and the numbers of implantations were nonsignificantly decreased in the high-dose group when compared to vehicle controls (Table 2). Uterine findings for the xylene-control, low-dose, and mid-dose groups were comparable to vehicle controls. Fetal weights were comparable for all groups.

Fetal malformations occurred sporadically and variations were noted with comparable frequencies in all groups. However, due to the small number of high-dose dams maintaining pregnancy, there were few litters yielding data at this dose level.

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

A. The study author concluded that the test material induced maternal body weight loss at all dose levels, and attributed the increased incidence of abortions at 70 mg/kg to the compound. The study author also stated that fetal examination data did not indicate an adverse effect in any dose group.

B. A quality assurance statement was signed and dated January 14, 1986.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

A. Test Material Analyses: We are unable to assess the adequacy of the dosing suspensions since no assay results were reported.

Maternal Data: In view of the clinical observations and body weight losses seen in this study, we consider the two deaths of undiagnosed causes to reflect toxicity at 70 mg/kg. We regard the remaining deaths, however, as incidental.

We assess that the dose-related increased incidences of decreased urination and defecation indicated toxicity at 5, 25, and 70 mg/kg and in the xylene controls. In addition, the body weight losses during the dosing period also reflected toxicity in these groups.

Developmental Data: We consider the slightly reduced pregnancy rates in the xylene-control group and all dosed groups to be incidental since they were within expected ranges. In addition, ammonium sulfide staining confirmed the absence of implantations in the nongravid uteri and reduced the probability that embryonic losses occurred after dosing initiation.

We consider the increased incidence of abortions at 70 mg/kg to be compound related. We attribute the apparent reduction in the numbers of implantations and, consequently, live fetuses at this dose level to the larger litter sizes of dams that aborted, and to the fact that their data were eliminated from the data pool.

TABLE 2. Summary of Mating Results, Uterine Findings, and Fetal Weights of Rabbits Gavaged with C-9211

Dose Level (mg/kg)	Inseminated	Not Pregnant	Died[a]	Aborted	With Live Fetuses at Cesarean
0[v]	20	0	1	0	19
0[x]	20	3	1[b]	2[b,c]	15
5	20	2	0	1	17
25	20	3	3	2[c]	12
70	20	2	5	6	7

Dose Level (mg/kg)	Corpora Lutea	Implantations	Live Fetuses	Fetal Weight (g)
0[v]	11.0±1.7	7.4±2.6	6.3±2.2	42.3±6.2
0[x]	11.5±2.9	6.9±3.5	6.1±3.1	40.3±9.8
5	10.6±2.9	5.9±2.6	5.5±2.5	44.6±8.4
25	10.3±1.9	6.8±2.1	5.8±2.0	44.0±6.5
70	10.9±3.8	4.4±2.1[d]	3.3±2.1*	42.7±6.4

[a]All females that died were pregnant.

[b]One dam that aborted and died is tabulated under both categories.

[c]Includes one dam that "aborted" on day 29.

[d]A value of 5.9±2.7 was obtained by the reviewers after incorporating the data from dams that aborted.

[v]Vehicle control.

[x]Xylene control.

*Significantly different from vehicle-control value ($p \leq 0.05$).

MANUFACTURING PROCESS INFORMATION IS NOT INCLUDED

When the implantation data from dams that aborted (reported in CBI Table 12) were included in the calculations (by the reviewers), the mean (±S.D.) number of implantations/litter was 5.9±2.7, rather than 4.4±2.1, in the high-dose group, versus 7.4±2.6 for vehicle controls.

Due to the low number of litters yielding data, we do not consider the fetal examination data in the high-dose group to be conclusive. In the remaining groups, however, we assess that the fetal examination findings did not indicate any adverse developmental effects.

Data from the xylene-control group indicate that [redacted] contributed slightly to the maternal toxicity of the test material; however, developmental toxicity was not demonstrated for the [redacted] concentrations used in this study.

B. There were no major discrepancies between the reviewers and the study author in the interpretation of the results.

C. We regard the absence of chemical analytical data of the dosing suspensions to be a deficiency of the study report.

Item 15--see footnote 1.

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

APPENDIX A

Materials and Methods

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Page _____ is not included in this copy.

Pages 13 through 21 are not included in this copy.

The material not included contains the following type of information:

_____ Identity of product inert ingredients.

_____ Identity of product impurities.

_____ Description of the product manufacturing process.

_____ Description of 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 document is not responsive to the request.

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EPA: 68-02-4225
DYNAMAC No. 290-A
July 14, 1987

DATA EVALUATION RECORD

C9211M

Mutagenicity--In vitro Chromosome Aberrations in Chinese Hamster Ovary Cells

STUDY IDENTIFICATION: Ivett, J. L. and Myhr, B. C. Clastogenic evaluation of antifoulant C9211M, experimental, lot No. RCL 2030 in an in vitro cytogenetic assay measuring chromosome aberration frequencies in Chinese hamster ovary (CHO) cells. (Unpublished study No. 85RC-64 prepared by Litton Bionetics, Inc., Kensington, MD, for Rohm and Haas Co., Spring House, PA; dated January 30, 1986.) Accession No. 262473.

APPROVED BY:

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

Signature: [signature]

Date: 7-14-87

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MANUFACTURING PROCESS INFORMATION IS NOT INCLUDED

1. CHEMICAL: Antifoulant C9211M; 4,5-dichloro-2-octyl-3(2H)isothiazolone.

2. TEST MATERIAL: Antifoulant C9211M, TD85-84, from lot No. RCL 2030, was described as an amber liquid that contained ~40% active ingredient (a.i.) in [redacted].

