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

005418

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Mancozeb - Review of Mutagenicity Data Submitted on Mancozeb and ETU in Response to Data Call-In Notice of January 17, 1983 - ID #707-78, Accession No. 259044

Caswell No. 913A

FROM: Irving Mauer, Ph.D.
Toxicology Branch
Hazard Evaluation Division (TS-769C)

TO: Arvella Farmer, PM 65
Special Review Branch
Registration Division (TS-767C)

and

Henry M. Jacoby, PM 21
Fungicide-Herbicide Branch
Registration Division (TS-767C)

THRU: Jane E. Harris, Ph.D.
Head, Section VI
Toxicology Branch
Hazard Evaluation Division (TS-769C)

Registrant: Rohm & Haas

Action Requested:

(870) Review and evaluate the following mutagenicity studies in response to the Data Call-In Notice dated January 17, 1983. Individual Data Reviews are attached to this memorandum.

Study Title (No. and Date)	Reported Result	Reviewer Evaluation
lian Cell Transformation Assay in C3H 10T 1/2 Cells with enethiourea (No. 84R-056, November 19, 1984).	Negative	Acceptable
lian Cell Transformation Assay for Promotion in C3H 10T 1/2 with Ethylenethiourea (No. 84R-298, May 29, 1985).	Negative	Unacceptable
bial Mutagen (Ames) Test with Dithane M-45 Fungicide (S-9 red from Aroclor 1254-induced Fischer 344 rats) (No. 84R-0059, 21, 1984).	Negative	(Incomplete)*
bial Mutagen (Ames) Test with Dithane M-45 Fungicide (S-9 red from Aroclor 1254-induced B6C3F1 mice) (No. 84R-0060, 21, 1984).	Negative	(Incomplete)*
Mediated Assay in Mice with Dithane M-45 Fungicide 84RC-25B, September 26, 1984).	Negative	Unacceptable
Mediated Assay in Mice with Dithane M-45 Fungicide (Repeat) (No. 84RC-48, July 1, 1985).	Negative	Acceptable
Mutation Assay in CHO Cells with Dithane M-45 Fungicide 84R-207, February 11, 1985).	Negative	(Incomplete)*
eduled DNA Assay in Rat Hepatocytes with Dithane M-45 Fungicide 84R-280, May 29, 1985).	Negative	Inconclusive**
enetic Study in Fischer-344 Rats with Dithane M-45 Fungicide 84R-246, December 21, 1984).	Negative	Acceptable
r Chromatid Exchange Assay in CHO Cells with Dithane M-45 cide (No. 84RC-60, March 1985).	Positive	Acceptable
lian Cell Transformation Assay in C3H 10T 1/2 Cells with ne M-45 Fungicide (No. 84R-055, November 19, 1984).	Negative	Acceptable
lian Cell Transformation Assay for Promotion in C3H 10T 1/2 with Dithane M-45 Fungicide (No. 84R-297, May 29, 1985).	Negative	Unacceptable

ceptable with S-9 activation. Although initially declared "unacceptable under nactivated conditions" because no positive controls were included for that part of e assay, the sensitivity of the test system to respond was demonstrated adequately the activated assays. Hence these studies are ACCEPTABLE.

esumptively positive; procedural problems indicate assay should be repeated.

Two additional studies were included with this submission (Accession No. 259044), but were not reviewed at this time, namely (as listed in the registrant's Table of Contents):

"Section 2. Acute Oral LD50 in B6C3F1 Mice With Dithane M-45 Fungicide" (R & H Report No. 83R-213A, dated September 24, 1984).

"Section 3. Acute Oral LD50 in Fischer-344 Rats With Dithane M-45 Fungicide" (R & H Report No. 83R-213B, dated September 24, 1984).

These two acute studies will be evaluated for inclusion in the MANCOZEB REGISTRATION STANDARD.

005418

EPA: 68-02-4225
DYNAMAC No. 009
April 3, 1986

(1)

DATA EVALUATION RECORD

MANCOZEB

Mutagenicity

STUDY IDENTIFICATION: Mutagenicity Overview on the Pesticide Mancozeb.

REVIEWED BY:

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

Signature: I. Cecil Felkner
Date: 4-3-86

Nancy E. McCarroll, B.S.
Independent Reviewer
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APPROVED BY:

William L. McLellan, Ph.D.
Genetic Toxicology
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Signature: William L. McLellan
Date: 4-3-86

Irving Mauer, Ph.D.
EPA Reviewer

Signature: Irving Mauer
Date: 04-04-86

Jane Harris, Ph.D.
EPA Acting Section Head

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Date: 04-04-86 /JEH

TEST CHEMICALS: Mancozeb; Dithane M-45; coordination product of zinc and manganese ethylene bis-dithiocarbamate, and ethylenethiourea (ETU), a principal metabolic derivative.

STUDY/ACTION TYPE: Overview--Registration Action.

MUTAGENICITY OVERVIEW ON THE PESTICIDE MANCOZEB

Introduction: Under FIFRA Guideline Subdivision F: Pesticide Assessment Guidelines: Hazard Evaluation - Human and Domestic Animals, dated 11-30-82, an overview (Section 80-1) is required for the various subdivisions of toxicology. "This subdivision details the toxicity data recommended to support the registration of pesticide products," and should meet the requirements of good laboratory practice (40 CFR Part 160), if applicable.

For each test substance, bioassays must be performed to assess the "potential to affect the qualitative or quantitative integrity of human genetic material." A battery of tests to assess mutagenicity is therefore required with the objectives of:

1. Detecting, with great sensitivity, the capacity of a test material to alter cellular genetic material.

2. Determining the relevance of genetic alterations to mammals.

3. Incorporating positive genetic findings into the risk assessments for heritable effects, carcinogenicity, and other possible health hazards.

There are three categories of genetic effects that must be addressed by the test battery:

1. Gene mutations.

2. Structural chromosomal aberrations.

3. Other mutagenic mechanisms (e.g., direct DNA damage, microtubule/spindle fiber inhibition) as deemed appropriate for the test material.

Mutagenicity data as required by 40 CFR Section 158.135 are submitted to support the registration of each manufacturing-use product and of certain end-use products. The assays are performed with the technical grade of each active ingredient in the product. The product should be tested in nonactivated and metabolically activated in vitro assays, and should also be assayed using in vivo mammalian systems with all appropriate positive and negative controls.

SUMMARY OF STUDY EVALUATIONS:

Category 1: Gene Mutation. There were five unpublished studies submitted by the registrant and one published report submitted by the reviewer which were in this category. They are summarized in Table 1 and discussed in this section according to assay type.

Salmonella typhimurium/microsome (Ames): Report Nos. 84R0059 and 84R0060 stated that Dithane M-45 did not induce mutations in any tester strains with or without S9 activation in a dose range of 2.5 to 250 µg/plate. The assays using S9 were conducted properly and therefore considered acceptable; however, the nonactivated assays performed in these studies did not include direct-acting positive controls and were not considered acceptable.

Host-mediated assay in mice (Salmonella typhimurium TA1530): Report No. 84RC-258, using S. typhimurium TA1530 as the detector in a host-mediated assay in mice, showed that Dithane M-45 was not mutagenic when the host was dosed at 0.5 to 5 mg/kg. However, the doses were inadequate; therefore, the assay was considered unacceptable. Report No. 84RC-43 described the use of a dose range of 0.5 to 5 g/kg in an otherwise identical host-mediated assay. Dithane M-45 was also negative for a mutagenic response and the assay was considered acceptable.

Mammalian cell mutagenicity--CHO/HGPRT: Report No. 84R-207 stated that Dithane M-45 was not mutagenic in the CHO/HGPRT in vitro assay either at 0.5 to 15 µg/mL in the nonactivated assay or at 0.25 to 45 µg/mL in the S9-activated assay. The S9-activated assay was properly conducted and therefore acceptable; however, the nonactivated assay was unacceptable because the positive control, ethylmethane sulfonate, had to be used at a level that was approximately 7-fold higher than the highest nonactivated dose, 15 µg/mL of test material.

Published data on Bacillus subtilis and Salmonella typhimurium (liquid preincubation) assays: Using a dose range of 1 to 25 µg/plate in the liquid preincubation assay with B. subtilis and S. typhimurium, Shiau et al. (1980)[1] reported that Dithane Technical (zinc ethylenebisdithiocarbamate) was mutagenic in both B. subtilis TKJ6321 and S. typhimurium TA1535. Although mutagenesis in the Salmonella mutant was not as strong as in B. subtilis, it could be demonstrated at nonactivated concentrations of 1, 5, and 10 µg/plate. In B. subtilis TKJ6321, a dose-responsive increase was observed in the nonactivated system at this same dose range, with a decline due to cytotoxicity at 25 µg/plate. At the highest concentration, approximately 45,000 His^+ revertants/10^8 cells was reported, and the calculated potency was 38.4 revertants/nanomole. In the presence of S9, Dithane was reported to show a "marked reduction" in the mutagenic response.

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Overall, the unpublished reports showed that Dithane M-45 was not mutagenic either in vitro using bacteria and mammalian cells with or without S9 activation, or in the host mediated assay. However, none of the assays were acceptable for the in vitro nonactivated system. Since published data using the liquid preincubation nonactivated system for both B. subtilis and S. typhimurium were positive, and in fact showed that Dithane was a very potent mutagen, inducing 38.4 revertants/nanomole, the test material should be considered mutagenic. It is classified as a base-pair mutagen on the basis of its positive activity for S. typhimurium strain TA1535 and B. subtilis strain TKJ6321. If all the unpublished and published data are considered, there are no data gaps for Category 1.

Category 2: Structural Chromosomal Aberrations.

Sister chromatid exchange: Study No. 84RC-60 reported that Dithane M-45 caused an increase in sister chromatid exchange in CHO-WB1 cells which was dose responsive in two assays in the range of 7.5 to 17.5 µg/mL in the nonactivated system (positive response beginning at 7.5 µg/mL), and inconclusive but presumed positive in the range of 10 to 17.5 µg/mL in the S9-activated assay (positive response beginning at 10 µg/mL). The assays were conducted properly and, therefore, acceptable.

In vivo cytogenic study in rats: The report No. 84R-246 stated that Dithane M-45 did not cause a significant increase in chromosomal aberrations in bone marrow cells of male rats exposed to 4.4 g/kg (maximum tolerated dose) sampled over the entire mitotic cycle. This data was acceptable; however, the study did not include female animals and was, therefore, deficient.

Except for the data gap on chromosomal aberrations in female animals, there is sufficient data to fulfill the requirements in Category 2. Since a positive response was obtained in the SCE assay in the nonactivated system and a presumptive positive in the S9-activated system, we presume that the test material causes structural chromosomal aberrations and that the frequency of inducing these aberrations could be reduced in either the presence of an exogenous or endogenous mammalian metabolic system.

Category 3: Other Mutagenic Mechanisms.

Unscheduled DNA synthesis in rat hepatocytes: The report No. 84R-280 stated that Dithane M-45 did not induce unscheduled DNA synthesis in isolated Fischer rat hepatocytes. However, we conclude that the net nuclear grain counts at 1.0, 2.5, and 5.0 µg/mL, although complicated by a higher than usual cytoplasmic grain count for the solvent and a decrease at 10 µg/mL, were sufficient to presume a positive but inconclusive UDS response.

DNA-damage in Bacillus subtilis: Shiau et al. (1980)[1] published data showing that Dithane technical caused DNA damage in several repair-deficient strains of B. subtilis at 50 µg/plate in the nonactivated system, but that S9 metabolism reduced the effect so that 300 µg/plate was required for a positive response. This data is supported by the

thesis work of Lee[2] (1980) who demonstrated DNA damage in B. subtilis strains hcr-9, fh2006-7, and mc-1 in the nonactivated system at all doses between 50 and 300 µg/plate. In addition, a positive DNA-damaging effect in a liquid incubation assay was demonstrated at all doses from 10 to 50 µg/mL. In an attempt to define the mechanism of Dithane action, Lee also showed that the DNA derived from B. subtilis cultures treated with 10 µg/mL of Dithane was damaged, and therefore had reduced DNA transformation activity in the histidine gene; furthermore a 1:1 ratio of Dithane mixed with purified DNA, damaged the DNA and specifically reduced DNA transformation for the histidine locus.

Hume[3] (1980) reported that nonactivated Dithane was negative in the phage induction assay (β-galactosidase induction) in Escherichia coli B13 (λ+) when doses ranging from 50 to 300 µg/plate were assayed in a semiquantitative spot test, but it was weakly positive at 50 µg/mL in the liquid (tube) assay.

In vitro transformation assays in C3H/10T1/2 mouse fibroblasts: Report 85R-055 stated that Dithane M-45 at doses ranging from 0.05 to 0.5 µg/mL did not induce transformed foci in C3H/10T1/2 mouse fibroblasts; likewise, ethylenethiourea (ETU) was reported to be negative in this system at doses ranging from 100 to 1,000 µg/mL (Report 84-R-056). These studies were properly conducted and therefore acceptable. However, study Nos. 84R-297 and 84R-298, designed to assess promoter activity in C3H/10T1/2 mouse fibroblasts, were unacceptable because only one dose of Dithane M-45 (0.1 µg/mL) or ETU (333 µg/mL) was used.

SUMMARY TABLE:

A one-liner table has been included in this overview. It identifies the individual studies, specifies the dose range, presents the reviewers' evaluations, places each study in its proper category, and classifies the studies according to their acceptability.

CONCLUSION:

If all studies submitted by the registrant and published articles are considered, testing in all three genetic effect categories are fulfilled. Although there were many studies submitted in which negative responses were obtained, there is sufficient evidence which shows that Dithane is mutagenic. Since this test material was shown to induce base-pair mutations in B. subtilis and S. typhimurium,[1] induce sister chromatid exchange (study No. 84RC-60), presumably induce unscheduled DNA synthesis (study No. 84R-280), induce DNA damage in B. subtilis,[1,2] induce β-galactosidase synthesis in the E. coli B13 (λ+) system,[3] and directly interact with DNA of B. subtilis,[2] it must be considered to have a significant genotoxic potential. The negative responses in some of the in vitro assays could have been contributed, at least in part, by the problems of solubility; the test material is very insoluble in water, therefore, using DMSO as the solubilizer was required.[1,2,3] In

addition, it appears that the S. typhimurium/microsome assays (study Nos. 84R0059 and 84R0060) lacked sensitivity and perhaps even specificity for detecting a gene mutation response; however, liquid preincubation and the use of DMSO as a solubilizer appeared to improve the sensitivity of the assay.[1] The inconclusive result in UDS assays could also be attributed to a failure in solubilizing the test material. When the transformation assays were performed, there was no indication that either Dithane or ETU induced or promoted transformed foci; these results are not surprising considering that the test materials may not have reached the target site under the conditions of the assays. In addition, transformation is considered to be a multistage phenomenon and the requirements to cause cell transformation would likely be less probable than induction of a point mutation, DNA interaction, or a clastogenic effect.