3. STUDY/ACTION TYPE: Mutagenicity--In vitro chromosome aberrations in Chinese hamster ovary cells.

4. STUDY IDENTIFICATION: Ivett, T. L. and Myhr, B. C. Clastogenic evaluation of antifoulant C9211M, experimental, lot No. RCL 2030 in an in vitro cytogenetic assay measuring chromosome aberration frequencies in Chinese hamster ovary (CHO) cells. (Unpublished study No. 85RC-64 prepared by Litton Bionetics, Inc., Kensington, MD, for Rohm and Haas Co., Spring House, PA; dated January 30, 1986.) Accession No. 262473

5. REVIEWED BY:

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Principal Reviewer
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Date: 7-14-87

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Carlos Rodriquez, M.S.
EPA Reviewer

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Date: 7/24/87

Judith Hauswirth, Ph.D.
EPA Section Head

Signature: Judith W. Hauswirth
Date: 7/27/87

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7. CONCLUSIONS:

A. Antifoulant C9211M, containing ~40% a.i. in [redacted] was assayed without metabolic activation at four doses (125, 250, 375, and 500 ng/mL and with S9 activation at 2.25, 3.00, 7.50, 15.0, 22.5, and 30 µg/mL. The test material induced cytotoxic effects in Chinese hamster ovary (CHO) cells, but was not clastogenic.

B. The study is acceptable; Antifoulant C9211M is not clastogenic in this test system.

Items 8 through 10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):

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

1. Test Material: Antifoulant C9211M, from lot No. RCL 2030, was described as an amber liquid that contained ~40% a.i. in [redacted] The test material was reported by the sponsor to be poorly soluble in water. Based upon subsequent solubility determinations performed by the reporting laboratory, dimethylsulfoxide (DMSO) was selected as the solvent of choice for this study.

2. Cell Line: The Chinese hamster ovary cells (CHO-WBL) used in this assay were originally obtained from Dr. Sheldon Wolff, University of California, San Francisco, CA. The CHO cells were grown in McCoy's 5a medium, supplemented with 10% fetal calf serum (FCS), L-glutamine, and antibiotics for 24 hours prior to use.

3. S9 Fraction: The S9 fraction was derived from the livers of male rats induced with Aroclor 1254. The S9 reaction mixture contained 15 µL/ml rat liver S9.

4. Preliminary Cytotoxicity Assay: Prepared cell cultures, seeded at 0.3×10^6 cells/flask, were exposed with or without S9 activation to half-log dilutions of the test material ranging from 0.033 to 1000 µg/mL, the solvent control (DMSO), or the positive controls [mitomycin C (MMC) at 0.25 and 0.5 µg/mL/-S9 and cyclophosphamide (CP) at 20 and 25 µg/mL/+S9].

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

MANUFACTURING PROCESS INFORMATION IS NOT INCLUDED

In the nonactivated system, cells were exposed for 2 hours to the test material; 5-bromodeoxyuridine (BrdU; 10 µM) was added to the cultures, and incubation was continued for 23 hours. Cell monolayers were washed, refed with fresh complete medium containing BrdU, and reincubated in the presence of 0.1 µg/mL colcemid for 2.5 hours. In the S9-activated system, cultures were exposed for 2 hours without FCS. After exposure, cells were washed twice, refed with complete medium containing BrdU, (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 metaphase cells were collected by mitotic shake off. Cells were swollen in a hypotonic 0.075 M solution of potassium chloride and fixed in methanol:acetic acid (3:1), and slides were prepared. Estimation of cell-cycle delay was accomplished by staining the cells with the modified fluorescent-plus-Giemsa techniques of Perry and Wolff[2] and Goto et al.[3] One hundred cells from each dosage group were examined for the percentage of first division (M_1), between first and second divisions (M_{1+}), and beyond second division ($>M_2$) metaphase cells.

5. Cytogenetics Assay:

   a. Treatment: Prepared cultures (in duplicate), seeded at 1.5×10^6 cells, were exposed to test material doses ranging from 0.0375 to 0.5 µg/mL/-S9 and 1.5 to 30 µg/mL/+S9, the negative control (McCoy's 5a medium), solvent control (DMSO), or the positive controls, MMC (0.5 and 1.0 µg/mL) without S9 activation or CP (25 and 50 µg/mL) with S9 activation.

      In the nonactivated system, cells were dosed for 7.5 hours. Cultures were washed, refed medium containing colcemid, and reincubated for approximately 2.5 hours. Under S9-activated conditions, cells were exposed for 2 hours, washed, refed culture medium, and incubated for an additional 7.5 hours. Colcemid was added 2.5 hours before the cultures were harvested.

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[2]Perry, P. and Wolff, S. New Giemsa method for the differential staining of sister chromatids. Nature (1974) 251: 156-158.

[3]Goto, K., Maeda, S., Kano, Y., and Sugiyama, T. Factors involved in differential Giemsa-staining of sister chromatids. Chromosoma (1978) 66: 351-359.

Metaphase cells were collected and fixed. Slides were stained and coded.

b. Metaphase Analysis: One hundred morphologically normal cells per culture were scored for chromosome aberrations. Only 25 to 50 cells were scored from one of each positive control culture.

6. Statistical Methods: The data were evaluated for statistical significance at $p \leq 0.05$ by Fisher's exact test. The negative (culture medium) and solvent control data were pooled if no statistical differences were calculated in the Fisher's exact test.

7. Evaluation Criteria: No criteria to establish assay validity or a positive response were presented. 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.

B. Protocol: See Appendix B.

12. REPORTED RESULTS:

A. Preliminary Cytotoxicity Assay: Dose selection for the preliminary cytotoxicity assay was governed by compound solubility. At doses ≥1.0 mg/mL (final concentration in culture medium) a fine white precipitate formed; accordingly, a dose range of 0.033 to 1000 µg/mL was assayed in the preliminary study. Nonactivated doses ≥1 µg/mL were completely cytotoxic (100%). Below this level, slight reductions in monolayer confluency were observed but no interference with cell-cycle kinetics was seen (Table 1). In the presence of S9 activation, the test material was less cytotoxic but cytotoxicity was complete at doses ≥33.3 µg/mL. Below this level, slight reductions in monolayer confluency were reported, but progression through the cell cycle was essentially normal (Table 1).

Based on these results, doses ranging from 0.0375 to 0.5 µg/mL were selected for the nonactivated cytogenetic assay, and a dose range of 1.5 through 30 µg/mL was chosen for the S9-activated test. Under both conditions, treated cells were harvested at 10 hours postexposure.