Literature Cited

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[1] Shiau, S. Y., R. A. Huff, B. C. Wells, and I. C. Felkner. Mutation Research 71(1980): 169-179.

[2] Lee, M. C. DNA Transformation and Mutagenesis Studies on Dithane with Bacillus subtilis Repair-Deficient Strains. M. S. Thesis at Texas Tech University, supported by EPA Contract CR806904-01, 1980, pp. 1-21.

[3] Hume, S. H. Screening Pesticides with a Colorimetric Phage Induction Assay. M. S. Thesis at Texas Tech University, supported by EPA Contract CR806904-01, 1980.

Study / Lab / Study Date	Material*	No.	Dose	Conclusions (Evaluations)	Category	Classification
1. Mutagenicity Reverse Mutation in Salmonella/Rohm and Haas Co./ 84R0059/6-21-84	Dithane M-45, TD No. 83-224, lot No. 0842, 88% a.i.	259044	2.5-250 µg/plate	Not mutagenic for S9-activated; data inadequate for nonactivated but negative	1	S9--acceptable; nonactivated--unacceptable
2. Mutagenicity--Reverse Mutation in Salmonella/Rohm and Haas Co./ 84R0060/6-21-84	Dithane M-45, TD No. 83-224, lot No. 0842, 88% a.i.	259044	2.5-250 µg/plate	Not mutagenic for S9-activated; data inadequate for nonactivated but negative	1	S9--acceptable; nonactivated--unacceptable
3. Mutagenicity--Host-mediated Assay-Salmonella in mice/ Hazelton Labs./84RC-25B/9-26-84	Dithane M-45, 88% a.i.	259044	0.5-5.0 mg/kg	Inadequate dosage; negative result	1	Unacceptable
4. Mutagenicity--Host-mediated Assay-Salmonella in mice/ Hazelton Labs./84RC-48/1-1-85	Dithane M-45, 88% a.i.	259044	0.5-5.0 g/kg	Not mutagenic	1	Acceptable
5. Mutagenicity--CHO/HGPRT/ Rohm and Haas Co./84R-207/ 2-11-85	Dithane M-45, TD83-224, lot No. 0842, 88% a.i.	259044	0.25-45 µg/mL	Not mutagenic for S9-activated; data inadequate for nonactivated	1	S9--acceptable; nonactivated--unacceptable
6. Mutagenicity--Sister Chromatid Exchange in CHO/Litton Bionetics, Inc./84RC-60/3-85	Dithane M-45, TD83-224, lot No. 0842, 88% a.i.	259044	7.5-17.5 µg/mL (2 assays)	Mutagenic in nonactivated assay (7.5 µg/mL); presumed mutagenic (10 µg/mL) in S9-activated assay	2	Acceptable

*Dithane M-45 which is the Rohm and Haas technical coordination product of zinc and manganese ethylene bis-dithiocarbamate.

7. Mutagenicity--In vivo Cytogenetics in Rats/Rohm and Haas Co./ 84R-246/12 21-84	Dithane M-45,* TD No. 83-224, lot No. 0842, 88% a.i.	259044	4.4 g/kg	Not mutagenic in male rats; not tested in female rats	2	Acceptable in male rats; unacceptable in female rats
8. Mutagenicity--In vitro Unscheduled DNA Synthesis/Rohm and Haas Co./ 84R-280/5-29-85	Dithane M-45,* TD 83-224 lot No. 0842, 88% a.i.	259044	0.025-10.0 µg/mL	Presumptive positive at 1.0, 2.5, and 5.0 µg/mL	3	Inconclusive
9. Mutagenicity In vitro Transformation in C3H/101 1/2 Mouse Fibroblasts/Rohm and Haas Co./ 85R-055/11-19-84	Dithane M 45,* TD 83-224 lot No. 0842, 88% a.i.	259044	0.05 0.5 µg/ml	Negative at all doses	3	Acceptable
10. Mutagenicity--In vitro Transformation in C3H/101 1/2 Mouse Fibroblasts/Rohm and Haas Co./ 84R-056/11-19-84	Ethylenethiourea, TD 83-223, lot No. 088-36, 99.8% pure	259044	100-1000 µg/mL	Negative at all doses	3	Acceptable
11. Mutagenicity--In vitro Transformation Assay for Promotion in C3H/101 1/2 Mouse Fibroblasts/ Rohm and Haas Co./84R-297/ 5-29-85	Dithane M-45* TD 83-224, lot No. 0842, 88% a.i.	259044	0.1 µg/mL	Negative, but insufficient dose range	3	Unacceptable

*Dithane M-45 which is the Rohm and Haas technical coordination product of zinc and manganese ethylene bis-dithiocarbamate.

TABLE 1 One Liner Summary Table of Mutagenicity Studies with Mancozeb (Dithane M 45) and Ethylenethiourea (continued)

Study / Lab / Study Date	Material	Accession No.	Dose	Conclusion (Evaluations)	Genetic Effects Category	Classification
12. Mutagenicity - *In vitro* Transformation Assay for Promotion in C3H/10T 1/2 Mouse Fibroblasts/ Rohm and Haas Co./84R 298/ 5 29 85	Ethylenethiourea, ID 83-223, lot No. D88-36, 99.8% pure	259044	333 µg/mL	Negative, but insufficient dose range	3	Unacceptable
13. Mutagenicity and DNA damaging Activity for Several Pesticides Tested with *Bacillus subtilis* mutants. *Mutation Res.* 71(1980): 169 179/Texas Tech Univ. EPA Contract No. 68-01 3963	Dithane (technical) zinc and manganese ethylene-bisdithiocarbamate	N/A--Published data provided by reviewer/ sponsor	(a) 50-300 µg/ plate	(a) Positive DNA damage in *B. subtilis* at 50 µg/plate--non-activated and 300 µg/plate S9-activated	(a) 3	(a) Acceptable
			(b) 1-25 µg/plate (Liquid incubation using DMSO as solvent)	(b) Positive mutagen in *B. subtilis* IKJ 6321. Potency = 38.4 mutants/nanomole, nonactivated. Positive mutagen in *S. typhimurium* TA1535 at 2 µg/plate, nonactivated	(b) 1	(b) Acceptable
14. DNA damaging and Direct Effects on DNA in *Bacillus subtilis*/ Thesis, Texas Tech Univ.- EPA Contract CR806904-01; NCI Grant 1-R01-CA21020 02A1/8-80	Dithane (technical) zinc and mangansese ethylene-bisdithiocarbamate	N/A--Thesis data provided by reviewer/ sponsor	(a) 50-300 µg/ plate	(a) Positive DNA damage in *B. subtilis* strain *hcr-9*, *fh2006-7*, and *mc-1* at all levels, nonactivated	(a) 3	(a) Acceptable
			(b) 10-50 µg/mL (liquid)	(b) Positive at 10 µg/mL in *B. subtilis* *hcr-9*, *fh2006-7*, and *mc-1*, nonactivated	(b) 3	(b) Acceptable
			(c) 10 µg/mL	(c) DNA in whole cells damaged for gene transformation	(c) 3	(c) Acceptable
			(d) 100 µg/ 100 µg DNA	(d) Purified DNA damaged for gene transformation	(d) 3	(d) Acceptable

TABLE 1 One Liner Summary Table of Mutagenicity Studies with Mancozeb (Dithane M 45) and Ethylenethiourea (continued)

Study / Lab / Study Date	Material	Accession No.	Dose	Conclusions (Evaluations)	Genetic Effects Category	Classification
15. Mutagenicity Phage Induct Test for DNA Damage (Escherichia coli strain B13(λ+)B-galactosidase assay)./Thesis, Texas Tech Univ. EPA Contract CR806904 01/5-81	Dithane (technical) zinc and manganese ethylene-bisdithio-carbamate	N/A - Thesis data provided by re-viewer/ sponsor	50 300 µg/plate	Negative in plate assay	3	Acceptable
			1 50 µg/ml (liquid assay)	Weakly positive in tube assay at 50 µg/ml	3	Inconclusive presumptive positive

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EPA: 68-02-4225
DYNAMAC No. 009-A2
April 3, 1986

(4)

DATA EVALUATION RECORD

MANCOZEB

Mutagenicity--In vitro Transformation Assay
in C3H/10T 1/2 Mouse Fibroblasts with Ethylenethiourea

STUDY IDENTIFICATION: McGlynn-Kreft, A. M., and McCarthy, K. L. Ethylenethiourea mammalian cell transformation test. (Unpublished study No. 84R-056 prepared and submitted by Rohm and Haas Co., Spring House, PA; dated November 19, 1984.) Accession No. 259044.

APPROVED BY:

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

Signature: I. Cecil Felkner

Date: 4-3-86

1. CHEMICAL: Mancozeb; ethylenethiourea.

2. TEST MATERIAL: Ethylenethiourea prepared by Rohm and Haas, sample TD 83-223, lot No. DB 8-36, had a purity of 99.8 percent; its physical description was not reported.

3. STUDY/ACTION TYPE: Mutagenicity--In vitro transformation assay in C3H 10T/1/2 mouse fibroblasts with ethylenethiourea.

4. STUDY IDENTIFICATION: McGlynn-Kreft, A. M., and McCarthy, K. L. Ethylenethiourea mammalian cell transformation test. (Unpublished study No. 84R-056 prepared and submitted by Rohm and Haas Co., Spring House, PA; dated November 19, 1984.) Accession No. 259044.

5. REVIEWED BY:

Nancy E. McCarroll, B..
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Signature: Nancy E. McCarroll
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6. APPROVED BY:

I. Cecil Felkner, Ph.D.
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Date: 4-3-86

Irving Mauer, Ph.D.
EPA Reviewer

Signature: Irving Mauer
Date: 04-04-86

Jane Harris, Ph.D.
EPA Section Head

Signature: Irving Mauer for JEH
Date: 04/04/86

7. CONCLUSIONS:

A. Under the conditions of this assay and in the absence of S9 activation, ethylenethiourea at doses of 100, 330, and 1,000 µg/mL did not cause an increase in the number of transformed foci in C3H/10T 1/2 cells. The performance of the assay without an exogenous metabolic activation system is an acceptable practice because this cell line can metabolize certain chemicals to active carcinogens. This was adequately demonstrated by the positive response in this assay with the known procarcinogen, 7,12-dimethylbenzanthracene (DMBA).

B. The study is acceptable.

Items 8 through 10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):

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

1. Test Material: Ethylenethiourea, sample TD 83-223, lot No. DB 8-36, had a purity of 99.8 percent and was dissolved in dimethylsulfoxide (DMSO). The stock solution and required dilutions were prepared on the day of treatment.

2. Cell Line: Clone 8 of C3H/10T 1/2 mouse embryo fibroblast cells were obtained from Dr. Charles Heidelberger, University of Southern California, Los Angeles. Logarithmic phase cultures, grown from frozen stock cultures, were used as the target cell.

3. Cytotoxicity Assay: Prepared cells, seeded at a density of 200 cells per plate, were exposed in triplicate to an unspecified number of test material concentrations spanning at least a 4-log dose range. After a 24-hour exposure, the medium was removed and cells were incubated with growth medium for 9-10 days. Surviving colonies were fixed, stained, counted, and compared to the number of colonies in the solvent control.

4. Cell Transformation Assay: Based on the results of the cytotoxicity assay, three doses were selected for the cell transformation test.

a. Exposure: Prepared cultures, seeded with either 200 or 2,000 cells/plate, were treated with the three selected doses of the test material, solvent, or positive control.

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

After a 24-hour exposure, the medium was removed and cultures were refed with fresh growth medium. Cells seeded at the lower density were used to determine the plating efficiency. Surviving colonies on these plates were fixed, stained, and counted after 9-10 days of incubation. The remaining cultures, seeded at 2,000 cells/plate, were periodically refed with growth medium throughout the 6-week incubation period. At the conclusion of incubation, cells in the transformation plates were fixed, stained, and scored for transformed foci.

b. Scoring Transformed Foci: Foci were scored by the criteria of Reznikoff et al.[2] as follows:

Type I--Densely stained areas composed of tightly packed cells.

Type II--More densely stained areas than Type I, with piling up of cells and overlapping nuclei.

Type III--Highly polar, piling up of cells, composed of areas exhibiting crisscrossing at the interface of the focus and monolayer.

5. Evaluation Criteria: The assay was considered positive if a dose-response relationship was apparent or the incidence of plates with Type III foci at one dose was significantly higher than the historical untreated and solvent controls.

6. The incidence of plates with Type III foci was statistically compared to the historical untreated and solvent controls by the Fisher Exact test.

7. Evaluation Criteria for Positive Control: Results for the positive control were not analyzed by statistics; the protocol stated that the positive control group must yield an incidence of at least 15 percent of the plates with Type III foci for the positive control to be considered valid.

B. Protocol: See Appendix B.

12. REPORTED RESULTS:

Cytotoxicity Assay: The preliminary cytotoxicity assay was conducted with 14 concentrations of the test material ranging from 0.1 to 1000 µg/mL, two doses of the positive control, DMBA, and the solvent

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[2] C. A. Reznikoff, D. W. Brankow, and C. Heidelberger. Establishment and characterization of a cloned cell line C3H mouse embryo cells sensitive to postconfluence inhibition of division. Cancer Res. 33(1973): 3231-38.

control. Cytotoxicity was not evident at any test dose; percent surivival for 1.0 and 2.5 µg/mL DMBA was 59 and 56 percent, respectively.

Transformation Assay: Based on the preliminary cytotoxicity findings, doses selected for the transformation assay were 100, 330, and 1,000 µg/mL. Since cytotoxicity was not achieved, the number of transformation plates for the high dose was increased to 80. For the remaining test doses, media, and positive control, 20 replicates were plated; 30 replicates were used for the solvent control. Plating efficiency was determined from the counts of triplicate plates for all test doses and controls.

The test material was not cytotoxic. No Type III foci were found following exposure of the cells to the three selected doses of the test material; statistical analysis of these data were, therefore, not perfcrmed. Representative data from this assay are presented in Table 1.

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. The authors concluded, "This study demonstrates that ethylene-thiourea produces no adverse effects in the Mammalian Cell Transformation Test under the conditions specified."

B. A quality assurance statement was signed and dated November 14, 1984.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

It is our assessment that the study was properly conducted and the authors' interpretation of the data was correct. Although the test material was not cytotoxic, the assay was conducted at the maximum recommended dose for noncytotoxic compounds.[3] Since no validated exogenous metabolic activation system currently exists for this test, using the C3H/10T 1/2 transformation assay in the absence of S9 activation is acceptable.[4] The sensitivity of the test system to detect the induction of transformants was adequately demonstrated by the positive control (DMBA, 0.5 µg/mL). Hence, the assay system appeared to have the appropriate enzymes to metabolize DMBA to a form that is active for inducing cell transformation.

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[3] C. Heidelberger, A. E. Freeman, R. J. Pienta, A. Sivak, J. S. Bertram, B. C. Casto, V. C. Dunkel, M. W. Francis, T. Kakunaga, J. B. Little, and L. Schechtman. Cell transformation by chemical agents--a review and analysis of the literature. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutat. Res. 114(1983): 283-385.

[4] Ibid.

TABLE 1. Representative Results of the C3H/10T 1/2 Transformation Assay with Ethylenethiourea

ubstance	Dose (µg/mL)	% Survival[a]	No. Plates w/Type II Foci/ Total No. Replicates	% Replicates w/Tvpe II Foci	No. Plates w/Type III Foci/ Total No. Replicates	% Replicates w/Type III Foci
gative Control						
ulture Media	--	95	0/20	0	0/20	0
lvent Control						
imethylsulfoxide	--	100	0/30	0	0/30	0
sitive Control						
,12-dimethyl-benzanthracene	0.5	78	5/20	25	9/20	45[c]
st Material						
thylenethiourea	1000[b]	94	1/80	1.25	0/80	0

$$\frac{\text{No. of colonies with test dose}}{\text{No. of colonies with solvent control}} \times 100$$

ighest dose tested; values for lower concentrations (100 and 330 µg/mL) were omparable to the solvent control and, therefore, not selected as representative.

ositive by the authors' criterion; $\geq$ 15% increase in plates with Type III foci.

Item 15--see footnote 1.

16. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 1-7; Appendix B, Protocol, CBI pp. 15-17.

APPENDIX A
Materials and Methods

14504 MANCOZEB RIN 2617-93

Page_____ is not included in this copy.

Pages 13 through 33 are not included.

The material not included contains the following type of information:

____ Identity of product inert ingredients.

____ Identity of product impurities.

____ Description of the product manufacturing process.

____ Description of quality control procedures.

____ Identity of the source of product ingredients.

____ Sales or other commercial/financial information.

____ A draft product label.

____ The product confidential statement of formula.

____ Information about a pending registration action.

✓ 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.

005418

EPA: 68-02-4225
DYNAMAC No. 009-A12
April 3, 1986

(5)

DATA EVALUATION RECORD

MANCOZEB

Mutagenicity--*In vitro* Transformation Assay for Promotion in C3H/10T 1/2 Mouse Fibroblasts with Ethylenethiourea

STUDY IDENTIFICATION: McLeod, P. L. and Doolittle, D. J. Ethylenethiourea mammalian cell transformation test for promotion. (Unpublished study No. 84R-298 prepared and submitted by Rohm and Haas Co., Spring House, PA; dated May 29, 1985.) Accession No. 259044.

APPROVED BY:

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

Signature: I Cecil Felkner

Date: 7-12-86

005418

1. CHEMICAL: Mancozeb; ethylenethiourea.

2. TEST MATERIAL: Ethylenethiourea prepared by Rohm and Haas, sample TD 83-223, lot No. DB8-36, had a purity of 99.8 percent; its physical description was not reported.

3. STUDY/ACTION TYPE: Mutagenicity--in vitro transformation assay for promotion in C3H/10T 1/2 mouse fibroblasts with ethylenethiourea.

4. STUDY IDENTIFICATION: McLeod, P. L. and Doolittle, D. J. Ethylenethiourea mammalian cell transformation test for promotion. (Unpublished study No. 84R-298 prepared and submitted by Rohm and Haas Co., Spring House, PA; dated May 29, 1985.) Accession No. 259044.

5. REVIEWED BY:

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

Signature: Nancy E. McCarroll
Date: 4-3-86

Brenda Worthy, M.T.
Independent Reviewer
Dynamac Corporation

Signature: Brenda Worthy
Date: 4-3-86

6. APPROVED BY:

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

Signature: I. Cecil Felkner
Date: 4-3-86

Irving Mauer, Ph.D.
EPA Reviewer

Signature:
Date: 6/4-04-86

Jane Harris, Ph.D.
Acting EPA Section Head

Signature:
Date: 6/4-04-86

35

7. CONCLUSIONS:

A. Under the conditions of the assay, ethylenethiourea at 333 µg/mL did not cause an increase in the number of transformed foci either in initiated or uninitiated C3H/10T 1/2 cells at a presumed maximum tolerated noncytotoxic dose. However, the assay was conducted with only one test dose, which may not be sufficient to conclude that the test material is not an in vitro tumor promoter.

B. The study is unacceptable because a single dose level is not sufficient for establishing promoter action.

8. RECOMMENDATIONS:

Dr. Craig J. Boreiko,[1] a noted expert on initiation/promotion assays, recommends the use of more than one dose level because promoters frequently induce erratic and nondose-related effects. In lieu of established guidelines for initiation/promotion assays, we feel his suggestions should be considered. We, therefore, recommend that the test material should be assayed at more than one dose or the authors should justify the use of a single treatment level.

Items 9 and 10--see footnote 2.

11. MATERIALS AND METHODS (PROTOCOLS):

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

1. Test Material: Ethylenethiourea, sample TD 83-223, lot No. 088-36, had a purity of 99.8 percent and was dissolved in dimethylsulfoxide (DMSO).

2. Cell System: Clone 8 of C3H/10T 1/2 mouse embryo fibroblast cells was obtained from Dr. Charles Heidelberger, University of Southern California, Los Angeles. Logarithmic phase cultures, grown from frozen stock cultures, were used as the target cell.

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[1] Boreiko, C. J., Chemical Institute of Toxicology, Research Triangle Park, NC, personal communications.

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

3. Cytotoxicity Assays:

   a. Range-Finding Cytotoxicity Test: Prepared cells, seeded at a density of 200 cells per plate, were exposed in triplicate to three concentrations of the test material.

      Cells were exposed either for 24 hours or continuously throughout a 9-day incubation period. After the 24-hour exposure, the medium was removed and cells were incubated with growth medium for 8 days. Surviving colonies for both exposures were fixed, stained, and counted.

   b. Cytotoxicity Assay with the Initiating Agent: Cultures, seeded at a density of 2000 cells per plate, were exposed to the initiating agent, 0.5 µg/mL N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), for 4 hours. Five and 9 days after initiation, cell viability was determined from three control cultures. The remaining cultures were continuously refed with media containing the three selected test doses or the solvent control; viability was monitored daily for 4 days and on alternate days thereafter until termination of the cytotoxicity test, day 11 after initial treatment with the test compound. A growth curve was plotted (total number of cells vs. days post-treatment) to determine growth inhibition.

4. Cell Transformation Assay for Promotion: Based on the combined results of the cytotoxicity assays, a single test dose was selected for the cell transformation test with promotion.

   a. Exposure: The appropriate number of prepared cultures, seeded with either 200 or 2000 cells/plate, were treated with the following agents: 0.5 µg/mL MNNG, 0.5 percent acetone (MNNG solvent), 0.5 µg/mL 7,12-dimethylbenzanthracene (DMBA), or 1.0 µg/mL 3-methylcholanthrene (MCA). Exposure to the initiating agents, MNNG or acetone, was terminated at 4 hours; the exposure period for DMBA or MCA was 24 hours. Five days after dosing, initiated cultures were refed with media containing the selected dose of the material being assayed for promoter activity, the solvent controls, DMSO or acetone, or the known promoting agent, 0.25 µg/mL 12-o-tetradecanoylphorbol-13-acetate (TPA); this treatment was continued for 6 weeks (promotion phase). Therefore, a single dose of the test material was exposed to 20 replicates of untreated cells, 20 replicates of MNNG-initiated cells, or 20 replicates of acetone-treated cells. The remaining control groups, DMSO or TPA, were similarly added to 20 replicate untreated or preinitiated cultures. Cultures treated with DMBA or MCA were not exposed to promoting agents because these compounds do not require promoter action to transform cell cultures. Plating efficiency was

not determined for uninitiated or initiated cultures exposed to the test material, but was determined for selected controls. Surviving colonies on these plates were fixed, stained, and counted after 9-10 days of incubation. Throughout the approximately 6-week promotion phase, cells were continuously refed with media containing the test dose, solvent, or control promoters. At the conclusion of incubation, cells in the transformation plates were fixed, stained, and scored for transformed foci.

b. Scoring Transformed Foci: Foci were scored by the criteria of Reznikoff et al.[3] as follows:

Type I-- Densely stained areas, composed of tightly packed cells.

Type II-- More densely stained areas than Type I with piling up of cells and overlapping nuclei.

Type III-- Highly polar, piling up of cells, composed of areas exhibiting crisscrossing at the interface of the focus and monolayer.

5. Evaluation Criteria: The assay was considered positive if an increase in Type III foci was observed. If an appreciable increase in Type II foci occurred, the test would be repeated with more replicates and/or more or different test concentrations.

6. Evaluation Criteria for Positive Controls: The positive "complete" carcinogen control, DMBA, must yield an incidence of at least 15 percent of the plates with Type III foci to be considered valid evidence of test system sensitivity. Similarly, the promoter control, TPA, must increase the incidence of plates with Type III foci relative to the initiating agent alone for the assay to be considered acceptable. The percent increase was not specified.

7. The data were not statistically analyzed.

B. Protocol: See Appendix B.

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[3] Reznikoff, C. A., Brankow, D. W., and Heidelberger, C. Establishment and characterization of a cloned cell line C3H mouse embryo cells sensitive to postconfluence inhibition of division. Cancer Res. 33(1973): 3231-38.

12. REPORTED RESULTS:

Cytotoxicity Assays:

a. Range-Finding Cytotoxicity Test: The preliminary cytotoxicity assays were conducted with 100, 333, and 1000 µg/mL of the test material. Following a 24-hour and a 9-day continuous exposure, cytotoxicity was not evident at any dose tested.

b. Cytotoxicity Assay with Initiating Agent: Cultures preinitiated with 0.5 µg/mL MNNG were continuously exposed to 100, 333, or 1000 µg/mL of the test material. Based on the reported growth curve results, a 60 percent inhibition of cells was plotted at day 4 for the highest dose. By day 7, cell growth at this level was 22 percent less than the solvent control. At 333 µg/mL, growth inhibition (30 percent) was plotted for day 4; however, by day 7 cells exposed to 333 µg/mL recovered and exceeded the growth in the solvent control group. Throughout the remaining incubation period, cell growth at this test level consistently exceeded growth in the solvent control. Based on these findings, 333 µg/mL was selected for the transformation assay for promotion.

c. Transformation Assay with Promotion: The selected dose, 333 µg/mL, was continuously applied to untreated and acetone-/ or MNNG-pretreated cells. No foci were observed on untreated or acetone-treated test-dose-promoted plates. Fifteen percent of the plates containing cells initiated with MNNG, promoted with 333 µg/mL, had Type II foci; no Type III foci were scored. Representative results are shown in Table 1.

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. The authors concluded, "This study demonstrates that ethylenethiourea does not promote morphological transformation in the Mammalian Cell Transformation Test for Promotion under the conditions specified."

B. A quality assurance statement was signed and dated March 29, 1985.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

It is our assessment that a properly designed study was conducted, and the authors' interpretation of the data was correct. No *in vitro* assays to detect tumor promoters have been validated and probably must await the clearer understanding of tumor promotion mechanisms before their use in screening programs can be fully realized.

LE 1. Representative Results of the C3H/10T 1/2 Transformation Assay for Promotion with Ethylenethiourea (ETU)

Substance[a]	Promoter	No. Plates w/ Type II Foci/ Total No. Replicates	% Replicates with Type II Foci	No. Plates w/ Type III Foci/ Total No. Replicates	% Replicates with Type III Foci
gative Control					
	Media	0/20	0	0/20	0
lvent Control					
	DMSO (0.33%)	0/20	0	0/20	0
0.5%)	DMSO (0.33%)	0/20	0	0/20	0
µg/mL)	DMSO (0.33%)	2/20	10	0/20	0
sitive Control					
µg/mL)	--	6/20	30	8/20	40[b]
µg/mL)	--	12/20	60	10/20	50[b]
µg/mL)	TPA (0.25 µg/mL)	11/20	55	9/20	45[c]
est Substance					
	ETU (333 µg/mL)	0/20	0	0/20	0
0.5%)	ETU (333 µg/mL)	0/20	0	0/20	0
µg/mL)	ETU (333 µg/mL)	3/20	15	0/20	0

imethylsulfoxide
-methyl-N'-nitro-N-nitrosoguanidine
-o-Tetradecanoyl-phorbol-13-acetate
,12-Dimethylbenzanthracene
Methylcholanthrene

by the authors' criterion (≥ 15% increase in plates with Type III foci).

by the authors' criterion (increased incidence of plates with Type III foci as to initiating agents alone).

The authors stated, in accordance with Frazelle et al.[4], that "a non-toxic concentration of test compound is the maximum tolerated dose for assessing promoting activity in this assay."

We confirmed this statement with Frazelle and Boreiko[5] who indicated that the majority of promoters are noncytotoxic. Boreiko[6] recommended, however, that more than a single dose should be assayed (five doses of an unknown agent are routinely evaluated in his laboratory) since promoters frequently induce erratic and nondose-related effects. Although ethylenethiourea was negative at the selected concentration, it is possible that 333 µg/mL was not the effective level and tumor promotion could have been detected if more doses were evaluated.

No established guidelines exist for initiation and promotion assays. Since Dr. Boreiko is a recognized expert in this area, his recommendation should be considered appropriate in lieu of published guidelines. It is our assessment, therefore, that the results reported by the authors are insufficient to support the conclusion that ethylenethiourea does not promote neoplastic transformation in this assay.

The ability of the known tumor promoter, TPA (0.25 µg/mL), to induce neoplastic transformation in initiated cells was demonstrated. Similarly, the direct induction of transformants by DMBA (0.5 µg/mL) and MCA (1.0 µg/mL) was adequately shown. Although no criteria were presented to evaluate a positive effect with MCA, we assumed that the criterion reported for DMBA (≥ 15% increase in plates with Type III foci) applied to both polycyclic aromatic hydrocarbons.

Item 15--see footnote 2.

16. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 1-8, page 5 missing; Appendix B, Protocol, CBI pp. 17-30.

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[4] Frazelle, J. H., Abernethy, D. J., and Boreiko, C. J. Determination of cell culture conditions optimal for the study of initiation and promotion in C3H 10T 1/2 cells. Environmental Mutagenesis 4(1982): 331-332.

[5] Sanchez, J. H. (nee Frazelle) and Boreiko, C. J., Chemical Institute of Toxicology, Research Triangle Park, NC, personal communications.

[6] Ibid.

APPENDIX A

Materials and Methods

14504 MANCOZEB RIN 2617-93

Page_____ is not included in this copy.

Pages 43 through 64 are not included.

The material not included contains the following type of information:

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____ Identity of product impurities.

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____ 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.

✓ FIFRA registration data.