TABLE 1. Representative Results from the Preliminary Test for Delay of Cell-Cycle Progression with Antifoulant C9211M

Substance	Dose/ mL	S9 Activation	% Cells[a] M_1	% Cells[a] M_{1+}	% Cells[a] $>M_2$	Monolayer Confluency (%)
Negative Control						
Culture Medium		-	2	1	97	100
		+	2	5	93	100
Solvent Control						
Dimethylsulfoxide	10 µL	-	1	8	91	100
		+	2	4	94	100
Positive Control						
Mitomycin C	0.25 µg	-	96	4	--	88
Cyclophosphamide	20.0 µg	+	14	70	16	36
Test Material						
Antifoulant C9211M	0.33 µg[b]	-	--	10	90	75
	1.0 µg	-	--	--	--	0
	10.0 µg[b]	+	2	23	75	86
	33.3 µg	+	--	--	--	0

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

[b]Highest noncytotoxic dose; lower doses (0.033 and 0.1 µg/mL/-S9 and 3.3 and 1.0 µg/mL/+S9) caused little or no cytotoxicity.

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B. Cytogenetic Assay:

1. Nonactivated Test Material: A slight (25%) reduction in monolayer confluency was reported for the highest dose (0.5 µg/mL). Results of metaphases examined from the 0.125-, 0.25-, 0.375-, and 0.5-µg/mL dose groups indicated that no increase in aberration frequency, percent cells with aberrations, or percent cells with >1 aberrations were scored at any test dose. The positive control, MMC at 1.0 µg/mL, induced a significant ($p < 0.01$) increase in the percent cells with aberrations. Representative results are presented in Table 2.

2. S9-Activated Test Material: No cells survived treatment with 22.5 and 30 µg/mL. Metaphase cells were examined from cultures exposed to 2.25, 3.0, 7.5, and 15.0 µg/mL of the test material.

   The frequency of aberrations, percent cells with aberrations, and percent cells with >1 aberration for all test groups were not significantly different when compared to the negative control. Significant ($p < 0.01$) increases in aberrations were scored for cells exposed to CP at 25 µg/mL.

   Representative results are presented in Table 2.

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. The study authors stated, "The test article, Anti-foulant C9211M, is considered negative for inducing chromosomal aberrations in Chinese hamster ovary cells under both the metabolic activation and nonactivation conditions of this assay."

B. A quality assurance statement was signed and dated January 22, 1986.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

We assess that the study was properly conducted and that the authors interpreted the data correctly. In both the presence and absence of S9 activation, antifoulant C9211M was assayed up to cytotoxic levels with no indication of a clastogenic effect. The results also suggest that S9 activation mitigates the cytotoxicity of the test material. By contrast, both the nonactivated and S9-activated positive controls induced significant ($p < 0.01$) increases in chromosome aberrations, indicating that the sensitivity of the assay to detect a clastogenic response was adequate.

Item 15--see footnote 1.

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TABLE 2. Representative Results of the CHO Cell *in vitro* Cytogenetic Assay with Antifoulant C9211M following a 10-Hour Cell Harvest

Substance	Dose/ mL	S9 Activation	No. of Cells Scored	No. of Aberrations per Cell	% Cells with Aberrations	% Cells with >1 Aberrations
Pooled Negative Control						
McCoy's 5a medium	--	-	200	>0.03	3.0	0.5
Dimethylsulfoxide		+	200	>0.08	6.5	3.5
Positive Control						
Mitomycin C	1.0 µg	-	25	0.40	36.0*	4.0
Cyclophosphamide	25.0 µg	+	25	0.36	36.0*	0.0
Test Material						
C9211M	0.5 µg[a]	-	200	0.02	2.0	0.0
	15.0 µg[b]	+	200	0.03	3.0	0.0

[a]Highest nonactivated dose; 25% reduction in monolayer confluency reported at this level. Results for lower doses (0.125, 0.25, and 0.375 µg/mL) were comparable to the pooled negative control values.

[b]Highest S9-activated dose scored; no cells survived treatment with 22.5 and 30 µg/mL. Results for lower doses (2.25, 3.0, and 7.5 µg/mL) were comparable to the pooled negative control values.

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

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16. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 3-6; Appendix B, Protocol, CBI Protocol pp. 1-15.

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APPENDIX A

Materials and Methods

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Page _____ is not included in this copy.

Pages 32 through 51 are not included in this copy.

The material not included contains the following type of information:

_____ Identity of product inert ingredients.

_____ Identity of product impurities.

_____ Description of the product manufacturing process.

_____ Description of 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.

CONFIDENTIAL BUSINESS INFORMATION
DOES NOT CONTAIN
NATIONAL SECURITY INFORMATION (EO 12065)

EPA: 68-02-4225
DYNAMAC No. 290-B
July 10, 1987

DATA EVALUATION RECORD

C9211

Mutagenicity--CHO/HGPRT Point Mutation Assay

STUDY IDENTIFICATION: Foxall, S., Doolittle, D. J., and McCarthy, K. L. C9211M CHO/HGPRT gene mutation assay. (Unpublished study No. 86R-0001 prepared and submitted by Rohm and Haas Company, Spring House, PA; dated March 21, 1986.) Accession No. 262498.

APPROVED BY:

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

Signature: I. Cecil Felkner

Date: 7-10-87

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MANUFACTURING PROCESS INFORMATION IS NOT INCLUDED

1. CHEMICAL: C9211; antifoulant C9211M; 4,5-dichloro-2-octyl-3(2H)isothiazolone.

2. TEST MATERIAL: C9211M, lot No. RCL 2030, TD85-84, was described as a tan solid in [redacted] the mixture had a purity of 40% active ingredient (ai).

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

4. STUDY IDENTIFICATION: Foxall, S., Doolittle, D. J., and McCarthy, K. L. C9211M CHO/HGPRT gene mutation assay. (Unpublished study No. 86R-0001 prepared and submitted by Rohm and Haas Company, Spring House, PA; dated March 21, 1986.) Accession No. 262498.