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005418

EPA: 68-02-4225
DYNAMAC No. C09A-4a
April 3, 1986

(6)

DATA EVALUATION RECORD

MANCOZEB

Mutagenicity--Salmonella/Mammalian-Microsome Assay

STUDY IDENTIFICATION: Chism, E. M. and Kunze, E. Dithane M-45 microbial mutagen assay. (Unpublished study No. 84R 0059 prepared and submitted by Rohm and Haas Company, Spring House, PA; dated June 21, 1984.) Accession No. 259044.

APPROVED BY:

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

Signature: I. Cecil Felkner

Date: 4-3-86

005418

1. CHEMICAL: Mancozeb; Dithane M-45, coordination product of zinc and manganese ethylene bis-dithiocarbamate.

2. TEST MATERIAL: Dithane M-45 prepared by Rohm and Haas Co., from TD No. 83-224, lot No. 0842, was described as a gray-tan powder with 88 percent active ingredient.

3. STUDY/ACTION TYPE: Mutagenicity--Salmonella/mammalian-microsome assay.

4. STUDY IDENTIFICATION: Chism, E. M. and Kunze, E. Dithane M-45 microbial mutagen assay. (Unpublished study No. 84R 0059 prepared and submitted by Rohm and Haas Company, Spring House, PA; dated June 21, 1984.) Accession No. 259044.

5. REVIEWED BY:

William L. McLellan, Ph.D.
Principal Reviewer
Dynamac Corporation

Signature: William L. McLellan
Date: 4-3-86

Brenda Worthy, M.T.
Independent Reviewer
Dynamac Corporation

Signature: Brenda Worthy
Date: 4-3-86

6. APPROVED BY:

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

Signature: I. Cecil Felkner
Date: 4-3-86

Irving Mauer, Ph.D.
EPA Reviewer

Signature: [illegible]
Date: 4/21-04-86

Jane Harris
EPA Section Head

Signature: [illegible]
Date: 04-04/86 JEH

7. CONCLUSIONS:

A. Under the conditions of the study Dithane M-45 did not cause increases in reversion in Salmonella typhimurium strains TA1535, TA1537, TA98, or TA100 using an S9 fraction from livers of Aroclor-1254-induced Fischer 344 rats or without activation. The maximum concentration used (250 µg/plate) inhibited growth of each strain with or without S9 activation. The positive controls, 2-anthramine and 2-acetamidofluorene, demonstrated the sensitivity of the assay to detect a mutagenic response with S9 activation only. However, no direct-acting mutagen positive controls were tested to demonstrate the sensitivity of the nonactivated system to detect a mutagenic response.

B. The study was acceptable with S9 activation but unacceptable in the nonactivated assay.

Items 8 through 10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):

A. Materials and Methods:

1. The test material, Dithane M-45, lot No. 0842, was 88 percent active ingredient. Concentration levels were calculated on a basis of 80 percent active ingredient. The diluent for suspension of the test material was water. Water was also the solvent control. The positive controls were 10 µg/plate 2-anthramine for strains TA1535, TA1537, and TA100 and 50 µg/plate 2-acetamidofluorene for strain TA98.

2. S. typhimurium strains TA1535, TA1537, TA98, and TA100 were obtained from Dr. Bruce Ames, University of California, Berkeley, and characterized for growth and histidine requirement at time of transfer. Strains TA98 and TA100 contained PKM 101 R-factor, confirmed by resistance to ampicillin.

3. The S9 preparation was from the livers of Aroclor-1254-induced Fischer 344 rats.

4. Mutagenicity testing was by the method of Ames et al.[2] The test compound was assayed at levels of 2.5, 7.5, 25, 75,

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

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

and 250 µg/plate in triplicate with and without S9 activation. Mean and standard deviations of reversions with the solvent control were calculated from 39 plates/strain.

5. Data were analyzed by the method of Mohn and Ellenberger.[3]

B. Protocol: See Appendix A.

12. REPORTED RESULTS:

A. Cytotoxicity: There was inhibition of growth of all the tester strains with Dithane M-45 at concentration levels of 75 or 250 µg/plate as indicated by a decrease in mean number of revertants compared to controls (Table 1).

B. Mutation Assay: There was no increase in revertants compared to controls at 2.5, 7.5, 25, 75, or 250 µg/plate with any of the tester strains. The positive controls gave the expected response in the S9-activated assay. No positive controls were included with the nonactivated assay (Table 1).

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. Dithane M-45 did not induce reverse mutations in S. typhimurium strains TA1535, TA1537, TA98, or TA100 with or without activation with Aroclor 1254 induced by S9 from liver of Fischer 344 rats. The maximum active ingredient concentration tested, 250 µg/plate, inhibited growth of each of the tester strains.

B. A quality assurance statement was not presented.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

It is our assessment that the authors' conclusions are supported by the data presented for the S9-activated assay only. It was not stated whether the test compound precipitated in the plates; since the test material is practically insoluble in aqueous or organic solvents, this might have been expected. Cytotoxicity was not measured directly; however, cytotoxicity was evident from the decrease in revertants at 75 and 250 µg/plate when compared to controls. Since no direct-acting mutagens were assayed, the authors failed to demonstrate the sensitivity of the nonactivated system to detect mutagenicity. Therefore, a conclusion cannot be made on the potential of the nonactivated test material to induce mutagenesis in the S. typhimurium tester strains.

---

[3] Mohn, G. R. and J. Ellenberger, in: Handbook of Mutagenicity Test Procedures (eds., Kilbey B. J., M. Legator, W. Nochols, and C. Ramels), Elsevier, Amsterdam, 1977.

TABLE 1. Mutagenicity Assay with Dithane M-45 Using S9 from Fischer 344 Rat Liver

Dose (µg/plate)	Mean Revertants/Plate of Bacterial Tester Strains							
	TA1535		TA1537		TA 98		TA100	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Dithane M-45								
2.5	26.0	20.0	9.7	12.0	25.7	43.7	112.0	100.3
7.5	29.3	16.0	8.3	8.7	32.0	43.0	113.7	89.3
25.0	26.7	11.7	7.0	8.7	26.7	37.7	100.3	89.7
75.0	0.0	7.0	0.0	0.7	3.0	19.3	4.0	21.0
250.0	0.0	5.0	0.0	0.0	0.0	2.3	0.0	0.0
Solvent control	30.5	17.8	9.6	11.4	29.4	52.1	106.6	115.2
Positive control								
2ANTH,[a] 10	32.7	419.0	10.3	176.7			91.2	1457.7
2AAF,[a] 50					30.7	1734.8		

[a]2ANTH is 2-anthramine and 2AAF is 2-acetamidofluorene.

Item 15--see footnote 1.

16. CBI APPENDIX: Appendix A, Protocol.

APPENDIX A

Protocol

APPENDIX A

Protocol

14504 MANCOZEB RIN 2617-93

Page_____ is not included in this copy.

Pages 73 through 78 are not included.

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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.

005418

EPA: 68-02-4225
DYNAMAC No. 009A-4b
April 3, 1986

(7)

DATA EVALUATION RECORD

MANCOZEB

Mutagenicity--Salmonella/Mammalian-Microsome Assay

STUDY IDENTIFICATION: Chism, E. M. and Kunze, E. Dithane M-45 microbial mutagen assay. (Unpublished study No. 84R 0060 prepared and submitted by Rohm and Haas Company, Spring House, PA; dated June 21, 1984.) Accession No. 259044.

APPROVED BY:

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

Signature: I. Cecil Felkner

Date: 4-3-86

1. CHEMICAL: Mancozeb; Dithane M-45, coordination product of zinc and manganese ethylene bis-dithiocarbamate.

2. TEST MATERIAL: Dithane M-45, prepared by Rohm and Haas Co. from TD No. 83-224, lot No. 0842, was described as a gray-tan powder with 88 percent active ingredient.

3. STUDY/ACTION TYPE: Mutagenicity--Salmonella/mammalian-microsome assay.

4. STUDY IDENTIFICATION: Chism, E. M. and Kunze, E. Dithane M-45 microbial mutagen assay. (Unpublished study No. 84R 0060 prepared and submitted by Rohm and Haas Company, Spring House, PA; dated June 21, 1984.) Accession No. 259044.

5. REVIEWED BY:

William L. McLellan, Ph.D.
Principal Reviewer
Dynamac Corporation

Signature: William L. McLellan
Date: 4-3-86

Brenda Worthy, M.T.
Independent Reviewer
Dynamac Corporation

Signature: Brenda Worthy
Date: 4-3-86

6. APPROVED BY:

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

Signature: I. Cecil Felkner
Date: 4-3-86

Irving Mauer, Ph.D.
EPA Reviewer

Signature: Irving Mauer
Date: 04-04-86

Jane Harris, Ph.D.
EPA Section Head

Signature: Jane Harris
Date: 04-04-86 / JEH

7. CONCLUSIONS:

A. Under the conditions of the study, Dithane M-45 did not cause increases in reversion in Salmonella typhimurium strains TA1535, TA1537, TA98, or TA100 using an S9 fraction from livers of Aroclor-1254-induced $B6C3F_1$ mice or without activation. The maximum concentration used (250 µg/plate) inhibited growth of each strain with or without S9 activation. The positive controls, 2-anthramine and 2-acetamidofluorene, demonstrated the sensitivity of the assay to detect a mutagenic response with S9 activation only. However, no direct-acting mutagen positive controls were tested to demonstrate the sensitivity of the nonactivated system to detect a mutagenic response.

B. The study was acceptable with S9 activation but unacceptable in the nonactivated assay.

Items 8 through 10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):

A. Materials and Methods:

1. The test material, Dithane M-45, lot No. 0842, was 88 percent active ingredient. Concentration levels were calculated on a basis of 80 percent active ingredient. The diluent for suspension of the test material was water. Water was also the solvent control. The positive controls were 10 µg/plate 2-anthramine for strains TA1535, TA1537, and TA100 and 50 µg/plate 2-acetamidofluorene for strain TA98.

2. S. typhimurium strains TA1535, TA1537, TA98, and TA100 were obtained from Dr. Bruce Ames, University of California, Berkeley, and characterized for growth and histidine requirement at time of transfer. Strains TA98 and TA100 contained PKM 101 R-factor, confirmed by resistance to ampicillin.

3. The S9 preparation was from the livers of Aroclor-1254-induced $B6C3F_1$ mice.

---

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

4. Mutagenicity testing was by the method of Ames et al.[2] The test material was assayed at levels of 2.5, 7.5, 25, 75, and 250 µg/plate in triplicate with and without S9 activation. Mean and standard deviations of reversions with the solvent control were calculated from 39 plates/strain.

5. Data were analyzed by the method of Mohn and Ellenberger.[3]

B. Protocol: See Appendix A.

12. REPORTED RESULTS:

A. Cytotoxicity: There was inhibition of growth of all the tester strains with Dithane M-45 at concentration levels of 75 or 250 µg/plate as indicated by a decrease in mean number of revertants compared to the respective vehicle controls (Table 1).

B. Mutation Assay: There was no increase in revertants compared to controls at 2.5, 7.5, 25, 75, or 250 µg/plate with any of the tester strains. The positive controls gave the expected response in the S9-activated assay. No positive controls were included with the nonactivated assay (Table 1).

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. Dithane M-45 did not induce mutagenicity in S. typhimurium strains TA1535, TA1537, TA98, or TA100 with or without activation by Aroclor-1254-induced Fischer 344 rat liver S9. The maximum active ingredient concentration tested, 250 µg/plate, inhibited growth of each of the tester strains.

B. A quality assurance statement was not presented.

---

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

[3] Mohn, G. R. and J. Ellenberger, in: Handbook of Mutagenicity Test Procedures (eds., Kilbey B. J., M. Legator, W. Nichols, and C. Ramels), Elsevier, Amsterdam, 1977.

TABLE 1. Mutagenicity Assay with Dithane M-45 Using S9 from Fischer 344 Rat Liver

Dose (µg/plate)	Mean Revertants/Plate of Bacterial Tester Strains							
	TA1535		TA1537		TA98		TA100	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Dithane M-45								
2.5	29.3	18.7	9.7	10.3	25.7	29.3	125.3	99.7
7.5	25.7	17.3	8.7	9.3	22.0	26.3	120.3	105.3
25.0	30.7	13.0	6.3	7.0	25.3	17.3	100.7	79.7
75.0	0.0	8.0	1.3	0.0	0.7	14.0	5.3	10.7
250.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Solvent control	28.7	20.9	8.2	10.5	24.2	33.8	117.7	104.5
Positive control								
2ANTH,[a] 10	31.8	476.2	11.2	180.8			117.7	1099.3
2AAF,[a] 50					21.3	2008.0		

[a]2ANTH is 2-anthramine and 2AAF is 2-acetamidofluorene.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

It is our assessment that the authors' conclusions are supported by the data presented for the S9-activated assay only. It was not stated whether the test compound precipitated in the plates; since the test material is practically insoluble in aqueous or organic solvents, this might have been expected. Cytotoxicity was not measured directly; however, cytotoxicity was evident from the decrease in revertants at 75 and 250 µg/plate when compared to controls. Since no direct-acting mutagens were assayed, the authors failed to demonstrate the sensitivity of the nonactivated system to detect mutagenicity. Therefore, a conclusion cannot be made on the mutagenic potential of the nonactivated test material in the S. typhimurium tester strains.

Item 15--see footnote 1.

16. CBI APPENDIX: Appendix A, Protocol.

APPENDIX A

Protocol

14504 MANCOZEB RIN 2617-93

Page_____ is not included in this copy.

Pages 86 through 91 are not included.

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____ 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.

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005418

EPA: 68-02-4225
DYNAMAC NO. 005418
April 8, 1986

(8)

DATA EVALUATION RECORD

MANCOZEB

Mutagenicity--Host-Mediated Assay in Mice

STUDY IDENTIFICATION: McCarroll, N. E., and Farrow, M. G. Host mediated assay in mice with compound Dithane M-45. (Unpublished study No. 84RC-258 prepared by Hazelton Laboratories, Vienna, VA, for Rohm and Haas Company, Spring House, PA; dated September 26, 1984.) Accession No. 259044.

APPROVED BY:

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

Signature: [signature]

Date: 4-8-86

005418

1. CHEMICAL: Mancozeb; Dithane M-45, coordination product of zinc and manganese ethylene bis-dithiocarbamate.

2. TEST MATERIAL: Dithane M-45, a pale yellow powder, stored at room temperature, had a purity of 88 percent active ingredient; prepared by Rohm and Haas Co.

3. STUDY/ACTION TYPE: Mutagenicity--host-mediated assay in mice.

4. STUDY IDENTIFICATION: McCarroll, N. E., and Farrow, M. G. Host mediated assay in mice with compound Dithane M-45. (Unpublished study No. 84RC-25B prepared by Hazelton Laboratories, Vienna, VA, for Rohm and Haas Company, Spring House, PA; dated September 26, 1984.) Accession No. 259044.