5. REVIEWED BY:

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Principal Reviewer
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Date: 7-10-87

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Date: 7-10-87

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EPA Reviewer

Signature: Carlos G. Rodriquez
Date: 7/27/87

Judith Hauswirth, Ph.D.
EPA Section Head

Signature: Judith G. Hauswirth
Date: 7/27/87

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MANUFACTURING PROCESS INFORMATION IS NOT INCLUDED

7. CONCLUSIONS:

A. Under the conditions of the assay, C9211M assayed at seven nonactivated doses from 0.5 to 6 µg/mL and four S9-activated doses ranging from 4 to 6 µg/mL did not induce an increase in the mutation frequency (MF) of Chinese hamster ovary (CHO) cells at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus. The positive controls, ethylmethanesulfonate (EMS) and 7,12-dimethylbenzanthracene (DMBA), induced gene mutations in the CHO cells, demonstrating the sensitivity of the assay.

B. The study is acceptable.

Items 8-10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):

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

1. Test Material: C-9211M, lot No. RCL 2030, TD85-84, was described as a tan solid dissolved [redacted] the mixture had a purity of 40% ai. The test material was further diluted to test dose levels in dimethylsulfoxide (DMSO), the solvent control.

2. Cell Line: The CHO cells used in the study were from the BH_4 subclone of the CHO-K_1 cell line developed by Dr. Abraham Hsie. Stock cultures were held frozen in liquid nitrogen. Growing cultures were periodically analyzed for mycoplasma contamination, karyotype stability, 6-thioguanine (6-TG) sensitivity, and aminopterin resistance. One week prior to initiation of an assay, cells were grown in Ham's nutrient medium F-12 with hypoxanthine and supplemented with 10% calf serum (heat inactivated).

3. S9 Fraction: The assay was performed with S9 fractions prepared from the livers of male Sprague Dawley rats induced with Aroclor 1254. The S9 mix contained (per mL): 0.8 mg NADP, 1.5 mg glucose-6-phosphate, 1.05 mg magnesium chloride, and the appropriate S9 fraction.

4. Positive Controls: Ethylmethanesulfonate (EMS) at 100 nL/mL and 7,12-dimethylbenzanthracene (DMBA) at 7 µg/mL were used as the nonactivated and S9-activated positive controls, respectively.

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

5. Preliminary Cytotoxicity Study: Cultures, seeded at 5×10^5 cells/plate, were exposed to 11 doses of test material ranging from 0.1 to 1000 µg/mL for 5 hours with S9 activation or for 18 to 20 hours without activation. These doses spanned a minimum of a 4-log concentration range; parallel cultures were also treated with the solvent or positive controls. All cultures were incubated at 37°C, relative humidity >90%, in 5% CO_2/air atmosphere. Two days after seeding, cells were subcultured with fresh growth medium (hypoxanthine free), and cytotoxicity was determined by the plating efficiency of the test material relative to the solvent control.

6. CHO Mutation Assay: Based on the cytotoxicity data, at least four doses with or without S9 activation were selected for the CHO assay. Doses were selected to span a toxicity range of 10 to 90 percent cell survival. Doses for a repeat assay were to be selected on the basis of results from the initial assay. The following parameters were followed:

   a. Dosing: Duplicate cell cultures were prepared and dosed with the appropriate level of test material, solvent, or positive control with or without S9 activation, as described in the cytotoxicity assay. To terminate exposure, the cultures were washed with a buffered saline solution. For cytotoxicity assessment, 200 cells were plated and the remaining cells, seeded at a density of 1×10^6, were subcultured for the mutation expression period.

   b. Mutation Expression Period: Cells used for mutation expression were subcultured twice during the 8-day expression period to maintain cells in logarithmic growth.

   c. Mutant Selection: Selection of 6-thioguanine resistant mutants (6-TG^r) was accomplished by plating 2×10^5 cells (five replicates) from each treatment group into media containing 10 µM 6-TG. Cell survival (at selection) for each treatment group was assessed from four plates, seeded with 200 cells/plate in medium free of 6-TG. Selection and survival plates were incubated for 7 days, fixed, stained, and counted. Mutation frequency (MF) was calculated as the number of 6-TG^r mutants/10^6 survivors.

7. Evaluation Criteria: The test material was considered positive if there was a significant and reproducible, dose-related increase in MF relative to the solvent control. If an increase in the MF occurred at one dose level, then the result would have to be reproduced in an independent assay.

B. Protocol: See Appendix B.

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12. REPORTED RESULTS:

A. CHO Mutation Assay--Without S9 Activation:

1. Preliminary Cytotoxicity Study: C9211M was assayed at 0.1, 1, 5, 10, 25, 50, 100, 150, 200, 500, and 1000 µg/mL without S9 activation. Cytotoxicity results, as assessed by plating efficiency, ranged from 114% survival at 0.1 µg/mL to 1.7% at 5 µg/mL. No cells survived doses ≥10 µg/mL.

2. Mutation Assay (No. 1): Based on the preliminary cytotoxicity findings, the test material was assayed at 0.5, 1, 2, 3, and 4 µg/mL in duplicate cultures. The results of the duplicate cultures were reported separately and referred to as replica No. 1 or 2. Cell survival, at the end of dosing, ranged from 97% at 0.5 µg/mL to 54% at 4 µg/mL. In replica Nos. 1 and 2, MFs for the solvent control were 0 and 3.6 6-TG^r mutants/10^6 survivors, respectively. The solvent control results were within the laboratory's historical range (0-82.6 mutants/10^6 survivors). In replica No. 1, the MFs for the test material doses (0.5, 1, 2, 3, and 4 µg/mL) were 0, 0, 2.8, 1.4, and 0 mutants/10^6 survivors; in replica No. 2, they were 0, 1.4, 1.2, 0, and 0 mutants/10^6 survivors. These results were comparable to the solvent control values.

   Although the test material did not cause an increase in mutants, a second (No. 2) assay was performed to achieve a wider range of cytotoxicity.

3. Mutation Assay (No. 2): At nonactivated doses of 3, 4, 4.5, 5, and 6 µg/mL, survival ranged from 87% at 3 µg/mL to 38% at 6 µg/mL; in one replicate (2) there was 23% survival at 4 µg/mL.