5. REVIEWED BY:

Brenda Worthy, M.T.
Principal Reviewer
Dynamac Corporation

Signature: Brenda Worthy
Date: 4-8-86

William L. McLellan, Ph.D.
Independent Reviewer
Dynamac Corporation

Signature: William L. McLellan
Date: 4-8-86

6. APPROVED BY:

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

Signature: I. Cecil Felkner
Date: 4-8-86

Irving Mauer, Ph.D.
EPA Reviewer

Signature: Irving Mauer
Date: 10-11-86

Jane Harris, Ph.D.
EPA Section Head

Signature: Jane E. Harris
Date: 10/15/86

7. CONCLUSIONS:

A. Under the conditions of the assay, a definitive conclusion cannot be made regarding the potential of Dithane M-45 for causing a mutagenic response in the host-mediated assay using mice; the dose range selected, 0.5 to 5 mg/kg, was not sufficient.

B. The study is unacceptable.

8. RECOMMENDATIONS:

A. It is recommended that the authors repeat the assay with a dose range that demonstrates some toxic or cytotoxic responses at the highest dose selected, thus demonstrating the appropriateness of the selected dose range.

B. It is also recommended that an evaluation criterion be included in the report.

11. MATERIALS AND METHODS (PROTOCOLS):

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

1. Test Material: Dithane M-45 was described as a pale yellow powder, stored at room temperature, with a purity of 88 percent active ingredient. The test material was dissolved in corn oil, the solvent control.

2. Test Animals: Eight-week-old male B6C3F1 mice were obtained from Charles River Breeding Laboratories, Inc., Portage, MI.

a. Animal Maintenance: The animals were acclimated to laboratory conditions for 3 weeks prior to the start of the study. They were individually housed in plastic shoe boxes in an environmentally controlled room (temperature, 73-85°F; relative humidity, 22-39 percent) on a 12-hour light/dark cycle. Food and water were available ad libitum.

b. Group Assignment: Sixty healthy animals were assigned to five treatment groups (20 mice/solvent control group, 10/dose group, and 10/positive control group) using a random number table. Animals were identified by toe clip/ear notch and with a unique species letter.

Items 9 and 10--see footnote 1.

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

9.4

c. Clinical Observation: Animals were observed once on the day of dosing for changes in appearance, behavior, and toxic or pharmacological signs. Body weights were taken prior to dosing.

3. Test Material Preparation and Administration:

a. The test material was prepared fresh on the day of dosing on a weight per volume basis to achieve 100 percent of the active ingredient per dose.

b. The test material at doses of 0.5, 2.0, and 5 mg/kg and the solvent control were administered via oral gavage. The positive control, 10 percent dimethylnitrosamine (DMN), was administered by intramuscular (im) injection.

4. Bacterial Inoculation: Two milliliters of a suspension of Salmonella typhimurium strain TA1530, containing 4.8 X 10^8 cells/mL, was administered to all animals intraperitoneally (ip) following test material administration.

5. Host-Mediated Assay:

a. Animal Sacrifice: All animals were sacrificed by cervical dislocation. The positive control group was sacrificed 2 hours after bacterial inoculation; the solvent and the three test material groups were sacrificed 4 hours after inoculation.

b. Bacterial Recovery: Animals were cleansed with ethanol and skinned for laparotomy. One milliliter of saline was injected ip through the abdominal musculature wall; the peritoneal cavity was aseptically opened, and the bacterial exudate was withdrawn.

c. Plating of Bacterial Exudate: The standard pour-plate technique was used. For revertant counts, undiluted peritoneal fluid was added in triplicate to complete top agar, containing histidine and biotin, mixed, and poured over Vogel-Bonner E minimal agar. Similarly, for total cell counts, 10-fold dilutions (10^{-1} to 10^{-8}) of peritoneal fluid were prepared in saline and the three highest dilutions were added to top agar and poured over Tryptone Soy Agar plates in triplicate. All plates were incubated at 37°C. After 48 hours, colonies were counted.

6. Evaluation Criteria: No specific criteria for a positive response were reported.

B. Protocol: See Appendix B.

12. REPORTED RESULTS:

Clinical observations were reported as normal for all animals, and no deaths occurred during the study. Pretreatment body weights were similar for all groups.

No increase was observed in the mutation frequency of bacterial colonies assayed in mice that were dosed orally with 0.5, 2.0, and 5 mg/kg of the test material when compared to the solvent control. However, the positive control, DMN administered im, caused a 50-fold increase in mutation frequency over the solvent control.

Representative results are presented in Table 1.

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. The authors concluded that "Dithane M-45 did not demonstrate a mutagenic response when tested in the Host Mediated mutation assay using Salmonella typhimurium strain TA1530 as the indicator strain and B6C3F$_1$ mice as the host."

B. A quality assurance statement was signed and dated September 26, 1984.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

It is our assessment that the authors interpreted the data correctly and that the test material by oral gavage did not cause a mutagenic response using S. typhimurium strain TA1530 in mice treated with Dithane M-45. By contrast, the positive control by injection caused a 50-fold increase in mutation frequency over the solvent control, therefore demonstrating that the assay system was capable of detecting a mutagenic response. However, the authors did not report any preliminary range finding or cytotoxicity data to support the selection of the dose range used in this assay, nor whether the compound was absorbed from the gastrointestinal tract and reached the indicator organism at an effective concentration. Since the highest dose tested, 5 mg/kg, did not elicit a toxic effect in the dosed mice or a cytotoxic response in the tester strain we concluded that the dose range selected was not high enough to determine the mutagenic potential of Dithane M-45 in the host-mediated assay.

Item 15--see footnote 1.

16. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 1-3; Appendix B, Protocol CBI (Appendix I) pp. 7-13.

TABLE 1. Representative Results of the Host-Mediated Assay With S. typhimurium Strain TA1530 in Mice Treated with Dithane M-45

Substance	Dose	Mean MCFU[a] ($\times 10^1$)	Mean CFU[b] ($\times 10^9$)	MF[c] ($\times 10^{-7}$)	Fold Increase Relative to Solvent Control
Solvent control					
Corn oil		5.8	2.95	0.2	-
Positive control					
10% Dimethylnitrosamine	0.1 mL	100.4	0.72	10	50[d]
Test material					
Dithane M-45	5 mg/kg[e]	4.14	2.45	0.2	1

[a]Mean mutant colony-forming units (MCFU)—calculated by reviewers.

[b]Mean colony-forming units (CFU)—calculated by reviewers.

[c]Mutation frequency = $\frac{\text{Mean MCFU}}{\text{Mean CFU}}$

[d]Positive response as reported by authors.

[e]Highest dose tested; lower doses (0.5 and 2 mg/kg) were comparable to the solvent controls.

APPENDIX A

Materials and Methods

14504 MANCOZEB RIN 2017-93

Page_____ is not included in this copy.

Pages 99 through 111 are not included.

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____ 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.

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005418

EPA: 68-02-4225
DYNAMAC No. 809-A6
April 8, 1986

(9)

DATA EVALUATION RECORD

MANCOZEB

Mutagenicity--Host-Mediated Assay in Mice

STUDY IDENTIFICATION: McCarroll, N.E. Host mediated assay in mice with compound Dithane M-45. (Unpublished study No. 85RC-48 prepared by Hazelton Laboratories, Vienna, VA, for Rohm and Haas Company, Spring House, PA; dated July 1, 1985.) Accession No. 259044.

APPROVED BY:

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

Signature: I. Cecil Felkner

Date: 4-7-86

005418

1. CHEMICAL: Mancozeb; Dithane M-45, coordination product of zinc and manganese ethylene bis-dithiocarbamate.

2. TEST MATERIAL: Dithane M-45, a pale yellow powder, stored at room temperature, had a purity of 88 percent active ingredient; prepared by Rohm and Haas Co.

3. STUDY/ACTION TYPE: Mutagenicity--host-mediated assay in mice.

4. STUDY IDENTIFICATION: McCarroll, N. E. Host mediated assay in mice with compound Dithane M-45. (Unpublished study No. 85RC-48 prepared by Hazelton Laboratories, Vienna, VA, for Rohm and Haas Company, Spring House, PA; dated July 1, 1985.) Accession No. 259044.

5. REVIEWED BY:

Brenda Worthy, M.T.
Principal Reviewer
Dynamac Corporation

Signature: Brenda Worthy
Date: 4-8-86

William L. McLellan, Ph.D.
Independent Reviewer
Dynamac Corporation

Signature: William L. McLellan
Date: 4-8-86

6. APPROVED BY:

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

Signature: I Cecil Felkner
Date: 4-7-86

Irving Mauer, Ph.D.
EPA Reviewer

Signature: Irving Mauer
Date: 04-11-86

Jane Harris, Ph.D.
EPA Section Head

Signature: Jane E Harris
Date: 4/15/86

7. CONCLUSIONS:

A. Under the conditions of the assay Dithane M-45 at doses of 500, 2000, and 5000 mg/kg did not induce a mutagenic response in the host-mediated assay in mice. The positive control, dimethylnitrosamine (DMN), however, caused a 16.6-fold increase in mutation frequency, demonstrating that the assay had adequate sensitivity to detect a mutagenic response.

B. The study is acceptable.

Items 8 through 10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):

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

1. Test Material: Dithane M-45 was described as a pale yellow powder, stored at room temperature, with a purity of 88 percent active ingredient. The test material was dissolved in corn oil, the solvent control.

2. Test Animal: Six-week-old male B6C3F1 mice were obtained from Charles River Breeding Laboratories, Inc., Portage, MI.

a. Animal Maintenance: The animals were acclimated to laboratory conditions for 13 days prior to study initiation. They were individually housed in plastic shoe boxes in an environmentally controlled room (temperature, 72-78°F; relative humidity, 18-45%) on a 12-hour light/dark cycle. Food and water were available ad libitum.

b. Group Assignments: Sixty healthy animals were assigned to five treatment groups (20 mice/control, 10/dose group, and 10/positive control) using a random card draw. Animals were identified by toe clip/ear notch and with a unique species letter.

c. Clinical Observation: Animals were observed once on the day of dosing for changes in appearance, behavior, once toxic or pharmacological signs. Body weights were taken prior to dosing.

---

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

3. Test Material Preparation and Administration:

   a. The test material was prepared fresh on the day of dosing on a weight per volume basis to achieve 100 percent of the active ingredient per dose.

   b. The test material at doses of 500, 2000, and 5000 mg/kg and the solvent control were administered via oral gavage. The positive control, 10% dimethylnitrosamine (DMN), was administered by intramuscular (im) injection.

4. Bacterial Inoculation: Two milliliters of a suspension of Salmonella typhimurium strain TA1530, containing 7.7×10^8 cells/mL, was administered to all animals intraperitoneally (ip) following test material administration.

5. Host-Mediated Assay:

   a. Animal Sacrifice: All animals were sacrificed by cervical dislocation. The positive control group was sacrificed 2 hours after bacterial inoculation; the solvent and the three test material groups were sacrificed 4 hours after inoculation.

   b. Bacterial Recovery: Animals were cleansed with ethanol and skinned for laparotomy. One milliliter of saline was injected ip through the abdominal musculature wall; the peritoneal cavity was aseptically opened, and the exudate was withdrawn.

   c. Plating of Bacterial Exudate: The standard pour-plate technique was used. For revertant counts, undiluted peritoneal fluid was added in triplicate to complete top agar, containing histidine and biotin, mixed, and poured over Vogel-Bonner E minimal agar. Similarly, for total cell counts 10-fold dilutions (10^{-1} to 10^{-8}) of peritoneal fluid were prepared in saline, and the three highest dilutions were added to top agar and poured over Tryptone Soy Agar plates in triplicate. All plates were incubated at 37°C. After 48 hours, colonies were counted.

6. Evaluation Criteria: No specific criteria for a positive response were reported.

B. Protocol: See Appendix B.

12. REPORTED RESULTS:

Clinical observations were reported as normal for all animals, and no deaths occurred during the study. Pretreatment body weights were similar for all groups.

No increase in mutation frequency was observed in the assay when mice were given Salmonella ip and dosed orally with 500, 2000, and 5000 mg/kg of the test material when compared to oral dosing with the solvent control. However, the positive control, DMN administered im, caused a 16.6-fold increase in mutation frequency over the solvent control.

Representative results are presented in Table 1.

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

A. The author concluded that "Dithane M-45 did not demonstrate a mutagenic response when tested in the Host Mediated mutation assay using Salmonella typhimurium strain TA1530 as the indicator strain and B6C3F1 mice as the host."

B. A quality assurance statement was signed and dated May 29, 1985.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

A. It is our assessment that the author interpreted the data correctly and Dithane M-45 did not induce a mutagenic response in the host-mediated assay at doses ranging from 500 to 5000 mg/kg. Although the highest dose tested did not elicit a toxic effect in the mice or a cytotoxic response in the S. typhimurium strain TA1530, the dose selected, 5000 mg/kg, in mice was assessed as more than adequate to test for a mutagenic response. In conjunction with this assessment, in an acute oral LD_{50} in mice with Dithane M-45 fungicide (Rohm and Haas report No. 83R-213A), it was reported that the oral LD_{50} was >5000 mg/kg.

The author did not report an evaluation criterion for determining a positive response; however, the positive control caused a 16.6-fold increase in mutation frequency over the solvent control, demonstrating that the test system was capable of detecting a mutagenic response.

Item 15--see footnote 1.

16. CBI APPENDIX:

Appendix A, Materials and Method, CBI pp. 4-8; Appendix B, Protocol, CBI (Appendix I) pp. 4-10.

005418

TABLE 1. Representative Results from the Host-Mediated Assay in Mice with Dithane M-45

Substance	Dose	Average MCFU[a] ($\times 10^{1}$)	Average CFU[b] ($\times 10^{10}$)	MF[c] ($\times 10^{-8}$)	Fold Increase Relative to Solvent Control
Solvent Control Corn oil		10.3	1.90	0.5	—
Positive Control 10% Dimethylnitrosamine	0.1 mL	63.1	0.76	8.3	16.6[d]
Test Material Dithane M-45	5000 mg/kg[e]	10.4	1.8	0.6	1.2

[a] Average mutant colony-forming units (MCFU)—calculated by reviewers.

[b] Average colony-forming units (CFU)—calculated by reviewers.

[c] Mutation Frequency = $\frac{\text{Average MCFU}}{\text{Average CFU}}$

[d] Positive response as reported by the author.

[e] Highest dose tested; lower doses (500 and 2000 mg/kg) were comparable to the solvent control.

APPENDIX A

Materials and Methods

14504 MANCOZEB RIN 2817-93

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005418

EPA: 68-02-4225
DYNAMAC No. 009A-7
April 3, 1986

(10)

DATA EVALUATION RECORD

MANCOZEB

Mutagenicity--CHO/HGPRT Point Mutation Assay

STUDY IDENTIFICATION: Foxall, S., Byers, M. J., and Scribner, H. E. Dithane M-45 CHO/HGPRT gene mutation assay. (Unpublished study No. 84R-207 prepared and submitted by Rohm and Haas Company, Spring House, PA; dated February 11, 1985.) Accession No. 259044.