   The MFs for the solvent controls were 0 and 1.24 mutants/10^6 survivors, respectively. The test material did not cause an increase in MF, and the results of both replicates were comparable to their respective controls.

   The positive control, EMS, caused a marked increase in MF (average = 331.2 mutants/10^6) over the solvent control.

   Representative results from the second assay are presented in Table 1.

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TABLE 1. Representative Results from CHO Assay No. 2 with C9211M--Without S9 Activation

Substance	Dose	Cytotoxicity After Dosing (% Survival)	Plating Efficiency at Selection (%)	Mutant Colonies/ Plate ±S.D.	6-TG^r Mutants Per 10^6 Survivors
Solvent Control					
Dimethylsulfoxide	--[a]	96	78.4	0.0±0.00	0
	--[b]	104	80.8	0.2±0.45	1.24
Positive Control					
Ethylmethanesulfonate	100 nL/mL[a]	73	70.5	48.6±6.5	344.60[c]
	100 nL/mL[b]	83	69.8	44.4±7.8	318.05[c]
Test Material					
C9211M	4 µg/mL[a,d]	66	75.3	0.0±0.00	0
	4 µg/mL[b,d]	23	72.9	0.0±0.00	0
	6 µg/mL[a,e]	36	70.5	0.2±0.45	1.42
	6 µg/mL[b,e]	46	68.3	0.0±0.00	0

[a] Results from replica No. 1.

[b] Results from replica No. 2.

[c] Positive response as assessed by authors.

[d] Dose level presented because of cytotoxicity--23% survival.

[e] Highest dose tested; other doses (3, 4.5, and 5 µg/mL) had results that were comparable to the solvent control.

B. CHO Mutation Assay--With S9 Activation (S9 at 1 mg protein/mL):

1. Preliminary Cytotoxicity Study: C9211M was assayed at 0.1, 0.5, 5, 10, 25, 50, 100, 150, 200, 500, and 1000 µg/mL with S9 activation. Cell survival was 82 and 92% at doses of 0.1 and 0.5 µg/mL, respectively. No cells survived at higher dose levels.

2. Mutation Assay: Based on the preliminary cytotoxicity results, dose levels of 4, 4.5, 5, and 6 µg/mL C9211M were assayed in duplicate cultures. Cell survival at the end of dosing ranged from 105% at 4 µg/mL to 58% at 6 µg/mL. In replica Nos. 1 and 2, the MFs for the solvent control were 4.43 and 12.42 mutants/10^6 survivors, respectively. The MFs for the test material doses were comparable to the solvent control values.

   The authors reported that the test material was not assayed at lower cytotoxicity levels, because of the extremely steep slope of the dose-response curve.

   The positive control, DMBA, caused an increase in MF (average = 104.8 mutants/10^6 survivors) over the solvent control.

   Representative results are presented in Table 2.

C. CHO Mutation Assay--Alternate S9 Concentrations: Since the test material did not cause an adverse effect with S9 at 1 mg protein/mL, the test material was assayed at 5 µg/mL with S9 concentrations of 0.3 and 2 mg of protein/mL.

   In the presence of S9 at 0.3 mg of protein/mL, the test material cell survival (replicate results) was 46 and 53%, respectively. The MFs for the solvent control was 0 and 4.87 mutants/10^6 survivors. The test material had MFs of 0 and 6.24 mutants/10^6, which was comparable to the solvent control values.

   In the presence of S9 at 2 mg of protein/mL, cell survival was 96% for both replicates. The MFs for the solvent controls were 1.05 and 1.28/10^6 survivors. The MFs for the test material were 6.98 and 3.14 mutants/10^6 for the replicates, respectively.

   No significant differences in MF were noted with the test material in the presence of alternate concentrations of S9 when compared to solvent control values.

   The positive control, DMBA at 7 µg/mL and activated by 2 mg S9 protein/mL, caused a marked increase in MF (average 265.5 mutants/10^6) when compared to solvent control values.

   Results are presented in Table 3.

TABLE 2. Representative Results from CHO Assay with C9211M with S9 Activation (1 mg protein/mL)

Substance	Dose	Cytotoxicity After Dosing (% Survival)	Plating Efficiency at Selection (%)	Mutant Colonies/ Plate ±S.D.	6-TG^r Mutants Per 10^6 Survivors
Solvent Control					
Dimethylsulfoxide	--[a]	99	90.3	0.8±0.84	4.43
	--[b]	101	88.6	2.2±1.90	12.42
Positive Control					
7,12-Dimethylbenzanthracene	7 µg/mL[a]	83	77.8	16.6±5.40	106.70[c]
	7 µg/mL[b]	78	77.8	16.0±3.70	102.83[c]
Test Material[d]					
C9211M	4 µg/mL[a]	105	94.4	0.2±0.45	1.06
	4 µg/mL[b]	90	85.7	0.2±0.45	1.17
	6 µg/mL[a]	67	94.1	0.0±0.00	0
	6 µg/mL[b]	58	81.0	0.0±0.00	0

[a]Results from replica No. 1.

[b]Results from replica No. 2.

[c]Positive response as assessed by authors.

[d]Lowest and highest doses tested; intermediate dose (4.5 and 5 µg/mL) results were comparable to the solvent control.

TABLE 2

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TABLE 3. Results from CHO Assay with C9211M--Alternate S9 Concentration

Substance	Dose	mg S9 Protein /mL	Cyto-toxicity After Dosing (% Survival)	Plating Efficiency at Selection (%)	Mutant Colonies/ Plate ±S.D.	6-TG^r Mutants Per 10^6 Survivors
Solvent Control						
Dimethylsulfoxide	--	0.3	95	102.1	0	0
	--	0.3	105	103.8	1.0±0.00	4.82
	--	2	105	94.8	0.2±0.45	1.05
	--	2	98	78.2	0.2±0.45	1.28
Positive Control						
7,12-Dimethylbenzan-thracene	7 µg/mL	2	102	96.5	53.4±6.10	276.68[a]
	7 µg/mL	2	112	71.0	35.6±6.80	250.70[a]
Test Material						
C9211M	5 µg/mL	0.3	46	105.1	0.0±0.00	0
	5 µg/mL	0.3	53	96.1	1.2±0.84	6.24
	5 µg/mL	2	96	86.0	1.2±0.45	6.98
	5 µg/mL	2	96	95.4	0.6±1.34	3.14

[a]Positive response as assessed by authors.

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13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. The study authors concluded that, "C9211M did not induce an increased mutation frequency at the HGPRT locus in CHO cells when tested from 0.5 to 6.0 µg/ml without metabolic activation. These treatments resulted in cell survival ranging from 23 to 97%. With a metabolic activation system at 1 mg S-9 protein/ml, C9211M did not induce mutations when tested from 4.0 to 6.0 µg/ml. These treatments resulted in 58 to 105% cell survival. In addition, C9211M at 5 µg/ml did not induce mutations when tested with a metabolic activation system containing 0.3 and 2.0 mg S-9 protein/ml. C9211M is not mutagenic in the CHO/HGPRT Gene Mutation Assay."

B. A quality assurance statement was signed and dated March 21, 1986.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

We assess that the study authors interpreted the data correctly and that C9211M at seven nonactivated doses ranging from 0.5 to 6 µg/mL or at four S9-activated doses that ranged from 4 to 6 µg/mL did not induce gene mutation in CHO/HGPRT cells.

The positive controls, EMS and DMBA, induced marked increases in the MFs of CHO cells, demonstrating the sensitivity of the assay to detect a mutagenic effect.

Item 15--see footnote 1.

16. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 6-12. Appendix B, Protocol, CBI pp. 26-39.

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APPENDIX A

Materials and Methods

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Pages 64 through 85 are not included in this copy.

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_____ 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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DRAFT

EPA: 68-02-4225 312
DYNAMAC No. 290-8
June 17, 1987

K Felkner (6/25/87)
see comments
(re = [redacted]

DATA EVALUATION RECORD

C9211

Mutagenicity--CHO/HGPRT Point Mutation Assay

-0001

STUDY IDENTIFICATION: Foxall, S., Doolittle, D. J., and McCarthy, K. L. C9211M CHO/HGPRT gene mutation assay. (Unpublished study No. 86R-001 prepared and submitted by Rohm and Haas Company, Spring House, PA; dated March 21, 1986.) Accession No. 262498.

APPROVED BY:

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

Signature: _____

Date: _____

000342

EPA Comments received 7/9/87 DRH

86

MANUFACTURING PROCESS INFORMATION IS NOT INCLUDED

1. CHEMICAL: C9211; antifoulant C9211M; 4,5-dichloro-2-octyl-3(2H)isothiazo one.

2. TEST MATERIAL: C9211M; Lot No. RCL 2030 VI085-84, was described as a tan solid in [redacted] the mixture had a purity of 40% active ingredient (ai).

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

4. STUDY IDENTIFICATION: Foxall, S., Doolittle, D. J., and McCarthy, K. L. C9211M CHO/HGPRT gene mutation assay. (Unpublished study No. 86R-001) prepared and submitted by Rohm and Haas Company, Spring House, PA; dated March 21, 1986.) Accession No. 262498.

5. REVIEWED BY:

Brenda Worthy, M.T.
Principal Reviewer
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Date: _____

Nancy E. McCarroll, B.S.
Independent Reviewer
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Date: _____

6. APPROVED BY:

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

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Carlos Rodriquez, M.S.
EPA Reviewer

Signature: _____
Date: _____

Judith Hauswirth, Ph.D.
~~Acting~~ EPA Section Head

Signature: _____
Date: _____

MANUFACTURING PROCESS INFORMATION IS NOT INCLUDED

006312

MANUFACTURING PROCESS INFORMATION IS NOT INCLUDED

7. CONCLUSIONS:

A. Under the conditions of the assay, C9211M assayed at seven nonactivated doses from 0.5 to 6 µg/mL and four S9-activated doses ranging from 4 to 6 µg/mL did not induce an increase in the mutation frequency (MF) of Chinese hamster ovary (CHO) cells at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus. The positive controls, ethylmethanesulfonate (EMS) and 7,12-dimethylbenzanthracene (DMBA), induced gene mutations in the CHO cells, demonstrating the sensitivity of the assay.

B. The study is acceptable; nevertheless, a potential concern about the assay is the [redacted] in the formulated product. The pronounced cytotoxic effects that were observed were probably due to [redacted] which is extremely cytotoxic and rarely used as a solvent in mammalian cell culture assays. We conclude, however, that within the constraints placed on the assay due to the test material formulation, C9211M containing 40% ai [redacted] was probably tested to the highest reasonable dose.

Items 8-10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):

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

1. Test Material: C-9211M, lot No. RCL 2030, T085-84, was described as a tan solid in [redacted] the mixture had a purity of 40% ai. The test material was ~~dissolved~~ dissolved in dimethylsulfoxide (DMSO), the solvent control, [illegible]

2. Cell Line: The CHO cells used in the study were from the BH_4 subclone of the CHO-K_1 cell line developed by Dr. Abraham Hsie. Stock cultures were held frozen in liquid nitrogen. Growing cultures were periodically analyzed for mycoplasma contamination, karyotype stability, 6-thioguanine (6-TG) sensitivity, and aminopterin resistance. One week prior to initiation of an assay, cells were grown in Ham's nutrient medium F-12 with hypoxanthine and supplemented with 10% calf serum (heat inactivated).

3. S9 Fraction: The assay was performed with S9 fractions prepared from the livers of male Sprague Dawley rats induced with Aroclor 1254. The S9 mix contained (per mL): 0.8 mg NADP, 1.5 mg glucose-6-phosphate, 1.05 mg magnesium chloride, and the appropriate S9 fraction.

---

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

4. Positive Controls: Ethylmethanesulfonate (EMS) at 100 nL/mL and 7,12-dimethylbenzanthracene (DMBA) at 7 µg/mL were used as the nonactivated and S9-activated positive controls, respectively.