APPROVED BY:

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

Signature: I. Cecil Felkner

Date: 4-3-86

005418

1. CHEMICAL: Mancozeb; Dithane M-45; coordination product of zinc and manganese ethylene-bis-dithiocarbamates.

2. TEST MATERIAL: Dithane M-45, lot No. 0842, TD 83-224, prepared by Rohm and Haas Co., had a purity of 88% active ingredient (ai).

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

4. STUDY IDENTIFICATION: Foxall, S., Byers, M. J., and Scribner, H. E. Dithane M-45 CHO/HGPRT gene mutation assay. (Unpublished study No. 84R-207 prepared and submitted by Rohm and Haas Company, Spring House, PA; dated February 11, 1985.) Accession No. 259044.

5. REVIEWED BY:

Brenda Worthy, M.T.
Principal Reviewer
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Signature: Brenda Worthy
Date: 4-3-86

Nancy E. McCarroll, B.S.
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Dynamac Corporation

Signature: Nancy E. McCarroll
Date: 4-3-86

6. APPROVED BY:

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

Signature: I. Cecil Felkner
Date: 4-3-86

Irving Mauer, Ph.D.
EPA Reviewer

Signature:
Date: 04-04-86

Jane Harris, Ph.D.
EPA Section Head

Signature:
Date: 04-04-86

7. CONCLUSIONS:

A. Under the conditions of the assay Dithane M-45, at doses from 0.5 to 15 µg/mL without activation or at doses from 0.25 to 45 µg/mL with S9 activation prepared from the livers of either Fischer 344 rats or B6C3F1 mice, did not induce a mutagenic response in the CHO/HGPRT gene mutation assay.

B. The study is acceptable under rat or mouse S9-activated test conditions, but unacceptable under nonactivated conditions because the sensitivity of the assay to detect mutagenic events within the range of test material doses was not demonstrated.

Items 8 through 10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):

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

1) Test Material: Dithane M-45, lot No. 0842, TD 83-224, had a purity of 88% ai; no further description was reported. The test material was dissolved in distilled water, the solvent control, and test concentrations were prepared fresh for each experiment.

2) Cell Line: The Chinese hamster ovary (CHO) cells used in the study were the BH_4 subclone of the $CHO\text{-}K_1$ cell line. Stock cultures were maintained frozen in liquid nitrogen. All cultures, both frozen and growing, were maintained in the absence of antibiotics. Growing cultures were periodically analyzed for mycoplasma contamination, karyotype stability, 6-thioguanine 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% fetal calf serum (heat inactivated).

3) S9 Fraction: The assay was performed with S9 fractions prepared from the livers of both male Fischer 344 rats and B6C3F1 mice induced with Aroclor 1254. The S9 mixes contained per mL: 0.8 mg NADH, 1.2 mg NADP, 1.5 mg G-6-P, 1.05 mg $MgCl_2$, and 1 mg protein/mL of the appropriate S9 fraction.

4) Preliminary Cytotoxicity Study: Cultures, seeded at 5×10^5 cells/plate, were exposed to an unspecified number of test doses for 5 hours with S9 activation or for 18 to 20 hours without activation. These doses spanned a minimum of a 4-log

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

concentration range; parallel cultures were also treated with solvent or positive control chemicals. All cultures were incubated at 37°C, > 90% relative humidity, 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.

5) CHO Mutation Assay: Based on the cytotoxicity data, at least four doses were selected for the CHO assay performed with or without S9 activation. Doses were selected to span a toxicity range of 10 to 90% cell survival. Doses for repeat trials were to be selected on the basis of results from the initial trial.

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

6) 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 MF occurred at one dose level, then the result was to be reproduced in an independent assay.

7) Although the statistical methods of Snee and Irr were cited in the references, the data were not statistically analyzed.

B. Protocol: See Appendix B.

12. REPORTED RESULTS:

A. CHO Mutation Assay--Without S9 Activation:

1) Cytotoxicity Study: Dithane M-45 was assayed at nine doses ranging from 0.05 to 1000 µg/mL without S9 activation. Cytotoxicity results, as assessed by plating efficiency, ranged from 88% survival at 0.1 µg/mL to 19% at 5 µg/mL. No cells survived at doses > 10 µg/mL. Dose levels of 0.05 and 0.5 µg/mL were not scored due to the lack of cytotoxicity.

2) Mutation Assay (#1): Based on the cytotoxicity findings, the test material was assayed with duplicate cultures at 0.5, 2, 3, and 6 µg/mL. The results of duplicate cultures were reported separately and referred to as replica 1 or replica 2. Cell survival at the end of treatment for cultures exposed to 6 µg/mL was 55%; below this level, survival was comparable to or higher than the solvent control.

Although the test material did not induce an increase in mutants/10^6 survivors, the toxicity range specified by the protocol (10 to 90% survival) was not achieved in assay #1; therefore, a repeat assay (#2) was performed.

3) Mutation Assay (#2): At test material doses of 5, 7, and 9 µg/mL, survival ranged from 27% at 5 µg/mL (both replicas) to 45% at 9 µg/mL (average of replicas 1 and 2). Since no colonies were found on the test material or the negative control plates, the assay was again repeated (#3); additionally, the protocol requirements for minimum cell survival were not satisfied by assay #2.

4) Mutation Assay (#3): In assay #3, doses of 4, 5, 6, 10, and 15 µg/mL were tested. Average cell survival for both replicas ranged from 49% (4 µg/mL) to 25% (15 µg/mL). Results from replica 1 indicated that an increase in MF (24.7 mutants/10^6) occurred at 10 µg/mL relative to the concurrent solvent control (2.2 mutants/10^6) but not in comparison to the historical control. However, replica 2 for all test doses and the solvent control was lost due to a combination of technical errors, contamination, and cytotoxicity. Since replica 2 was not available, the assay was again repeated (#4).

5) Mutation Assay (#4): Dithane M-45 was assayed at doses of 8, 10, 12, and 15 µg/mL; these doses resulted in an average cell survival range of 28.5% (8 µg/mL) to 9% (15 µg/mL).

In replicas 1 and 2 the MFs for the solvent control were 1.3 and 6.6 $6TG^r$ mutants/10^6. The MFs for the test material at 8 and 10 µg/mL were comparable to the solvent control for both replicates. In replica 1 the MF at 15 µg/ml was 2.2 mutants/10^6. The result obtained in replica 2 at this dose was considered invalid because survival was <5%. There was an increase in MF at 12 µg/mL in replica 1; however, the response was not considered significant because the increase was not confirmed in replica 2.

The positive control, ethyl methanesulfonate (EMS), induced an average MF of 341 $6TG^r$ mutants/10^6 compared to the average MF ($3.95/10^6$) of the solvent control.

Representative results from the fourth nonactivated assay are presented in Table 1.

B. CHO Mutation Assay--With S9 Activation (Fischer 344 Rat Liver):

1) Cytotoxicity Study: Dithane M-45 was assayed at five doses ranging from 0.1 to 1000 µg/mL in the presence of rat S9 activation. CHO cell survival ranged from 109% at 0.1 µg/mL to 41% at 20 µg/mL. No cells survived at the highest dose tested, 1000 µg/mL.

2) Mutation Assay (#1): Based on the cytotoxicity study, the test material was assayed at doses of 2, 10, 30, 45, and 60 µg/mL. Percent survival at 2 µg/mL was 51% (replica 1) and 33% (replica 2). No cells survived treatment with 60 µg/mL. However, due to extreme toxicity or contamination, the majority of cultures in replica 2 were lost; therefore, the assay was repeated.

3) Mutation Assay (#2): In assay #2, three doses of Dithane M-45 at 0.25, 0.5, and 1.0 µg/mL were tested. Percent survival at all test doses was >60 percent. Although there were no increases in MF induced by the test material in the independent CHO cultures, the positive control, 7-12-dimethylbenzanthracene (DMBA) (7 µg/mL), was nonmutagenic in replica 2. Accordingly, the assay was repeated.

4) Mutation Assay (#3): In assay #3, CHO cells were dosed with 0.5, 1, and 2 µg/mL of the test material. There were no increases in $6TG^r$ MF at any test material dose; however, the MFs for the positive control were low and did not meet assay acceptance criteria. The assay was therefore repeated.

5) Mutation Assay (#4): A fourth rat S9-activated assay was conducted with the test material at doses of 1, 2, 10, 30, and 45 µg/mL. Average relative cell survival ranged from 96% (1 µg/mL) to 53.5% (45 µg/mL).

TABLE 1. Representative Replicate Results from CHO Assay with Dithane M-45 without Activation (Assay #4)

	Dose	Replica	Toxicity after Dosing (% Survival)	Plating Efficiency at Selection (%)	6TG^r Mutant Frequency per 10^6 Survivors
Solvent Control					
Distilled water	—	1	103	78.8	1.3
		2	97	91.3	6.6
Positive Control					
Ethyl methanesulfonate	100 nL/mL	1	62	85.7	301[a]
		2	66	74.2	380[a]
Test Material					
Dithane M-45	12 µg/mL	1	17	90.6	45.2[b]
		2	9	80.9	8.6
	15 µg/mL[c]	1	15	90.1	2.2
		2	3	73.5	0[d]

[a] Significantly positive as assessed by authors.

[b] The increased MF result in replica 1 was not considered significant because it was not confirmed by replica 2.

[c] Highest dose tested; lower doses (from 0.5 to 10 µg/mL) were comparable to the solvent control.

[d] This result was considered invalid because survival was <5%.

In replicas 1 and 2 $6TG^r$ MFs for the solvent control were 5.24 and 6.67/10^6 survivors, respectively. The MFs for the test material at 1, 2, 30, and 45 µg/mL in both replicas were comparable to solvent control values. Although an increase in MF of 33.86 $6TG^r/10^6$ survivors was observed at 10 µg/mL in replica 2; in replica 1, the MF at this dose was 5.78; therefore, the increase was not considered significant because this response was not confirmed. Representative results from the fourth rat S9-activated assay are presented in Table 2.

6) Additional Studies with Various Rat S9 Concentrations: Because there were no mutagenic responses observed with Dithane M-45 in the presence of S9 at 1 mg protein/mL, 1 µg/mL of the test material was assayed using 0.3 and 2 mg protein/mL S9 in duplicate.

Treatment of CHO cells with Dithane M-45 at 1 µg/mL in the presence of S9 at 0.3 mg protein/mL resulted in an MF of 5.35/10^6 survivors (the duplicate was contaminated), and the MF for the solvent control was 1.16/10^6 survivors; however, the positive control was contaminated.

Dithane M-45 at 1 µg/mL in the presence of 2 mg S9 protein/mL resulted in MFs of 10.95 and 7.01 mutants/10^6 survivors; however, the solvent control was contaminated. The MF for the positive control, DMBA, was 252.38 mutants/10^6 survivors. The authors reported, "No difference in mutant frequency was observed at the two different rat S9 concentrations."

C. Mutation Assay--With S9 Activation (B6C3F1 Mice Liver):

1) Cytotoxicity Assay: A cytotoxicity assay, as specified in the protocol, was not performed.

2) Mutation Assay: Dithane M-45 was assayed at 1, 4, 8, 12, and 16 µg/mL with 1 mg S9 protein/mL. The average relative cell survival for the two replicas ranged from 90.5 percent (1 µg/mL) to 5 percent (16 µg/mL).

The MFs of the solvent controls were 4.03 and 4.28 mutants/10^6 survivors in replicas 1 and 2, respectively. Treatment of CHO cells with 1, 4, 8, 12, and 16 µg/mL of the test material induced MFs of 1.06, C. 5.33, 0, and 0/10^6 in replica 1 and 1.12, 3.20, 1.26, 4.16, and 0 mutants/10^6 survivors in replica 2, respectively; the positive control, DMBA at 7 µg/mL, induced MFs of 141.89 and 97.10 mutants/10^6 survivors.

TABLE 2. Representative Replicate Results from CHO Assay with Dithane M-45 and Fischer 344 Rat Liver S9 Activation (Assay #4)

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	Dose	Replica	Toxicity after Dosing (% Survival)	Plating Efficiency at Selection (%)	5TG^r Mutant Frequency per 10^6 Survivors
Solvent Control					
Distilled water	--	1	100	76.4	5.24
		2	58	105.0	6.67
Positive Control					
DMBA[a]	7 µg/mL	1	127	77.9	252.89[b]
		2	125	66.9	218.24[b]
Test Material					
Dithane M 45	10 µg/mL	1	87	86.5	5.78
		2	106	82.7	33.86[c]
	45 µg/mL[d]	1	53	98.1	0
		2	54	65.4	0

[a] DMBA = 7,12-dimethylbenzanthracene.

[b] Significantly positive as assessed by authors.

[c] The increased MF result in replica 2 was not considered significant because it was not confirmed by replica 1.

[d] Highest dose tested; lower doses (from 0.5 to 12 µg/mL) were comparable to the solvent control.

Representative results are presented in Table 3.

3) Additional Studies with Various Mouse S9 Concentrations: Using Dithane M-45 at doses of 4 and 12 µg/mL in the presence of S9 at 0.3 and 2 mg protein/mL, there were no appreciable increases in MFs when compared to the solvent control.

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. The authors concluded that under the conditions of the study, Dithane M-45 did not induce mutations at the HGPRT locus in CHO cells when cultures were tested in the absence of metabolic activation or in the presence of either Fischer 344 rat liver S9 or B6C3F1 mouse liver S9.

B. A quality assurance statement was signed and dated February 11, 1985.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

There were wide variations in cytotoxicity, plating efficiency, and MFs throughout the many repeated studies reported by the authors. We assess, however, that these erratic results were not necessarily indicative of poor laboratory performance, but were probably due to inherent technical problems with the CHO/HGPRT assay. In contrast to mammalian cell assays in suspension (e.g., mouse lymphoma assay), unique problems arise when mutational assays are conducted using monolayer cultures. Several of the major problems outlined below must be considered in order to carefully interpret CHO/HGPRT assay data:

A. Prior to treatment, cells are seeded at a specific density for 24 hours, the assumption being that all cultures grow logarithmically and grow to an equal cell population in all plates by 24 hours. Variability in log-phase growth or cell culture density can, therefore, affect initial and selection cytotoxicity as well as cloning efficiency.

B. Trypsinization of cells after treatment further compromises reproducibility relative to cell survival and cloning efficiency.

C. Reproducibility can also be affected by a low or erratic background MF. In this series of experiments, background MFs ranged from 0 to 21.4 mutants/10^6, which fell within the range considered acceptable by the U.S. Environmental Protection Agency Gene-Tox Program.[2] Therefore, an increase in a test dose

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[2] Hsie, A. W., Casciano, D. A., Couch, D. B., Krahn, D. F., O'Neill, J. P., and Whitfield, B. L. The use of Chinese hamster ovary cells to quantify specific locus mutation and to determine mutagenicity of chemicals, Mutat. Res. 86:193-214, 1981.