5. Preliminary Cytotoxicity Study: Cultures, seeded at 5 X 10^5 cells/plate, were exposed to 11 doses of test material ranging from 0.1 to 1000 µg/mL for 5 hours with S9 activation or for 18 to 20 hours without activation. These doses spanned a minimum of a 4-log concentration range; parallel cultures were also treated with the solvent or positive controls. All cultures were incubated at 37°C, relative humidity >90%, in 5% CO_2/air atmosphere. Two days after seeding, cells were subcultured with fresh growth medium (hypoxanthine free), and cytotoxicity was determined by the plating efficiency of the test material relative to the solvent control.

6. CHO Mutation Assay: Based on the cytotoxicity data, at least four doses with or without S9 activation were selected for the CHO assay. Doses were selected to span a toxicity range of 10 to 90 percent cell survival. Doses for a repeat assay were to be selected on the basis of results from the initial assay. The following parameters were followed:

   a. Dosing: Duplicate cell cultures were prepared and dosed with the appropriate level of test material, solvent, or positive control with or without S9 activation, as described in the cytotoxicity assay. To terminate exposure, the cultures were washed with a buffered saline solution. For cytotoxicity assessment, 200 cells were plated and the remaining cells, seeded at a density of 1 X 10^6, were subcultured for the mutation expression period.

   b. Mutation Expression Period: Cells used for mutation expression were subcultured twice during the 8-day expression period to maintain cells in logarithmic growth.

   c. Mutant Selection: Selection of 6-thioguanine resistant mutants (6-TG^r) was accomplished by plating 2 X 10^5 cells (five replicates) from each treatment group into media containing 10 µM 6-TG. Cell survival (at selection) for each treatment group was assessed from four plates, seeded with 200 cells/plate in medium free of 6-TG. Selection and survival plates were incubated for 7 days, fixed, stained, and counted. Mutation frequency (MF) was calculated as the number of 6-TG^r mutants/10^6 survivors.

7. Evaluation Criteria: The test material was considered positive if there was a significant and reproducible, dose-related increase in MF relative to the solvent control. If an increase in the MF occurred at one dose level, then the result would have to be reproduced in an independent assay.

B. Protocol: See Appendix B.

12. REPORTED RESULTS:

006312

A. CHO Mutation Assay--Without S9 Activation:

1. Preliminary Cytotoxicity Study: C9211M was assayed at 0.1, 1, 5, 10, 25, 50, 100, 150, 200, 500, and 1000 µg/mL without S9 activation. Cytotoxicity results, as assessed by plating efficiency, ranged from 114% survival at 0.1 µg/mL to 1.7% at 5 µg/mL. No cells survived doses ≥10 µg/mL.

2. Mutation Assay (No. 1): Based on the preliminary cytotoxicity findings, the test material was assayed at 0.5, 1, 2, 3, and 4 µg/mL in duplicate cultures. The results of the duplicate cultures were reported separately and referred to as replica No. 1 or 2. Cell survival, at the end of dosing, ranged from 97% at 0.5 µg/mL to 54% at 4 µg/mL. In replica Nos. 1 and 2, MFs for the solvent control were 0 and 3.6 6-TG^r mutants/10^6 survivors, respectively. The solvent control results were within the laboratory's historical range (0-82.6 mutants/10^6 survivors). In replica No. 1, the MFs for the test material doses (0.5, 1, 2, 3, and 4 µg/mL) were 0, 0, 2.8, 1.4, and 0 mutants/10^6 survivors; in replica No. 2, they were 0, 1.4, 1.2, 0, and 0 mutants/10^6 survivors. These results were comparable to the solvent control values.

   Although the test material did not cause an increase in mutants, a second (No. 2) assay was performed to achieve a wider range of cytotoxicity.

3. Mutation Assay (No. 2): At nonactivated doses of 3, 4, 4.5, 5, and 6 µg/mL, survival ranged from 87% at 3 µg/mL to 36% at 6 µg/mL; in one replicate (2) there was 23% survival at 4 µg/mL.

   The MFs for the solvent controls were 0 and 1.24 mutants/10^6 survivors, respectively. The test material did not cause an increase in MF, and the results of both replicates were comparable to their respective controls.

   The positive control, EMS, caused a marked increase in MF (average = 331.2 mutants/10^6) over the solvent control.

   Representative results from the second assay are presented in Table 1.

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TABLE 1. Representative Results from CHO Assay No. 2 with C9211M--Without S9 Activation

Substance	Dose	Cytotoxicity After Dosing (% Survival)	Plating Efficiency at Selection (%)	Mutant Colonies/ Plate ±S.D.	6-TG^r Mutants Per 10^6 Survivors
Solvent Control					
Dimethylsulfoxide	--[a]	96	78.4	0.0±0.00	0
	--[b]	104	80.8	0.2±0.45	1.24
Positive Control					
Ethylmethanesulfonate	100 nL/mL[a]	73	70.5	48.6±6.5	344.68[c]
	100 nL/mL[b]	83	69.8	44.4±7.8	318.05[c]
Test Material					
C9211M	4 µg/mL[a,d]	66	75.3	0.0±0.00	0
	4 µg/mL[b,d]	23	72.9	0.0±0.00	0
	6 µg/mL[a,e]	36	70.5	0.2±0.45	1.42
	6 µg/mL[b,e]	46	68.3	0.0±0.00	0

[a]Results from replica No. 1.

[b]Results from replica No. 2.

[c]Positive response as assessed by authors.

[d]Dose level presented because of cytotoxicity--23% survival.

[e]Highest dose tested; other doses (3, 4.5, and 5 µg/mL) had results that were comparable to the solvent control.

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B. CHO Mutation Assay--With S9 Activation (S9 at 1 mg protein/mL):

1. Preliminary Cytotoxicity Study: C9211M was assayed at 0.1, 0.5, 5, 10, 25, 50, 100, 150, 200, 500, and 1000 µg/mL with S9 activation. Cell survival was 82 and 92% at doses of 0.1 and 0.5 µg/mL, respectively. No cells survived at higher dose levels.

2. Mutation Assay: Based on the preliminary cytotoxicity results, dose levels of 4, 4.5, 5, and 6 µg/mL C9211M were assayed in duplicate cultures. Cell survival at the end of dosing ranged from 105% at 4 µg/mL to 58% at 6 µg/mL. In replica Nos. 1 and 2, the MFs for the solvent control were 4.43 and 12.42 mutants/10^6 survivors, respectively. The MFs for the test material doses were comparable to the solvent control values.