TABLE 3. Representative Replicate Results from CHO Assay with Dithane M-45 and B6C3F1 Mice Liver S9 Activation

	Dose	Replica	Toxicity after Dosing (% Survival)	Plating Efficiency at Selection (%)	6TG^r Mutant Frequency per 10^6 Survivors
Solvent Control					
Distilled water	--	1	95	99.3	4.03
		2	105	93.5	4.28
Positive Control					
DMBA[a]	7 µg/mL	1	74	88.8	141.89[b]
		2	90	79.3	97.10[b]
Test Material					
Dithane M-45	16 µg/mL[c]	1	6[a]	81.1	0
		2	4[b]	79.8	0

[a] DMBA = 7,12-dimethylbenzanthracene.

[b] Significantly positive as assessed by authors.

[c] Highest dose tested; lower doses (from 1 to 12 µg/mL) were comparable to the solvent control.

with a low background MF may not be reproduced in a followup study if a higher but acceptable background MF is obtained.

The cumulative effects of the major technical problems outlined above are ultimately reflected in MF variations. For example, if plating efficiency is low and mutant counts are within an expected range, the MF will be high not because an induction in mutation has occurred but because of reduced plating efficiency. Therefore, the number of mutants at a given test dose relative to solvent and historical controls assumes greater importance and is probably the crucial parameter for evaluating results.

In light of the above considerations, it is our assessment that the authors interpreted the data correctly and that Dithane M-45 without activation and with rat or mouse S9 activation did not induce a mutagenic response.

The positive controls, EMS and DMBA, demonstrated the sensitivity of the assay to detect a mutagenic response in the absence or presence of S9 activation. However, the dose of EMS (100 nL/mL) used in all nonactivated studies was well above the highest concentration of the test material; therefore, the ability of the nonactivated test system to detect a mutagenic response in a concentration range comparable to the test material was not shown.

The results reported for Dithane M-45 with various rat S9 concentrations were not considered valid because in one assay the positive control was contaminated and in the other assay the solvent control was contaminated; therefore, the reported results could not be evaluated. However, this deficiency did not invalidate this study.

Item 15--see footnote 1.

16. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 1-12; Appendix B, Protocol, CBI pp. 34-47.

APPENDIX A

Materials and Methods

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EPA: 68-02-4225
DYNAMAC No. 009-A8
April 3, 1986

(11)

DATA EVALUATION RECORD

MANCOZEB

Mutagenicity--*In vitro* Unscheduled DNA Synthesis in Rat Hepatocytes

STUDY IDENTIFICATION: Byers, M. J. and Scribner, H. E. Dithane M-45, in vitro unscheduled DNA synthesis. (Unpublished study No. 84R-280 prepared and submitted by Rohm and Haas Co., Spring House, PA; dated May 29, 1985.) Accession No. 259044.

APPROVED BY:

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

Signature: I Cecil Felkner

Date: 4-3-86

172

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1. CHEMICAL: Mancozeb; Dithane M-45, a coordination product of zinc and manganese ethylene bis-dithiocarbamate.

2. TEST MATERIAL: Dithane M-45, prepared by Rohm and Haas Co. from lot No. 0842, TD No. 83-224, was 88.0 percent active ingredient and described as a yellow powder practically insoluble in water and most organic solvents.

3. STUDY/ACTION TYPE: Mutagenicity--in vitro unscheduled DNA synthesis in rat hepatocytes.

4. STUDY IDENTIFICATION: Byers, M. J. and Scribner, H. E. Dithane M-45, in vitro unscheduled DNA synthesis. (Unpublished study No. 84R-280 prepared and submitted by Rohm and Haas Co., Spring House, PA; dated May 29, 1985.) Accession No. 259044.

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7. CONCLUSIONS:

Unscheduled DNA synthesis (UDS) cannot be adequately evaluated in this study. Dithane M-45 had a presumptive positive response for UDS based on an increase in net nuclear grain counts at 1.0, 2.5, and 5.0 µg/mL; however, the increase was complicated by a rather high cytoplasmic grain count in the solvent control and a decrease in cytoplasmic grain count at the presumptive positive dose levels. The study should be repeated with a different rat hepatocyte preparation.

The study is inconclusive.

Items 8 through 10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):

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

The test compound was uniformly suspended in Williams' medium E without fetal calf serum at 4000 µg/mL, and 15 twofold dilutions were prepared so that when 1 mL of the preparation was added to a culture well containing 3 mL of medium plus fetal calf serum the final concentrations ranged between 0.025 and 1000 µg/mL.

Hepatocytes were isolated from adult male Fischer 344 rats by the method of Williams.[2] Cells (4×10^5) in 4 mL medium were added to 3.5-cm wells of microtiter plates and allowed to attach to plastic coverslips by incubation for 1.5 hours at 37°C. After attachment, medium was removed and the cells were exposed to test compound in 4 mL of medium containing 10 µCi/mL [^{3}H]-thymidine.

The negative controls contained 1 mL of distilled water instead of test compound, and the untreated controls (six wells) received only [^{3}H]-thymidine. The positive control (solvent not stated), 2-acetaminofluorene (2-AAF), was tested at 0.05 and 0.2 µg/mL.

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

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

Following overnight incubation, the cells were washed twice with saline G. Two coverslips from each treatment level were stained with trypan blue for toxicity assessment and four coverslips prepared for radioautography.[3] Slides were stored at -20°C in the dark for 7 days before developing and grain counting.

The assay was considered acceptable if the following criteria were met:

1. The values for the untreated and solvent control were consistent with reported literature values;

2. The positive control values indicated that the hepatocytes were capable of metabolic activation;

3. Slides from at least two treatment concentrations were scorable, and relative survival was greater than 50 percent.

The test material was evaluated positive for UDS if it exhibited a reproducible significant increase in net nuclear grains in the absence of cytotoxicity, as indicated by a decrease in cytoplasmic grains. Two or more consecutive test concentrations must exhibit a significant increase in net nuclear grains. If a positive result occurs at only one concentration, the assay should be repeated with a different hepatocyte preparation at several concentrations around the dose range of the unconfirmed positive to determine if the results were reproducible.

B. Protocol: See Appendix B.

12. REPORTED RESULTS:

Cytotoxicity: When Dithane M-45 was tested at 15 dose levels ranging from 0.025 to 1000 µg/mL, the highest dose that had sufficient cells for scoring was 5.0 µg/mL. Treatment concentrations greater than 10.0 µg/mL resulted in cells being stripped from the coverslips. The report stated that viabilities for the treatment groups 24 hours after initiation of treatment ranged from 88.2 percent for the 10-µg/mL group to 99 percent for the 0.025-µg/mL group. Survival relative to the control group is summarized in Table 1. Survival was notably decreased at 10.0 µg/mL.

UDS: Treatment at levels between 1.0 and 10.0 µg/mL produced an increase in net nuclear grains compared to negative controls. However, there was a decrease in cytoplasmic grain counts and an increase in nuclear grain counts.

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[3] Ibid.

TABLE 1. Summary Data for Rat Hepatocyte UDS Assay with Dithane M-45

Treatment Group (µg/mL)	Relative Survival[a]	Average Net Nuclear Grains	Estimated Grain Count[b]	
			Cytoplasm	Nucleus
Solvent Control	115	-4.8	19.8	14.0
	84.4	-5.4	—	—
Untreated Control	71.1	-4.2	—	—
Dithane M-45				
0.25	66.9	-2.6	16.8	14.3
0.50	75.8	-2.1	18.2	16.2
1.00	48.9	3.2	12.2	15.4
2.50	72.0	5.8	12.8	18.8
5.00	75.6	5.5	14.0	19.5
10.00	38.5	4.2	6.4	10.6
Positive Control (2-AAF)				
0.05	62.7	63.3	—	—
0.20	77.7	65.4	—	—

[a]The fraction of attached cells relative to combined solvent controls 24 hours after treatment.

[b]Estimated visually from Figure 1 of report; numerical data were not provided.

Statistical analysis of the data was presented in a memorandum. The data within a slide were not normally distributed, thus it was necessary to log transform the data for statistical analysis. When data were expressed as log (nuclear count/cytoplasmic count), there was a significant increase in the 1.0-, 2.5-, and 5-µg/mL groups ($p \leq 0.01$) compared to controls by groupwise comparison. There was no significant increase in total cell nuclear grain count when the data were log transformed ($p > 0.25$); however, log average cytoplasmic count was significantly decreased compared to controls ($p < 0.05$) at the 1.00- and 2.50-µg/mL levels, but not at the 5.00-µg/mL level. Data for the 10.00-µg/mL level were not included in the statistical analysis because of toxicity and solubility problems.

It was concluded that increases in the net nuclear grain count observed at dose levels of 1.00, 2.50, 5.00, and 10.00 µg/mL resulted from decreases in the cytoplasmic grain counts that were due to cytotoxicity and not to the induction of DNA repair mechanisms.

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. When freshly isolated hepatocytes from Fischer 344 rats were incubated for 18 hours in the presence of 10 µCi/mL [^{3}H]-thymidine and various concentrations of Dithane M-45 from 0.025 to 1000 µg/mL, excessive toxicity was found at concentrations above 10 µg/mL and 39 percent survival at 5 µg/mL. No increase in nuclear grains was seen at levels of 0.5, 1.0, 2.5, or 5 µg/mL but there was a dose-related decrease in cytoplasmic grains. Subsequent calculation of net nuclear grains per treatment group resulted in a dose-related increase; however, this did not result from genotoxicity but from cytotoxicity. Dithane M-45 did not induce UDS in isolated Fischer rat hepatocytes.

B. A quality assurance statement was signed and dated May 17, 1985.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

It is our assessment that the results of the assay are inconclusive but indicate a presumptive positive response; the assay should be repeated with a different hepatocyte preparation at levels between 1 and 5 µg/mL Dithane M-45.

The report authors indicated a significant ($p \leq 0.05$) increase in net nuclear grain counts at 1.0, 2.5, and 5.0 µg/mL Dithane M-45 but attributed this increase to a dose-related decrease in cytoplasmmic grain counts, which they interpreted to result from cytotoxicity. However, cytoplasmic background counts are in general lower with positive compounds, attributed to active uptake of thymidine into the nuclei of hepatocytes undergoing UDS.

Values for average net nuclear grains for solvent and untreated controls for this study ranged from -4.2 to -4.8. These negative values may be caused by unusually high cytoplasmic grain counts; however, the normal range for values in this testing laboratory was not discussed. Several testing laboratories use a net value of 0 to calculate a mean net nuclear grain count if the cytoplasmic count is greater than the nuclear grain count. Although numerical values for net nuclear grain counts were presented, no data for total nuclear grain counts or cytoplasmic grain count were presented. For this study, the cytoplasmic grain count estimated from Figure 1 of the report was 19.8. The expected value is generally less than 10.[4] When Probst et al. tested UDS with 10 different preparations of rat hepatocytes, in only one preparation was the cytoplasmic mean grain count above 10, and this high value (22) was attributed to insufficient washing of the cells.[5]

Because of these uncertainties, the assay should be repeated with a different preparation of hepatocytes, extra care should be taken in washing the cells, and a solvent other than water should be tested because of the insolubility of the test compound.

Item 15--see footnote 1.

16. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 1-7; Appendix B, Protocol, CBI pp. 17-22.

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[4] Probst, G. S., McMahon, R. E., Hill, L. E., Thompson, C. Z., Epp, J. K., and Neal, S. B. Chemically-induced unscheduled DNA synthesis in primary rat hepatocyte cultures: a comparison with bacterial mutagenicity using 218 compounds. Environ. Mutag. 3(1981): 11-32.

[5] Ibid.

APPENDIX A

Materials and Methods

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Pages 140 through 194 are not included.

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EPA: 68-02-4225
DYNAMAC No. 009-A9
April 18, 1986

(12)

DATA EVALUATION RECORD

MANCOZEB

Mutagenicity--*In Vivo* Cytogenetic Study in Rats

STUDY IDENTIFICATION: Sames, J. L., McLeod, P. L., and Doolittle, D. J. Dithane M-45 *in vivo* cytogenetic study in rats. (Unpublished study No. 84R-246 prepared and submitted by Rohm and Haas, Spring House, PA; dated December 21, 1984.) Accession No. 259044.

APPROVED BY:

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

Signature: _____

Date: _____

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005118

1. CHEMICAL: Mancozeb; Dithane M-45, coordination product of zinc and manganese ethylene bis-dithiocarbamate.

2. TEST MATERIAL: Dithane M-45, prepared by Rohm and Haas Co., TD 83-224, from lot No. 0842, contained 88.0 percent active ingredient.

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

4. STUDY IDENTIFICATION: Sames, J. L., McLeod, P. L., and Doolittle, D. J. Dithane M-45 in vivo cytogenetic study in rats. (Unpublished study No. 84R-246 prepared and submitted by Rohm and Haas, Spring House, PA; dated December 21, 1984.) Accession No. 259044.

5. REVIEWED BY:

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Principal Reviewer
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Date: 4-18-86

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Jane Harris, Ph.D.
EPA Section Head

Signature: Jane E Harris
Date: 4/24/86

7. CONCLUSIONS:

A. Under the conditions of this assay, the acute (one dose) or sub-acute (daily x 5 days) oral exposure of male rats to the maximum tolerated dose of Dithane M-45 (4400 mg/kg) did not cause a significant increase in chromosomal aberrations in bone marrow cells sampled over the entire mitotic cycle.

B. The study is acceptable.

Items 8 through 10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):

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

1. Test Material: Dithane M-45 (ethylene-bis-dithiocarbamate), TD83-224, lot No. 0842, was listed as 88.0 percent pure. The test material was dispersed in corn oil; prepared solutions of the test material were based on the active ingredient content of the test material.

2. Test Animal: Two hundred and forty-eight male Fischer-344 rats, weighing 137 to 158 g, were obtained from Charles River Kingston Breeding Farms, Kingston, NY.

3. Animal Maintenance: Prior to initiation of the study the animals were acclimated to laboratory conditions for 13 days, which included a 6-day quarantine period. Two hundred and forty animals were initially selected and randomly distributed among cages. Throughout the course of the study, animals were housed in an environment controlled for temperature (22.0-25.0°C), relative humidity (43-59 percent), and light (12 hours). With the exception of dosing on day 1, animals were permitted Purina Rodent Lab Chow Checkers ad libitum; water was available ad libitum at all times.

4. Assignment to Groups: One hundred and seventy males, weighing 160.0 to 200.7 g, were selected from the 240 animals. Animals were randomly assigned to treatment groups, housed in groups of two, ear tagged, and identified with a unique number.