   The authors reported that the test material was not assayed at lower cytotoxicity levels, because of the extremely steep slope of the dose-response curve.

   The positive control, DMBA, caused an increase in MF (average = 104.8 mutants/10^6 survivors) over the solvent control.

   Representative results are presented in Table 2.

C. CHO Mutation Assay--Alternate S9 Concentrations: Since the test material did not cause an adverse effect with S9 at 1 mg protein/mL, the test material was assayed at 5 µg/mL with S9 concentrations of 0.3 and 2 mg of protein/mL.

   In the presence of S9 at 0.3 mg of protein/mL, the test material cell survival (replicate results) was 46 and 53%, respectively. The MFs for the solvent control was 0 and 4.87 mutants/10^6 survivors. The test material had MFs of 0 and 6.24 mutants/10^6, which was comparable to the solvent control values.

   In the presence of S9 at 2 mg of protein/mL, cell survival was 96% for both replicates. The MFs for the solvent controls were 1.05 and 1.28/10^6 survivors. The MFs for the test material were 6.98 and 3.14 mutants/10^6 for the replicates, respectively.

   No significant differences in MF were noted with the test material in the presence of alternate concentrations of S9 when compared to solvent control values.

   The positive control, DMBA at 7 µg/mL and activated by 2 mg S9 protein/mL, caused a marked increase in MF (average 265.5 mutants/10^6) when compared to solvent control values.

   Results are presented in Table 3.

TABLE 2. Representative Results from CHO Assay with C9211M--S9 Activation (1 mg protein/mL)

Substance	Dose	Cytotoxicity After Dosing (% Survival)	Plating Efficiency at Selection (%)	Mutant Colonies/ Plate ±S.D.	6-TG^r Mutants Per 10^6 Survivors
Solvent Control					
Dimethylsulfoxide	--[a]	99	90.3	0.8±0.84	4.43
	--[b]	101	88.6	2.2±1.90	12.42
Positive Control					
7,12-Dimethylbenzanthracene	7 µg/mL[a]	83	77.8	16.6±5.40	106.70[c]
	7 µg/mL[b]	78	77.8	16.0±3.70	102.83[c]
Test Material[d]					
C9211M	4 µg/mL[a]	105	94.4	0.2±0.45	1.06
	4 µg/mL[b]	90	85.7	0.2±0.45	1.17
	6 µg/mL[a]	67	94.1	0.0±0.00	0
	6 µg/mL[b]	58	81.0	0.0±0.00	0

[a]Results from replica No. 1.

[b]Results from replica No. 2.

[c]Positive response as assessed by authors.

[d]Lowest and highest doses tested; intermediate dose (4.5 and 5 µg/mL) results were comparable to the solvent control.

TABLE 3. Results from CHO Assay with C9211M--Alternate S9 Concentration

Substance	Dose	mg S9 Protein /mL	Cyto-toxicity After Dosing (% Survival)	Plating Efficiency at Selection (%)	Mutant Colonies/ Plate ±S.D.	6-TG^r Mutants Per 10^6 Survivors
Solvent Control						
Dimethylsulfoxide	--	0.3	95	102.1	0	0
	--	0.3	105	103.8	1.0±0.00	4.82
	--	2	105	94.8	0.2±0.45	1.05
	--	2	98	78.2	0.2±0.45	1.28
Positive Control						
7,12-Dimethylbenzan-thracene	7 µg/mL	2	102	96.5	53.4±6.10	276.68[a]
	7 µg/mL	2	112	71.0	35.6±6.80	250.70[a]
Test Material						
C9211M	5 µg/mL	0.3	46	105.1	0.0±0.00	0
	5 µg/mL	0.3	53	96.1	1.2±0.84	6.24
	5 µg/mL	2	96	86.0	1.2±0.45	6.98
	5 µg/mL	2	96	95.4	0.6±1.34	3.14

[a]Positive response as assessed by authors.

MANUFACTURING PROCESS INFORMATION IS NOT INCLUDED

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. The study authors concluded that, "C9211M did not induce an increased mutation frequency at the HGPRT locus in CHO cells, when tested from 0.5 to 6.0 µg/ml without metabolic activation. These treatments resulted in cell survival ranging from 23 to 97%. With a metabolic activation system at 1 mg S-9 protein/ml, C9211M did not induce mutations when tested from 4.0 to 6.0 µg/ml. These treatments resulted in 58 to 105% cell survival. In addition, C9211M at 5 µg/ml did not induce mutations when tested with a metabolic activation system containing 0.3 and 2.0 mg S-9 protein/ml. C9211M is not mutagenic in the CHO/HGPRT Gene Mutation Assay."

B. A quality assurance statement was signed and dated March 21, 1986.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

We assess that the study authors interpreted the data correctly and that C9211M at seven nonactivated doses ranging from 0.5 to 6 µg/mL or at four S9-activated doses that ranged from 4 to 6 µg/mL did not induce gene mutation in CHO/HGPRT cells. There is, however, some concern about the effect of xylene in the assay system. In a related CHO cell cytogenetic study (report No. 85RC-64, accession No. 262473), it was reported that [redacted] of the test material formulation and that the test material (dissolved in DMSO) at doses ≥1 mg/mL (final concentration in culture medium) produced a fine white precipitant. Xylene is extremely cytotoxic in mammalian cell cultures; therefore, it is rarely recommended as a solvent control. We assess that the severe cytotoxicity observed in this study may have been due to the xylene. Hence, the insolubility of the test material and cytotoxic interference by xylene limit the usefulness of the assay. However, due to the constraint placed on the assay because of the test material formulation and the lack of a mutagenic response, we assess that the test material was probably tested to the highest reasonable dose.

The positive controls, EMS and DMBA, induced marked increases in the MFs of CHO cells, demonstrating the sensitivity of the assay to detect a mutagenic effect.

Item 15--see footnote 1.

16. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 6-12. Appendix B, Protocol, CBI pp. 28-39.

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APPENDIX A

Materials and Methods

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