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

5. Compound Preparation/Dosing Procedures:

a. Compound Preparation: Based on the active ingredient content of the test material, three concentrations were prepared as corn oil dispersions. The positive control, 1.0 mg/kg triethylenemelamine (TEM), was prepared in distilled water and administered intraperitoneally (ip). Dosing solutions were prepared daily; samples of dosing solutions used on the first and final dosing days were analyzed for test material concentration.

b. Dosing Procedures: The doses of the test material used in this assay (440, 1760, and 4400 mg/kg) were selected based on the highest concentration (5000 and 4400 mg/kg) tested in Fischer-344 rats in an acute oral toxicity study. Toxicity data to support this value were furnished by the sponsor. The appropriate dose levels of the test material, at a dosing volume of 10 mL/kg, were administered orally.

6. Compound Administration:

a. Acute Cytogenetic Study: Thirty animals per group received a single oral administration of the appropriate concentration of the test material or vehicle control. Animals were weighed before dosing and on days 2 and 3; toxic signs were monitored daily. Ten representative members of each group were sacrificed at 6, 24, and 48 hours after compound administration.

The positive control, TEM, was administered as a single dose (1.0 mg/kg, ip) to 10 animals. Animals in this group were sacrificed 18 hours postexposure.

b. Subacute Cytogenetic Study: Ten animals per group were orally administered a single daily dose of the appropriate concentration of the test material or vehicle control for 5 consecutive days. Animals were weighed prior to dosing, observed for toxic effects, and sacrificed 6 hours after the final dose administration.

c. Animal Sacrifice/Bone Marrow Harvest: Colchicine (1 mg/kg, ip) was injected 3 hours prior to the appropriate sacrifice interval; animals were sacrificed by CO_2 asphyxiation. Bone marrow cells were collected from both femurs by aspiration into 0.65 percent KCl. Aspirates were incubated 8-10 minutes at 37°C and centrifuged; the supernatants were discarded. The pellets were fixed three times in methanol:acetic acid (3:1), pipetted onto slides, flame dried, stained, mounted, and coded.

d. Slide Analysis: A maximum of 50 well-defined metaphases per animal were scored for the presence of cytogenetic abnormalities. Chromosomal aberrations were characterized as breaks, gaps, fragments, pulverized cells, translocations, or rearrangements. The number of chromosomes present in each metaphase spread was counted. Gaps were not included in the final analyses.

7. Evaluation Criteria: The data were evaluated for statistical significance ($p < 0.05$) by the Beta Binomial Model[2] and the Fisher Exact Test.

B. Protocol: See Appendix B.

12. REPORTED RESULTS:

A. Acute Cytogenetics Study: Toxic signs observed in the acute cytogenetics study following exposure to 4400 mg/kg Dithane M-45 included lethargy observed on day 1 and lethargy, ataxia, dyspnea, stained muzzle, and piloerection observed on day 2. One animal died immediately prior to the day 2 scheduled sacrifice; bone marrow was harvested. With the exception of one animal exhibiting piloerection, all toxicological signs subsided by day 3. Lethargy was only observed 1 day postexposure to 1760 mg/kg Dithane M-45; one animal with a stained muzzle was reported for days 2 and 3. Two animals in the low-dose group had yellow-stained anogenital areas on day 1 and piloerection was recorded for one animal on day 3.

Slight increases in the number of cells with aberrations were noted at the 4400-mg/kg dose after the 24- and 48-hour harvests; however, these increases were not statistically significant. Since no effect was detected following the acute exposure of male rats to 4400 mg/kg Dithane M-45, slides were not scored for the lower doses. Representative results are shown in Table 1. A reduction in analyzable metaphases was observed in the positive control group; however, the percent aberrant cells scored for this group was significantly higher ($p < 0.05$) than the appropriate control exposure group. Based on the results of a dose response study conducted at a later date with 0.1, 0.5, and 1.0 mg/kg TEM ip, the authors concluded that the reduced recovery of acceptable metaphase spreads was due to the higher than expected sensitivity of Fischer-344 rat bone marrow to TEM. Data were furnished to support this conclusion.

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[2] Williams, D. A., "The analysis of binary responses from toxicological experiments involving reproduction and teratogenicity," Biometrics 31 (1975): 949-954.

TABLE 1. Representative Results of the Acute and Subacute In Vivo Cytogenetic Study in Rats After Dithane M-45 Administration

Substance	Dose	Exposure[a] Time	No. of Male Animals Scored	No. of Metaphases Examined	No. of Cells with Aberrations[b]	Percent Aberrant Cells[c]
Vehicle Control						
Corn oil	10 mL/kg	6 h	10	500	2	0.4
		24 h	10	465	1	0.2
		48 h	10	500	6	1.2
		5 d	10	500	3	0.6
Positive Control						
Triethylenemelamine	1.0 mg/kg	18 h	10	85[d]	54	63.5*
Test Material						
Dithane M-45	4400 mg/kg[e]	6 h	10	477	2	0.4
		24 h	10	477	4	0.8
		48 h	10	500	8	1.6
		5 d	7	350	2	0.6

[a] Time after compound administration.

[b] Gaps not included

[c] Percent aberrant cells = $\frac{\text{No. of cells with aberrations}}{\text{No. of metaphases examined}} \times 100$

[d] Reduced recovery of acceptable metaphase spreads.

[e] Toxic signs observed at all observation intervals; three animals died in the subacute study.

* Significantly different from the 24-hour control value at $p < 0.05$ by Fischer Exact Test and Beta Binomial Model.

Note: Chromosome preparations for the 1760- and 440-mg/kg dosing groups were not scored.

B. Subacute Cytogenetic Study: Five consecutive daily administrations of 4400 mg/kg Dithane M-45 resulted in overt toxicity that intensified as the sequential dosing regime progressed. By day 4, two animals exposed to 4400 mg/kg Dithane M-45 were dead, 90 percent were lethargic, 80 percent exhibited piloerection, and 30 percent were dyspneic. Other toxic signs included stained muzzles, ataxia, diarrhea, and yellow-stained anogenital areas. Toxic signs reported for day 5 occurred at a comparable or in some cases lower frequency than day 4; one animal was found dead. The cumulative toxic effects of the mid dose (1760 mg/kg) were not readily apparent until day 3 (70 percent with piloerection). By day 5, all animals were lethargic, showed piloerection, and 40 percent had stained muzzles. The continuous 5-day exposure to 440 mg/kg Dithane M-45 resulted in animals with lethargy (days 1 and 5) and piloerection (100 percent on days 4 and 5).

No statistically significant increase in chromosomal aberrations resulted from the subacute exposure of the male rats to 4400 mg/kg Dithane M-45. Slides were not scored for the mid and low doses. Representative results are present in Table 1.

C. Dosing Solution Analysis:

Results of analyzing the dosing solution indicate that the dosing solutions prepared on days 1 and 5 were within 1 percent of the expected concentration.

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. The authors concluded, "No adverse effect occurred in bone marrow chromosomes of rats following either an acute or subacute dosing regime of Dithane M-45. Therefore Dithane M-45 does not represent a cytogenetic hazard under the conditions of this test."

B. A quality assurance statement was signed and dated December 30, 1984.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

It is our assessment that this study was well conducted and the authors' interpretation of the data was correct. Dose-related toxicological signs were evident in animals exposed to the three doses of the test material in both the acute and subacute dosing regimes, indicating that the selected dose range was appropriate for this study.

Although a reduced recovery of analyzable metaphase spreads for the positive control group was reported by the authors, the statistically significant increase in chromosomal aberrations in rats treated with

the positive control (TEM, 1.0 mg/kg, ip) adequately demonstrated the sensitivity of the test system to detect clastogenic agents. The authors further demonstrated in a later study that the reduction was related to the sensitivity of Fischer-344 rats to TEM.

Item 15--see footnote 1.

16. CBI APPENDIX:

Appendix A, Materials and Methods, CBI pp. 1-7; Appendix B, Protocol, CBI pp. 15-27.

APPENDIX A

Materials and Methods

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Page_____ is not included in this copy.

Pages 204 through 224 are not included.

The material not included contains the following type of information:

____ Identity of product inert ingredients.

____ Identity of product impurities.

____ Description of the product manufacturing process.

____ Description of quality control procedures.

____ Identity of the source of product ingredients.

____ Sales or other commercial/financial information.

____ A draft product label.

____ The product confidential statement of formula.

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(13)

EPA: 68-02-4225
DYNAMAC No. 009-A18
April 3, 1986

005418

DATA EVALUATION RECORD

MANCOZEB

Mutagenicity--Sister Chromatid Exchange Assay in
Chinese Hamster Ovary (CHO) Cells

STUDY IDENTIFICATION: Ivett, J. L. and Myhr, B. C. Mutagenicity evaluation of Dithane M-45 fungicide lot No. 0842 in an in vitro sister chromatid exchange assay in Chinese hamster ovary (CHO) cells. (Unpublished study No. 84RC-60 prepared by Litton Bionetics, Inc., Kensington, MD, for Rohm and Haas, Spring House. PA; dated March 1985.) Accession No. 259044.

APPROVED BY:

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

Signature: I. Cecil Felkner

Date: 4-3-86

005418

1. CHEMICAL: Mancozeb; Dithane M-45, coordination product of zinc and manganese ethylene bis-dithiocarbamate.

2. TEST MATERIAL: Dithane M-45, a yellow-powdered fungicide prepared by Rohm and Haas Co., was obtained from lot No. 0842 (TD 83-224); its purity was not reported.

3. STUDY/ACTION TYPE: Mutagenicity--sister chromatid exchange assay in Chinese hamster ovary (CHO) cells.

4. STUDY IDENTIFICATION: Ivett, J. L. and Myhr, B. C. Mutagenicity evaluation of Dithane M-45 fungicide lot No. 0842 in an in vitro sister chromatid exchange assay in Chinese hamster ovary (CHO) cells. (Unpublished study No. 84RC-60 prepared by Litton Bionetics, Inc., Kensington, MD, for Rohm and Haas, Spring House, PA; dated March 1985.) Accession No. 259044.

5. REVIEWED BY:

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Date: 84-4-8

Jane Harris, Ph.D.
Acting EPA Section Head

Signature:
Date: 84-4/10

7. CONCLUSIONS:

A. Under the conditions of this assay and in the absence of S9 activation, Dithane M-45 at doses of 7.5, 10.0, 12.5, and 15.0 µg/mL in the initial study and 15.0 and 17.5 µg/mL in a repeat assay induced statistically significant and dose-related increases in the incidence of sister chromatid exchanges (SCEs) per metaphase in Chinese hamster ovary (CHO) cells in vitro. Under conditions using mouse S9 activation, doses ranging from 10 to 17.5 µg/mL induced significant increases, which, however, were not dose related or confirmed in a repeat study. We assess that the test material produced a positive response for induction of SCE in the nonactivated assay and a presumptive, but inconclusive, positive response in the S9-activated system.

B. The study is acceptable.

Items 8 through 10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):

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

1. Test material: Dithane M-45, a fungicide, was obtained from lot No. 0842 (TD 83-224) and was described as a yellow powder with an unspecified purity. At the request of the sponsor, the test material was suspended in serum-free culture medium, the solvent of choice for this study.

2. Cell line: Chinese hamster ovary (CHO) cells, CHO-WB1, were obtained from Dr. S. Wolff, University of California, San Francisco. CHO cells used in this assay were grown for 24 hours in McCoy's supplemented medium prior to use.

3. The S9 fractions used for metabolic activation were prepared from the livers of Fischer 344 rats and B6C3F1 mice induced with Aroclor 1254.

4. Preliminary Cytotoxicity Assays:

a. Relative Growth Assay: Prepared cells (0.3×10^6 cells/25 cm^2 flask) were exposed to 10 doses of the test material for 2 hours in the absence or presence of rat and mouse S9 fractions. Cells were washed, incubated in fresh medium for 24 hours, and counted with a Coulter counter, and percent growth, relative to the solvent and the two lowest doses of the test material, was calculated.

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

b. Relative Cloning Efficiency: Cells were seeded at a density of 200 cells per plate, allowed a 16- to 18-hour attachment period, and exposed to 10 concentrations of the test material for 2 hours in the absence or presence of rat and mouse S9 fractions. Treated cells were washed, incubated in media for 7 days, and counted, and the number of colonies in dosed groups was compared to the control cultures.

5. Sister Chromatid Exchange (SCE) Assay:

a. Exposure: Exponentially growing cells, seeded at 1×10^6 per flask, were exposed in duplicate to three to five doses of the test material solvent or positive controls for 2 hours in the presence or absence of the appropriate S9 fraction. Cells were rinsed, refed media containing 10 µM BrdU, and incubated 26 hours. Colcemid (0.1 µg/mL) was added during the last 2-2.5 hours of incubation. Cytotoxicity was assessed in duplicate flasks for each test dose concurrent with the SCE assay.

b. Preparation of Chromosomes: Cells were collected by mitotic shake-off, treated with hypotonic solution, fixed, dropped onto slides, air dried, stained by a modified fluorescent-plus-Giemsa technique, mounted, and coded.

c. Slide Analysis: A maximum of 50 well-defined metaphases (25 cells/flask) were scored from the four highest doses of test material and the negative control; 25 cells were scored for the positive control.

6. Evaluation Criteria: The assay was considered positive if a) an approximate doubling in SCE frequency over the negative control was observed at one or more doses or b) in the absence of a doubling, if a statistically significant increase occurred at a minimum of three doses.

7. A t-test was used to statistically analyze the data.

B. Protocol: See Appendix A.

12. REPORTED RESULTS:

A. Preliminary Cytotoxicity Assays:

1. Relative Growth Assay: Doses of the test material ranging from 1 to 100 µg/mL were tested with or without rat and mouse S9 activation. Under all conditions of activation or nonactivation, relative survival increased as the test material concentration was decreased. The test material was

more cytotoxic at higher concentrations (≤10 percent survival at 20–100 µg/mL) without S9 activation. Slightly higher survival (8 to 12%) at comparable doses was recorded in the presence of mouse S9; however, at 10 µg/mL, 39 percent of the cells survived compared to 65 percent in the nonactivated assay. Cytotoxicity was markedly diminished in the presence of rat liver S9; survival ranged from 20 percent at 100 µg/mL to 71 percent at 20 µg/mL.

2. Relative Cloning Efficiency: Similar concentrations (1–100 µg/mL) were assayed with or without mouse and rat S9 to determine relative clonal survival. Cytotoxicity in suspension cultures as assessed by survival was more severe than in plated cells. No colonies were recovered from cells treated with doses up to and including 6.0 µg/mL under activated or nonactivated conditions. Percent survival at 4.0 µg/mL was 8.3 percent (-S9), 0 percent (+ mouse S9), and 7.9 percent (+ rat S9). At the two remaining doses (1 and 2 µg/mL) and in agreement with the relative growth assay findings, cytotoxicity was most pronounced without S9 activation and least apparent in the presence of rat-induced S9 fraction. Representative results from the two cytotoxicity assays are presented in Table 1.

B. SCE Assay: Based on the combined findings of the cytotoxicity assays, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, and 20 µg/mL were investigated in the nonactivated and rat or mouse S9-activated SCE assay. A suspension cytotoxicity assay with the above concentrations and under the same conditions was conducted in parallel with the SCE assay; cells were visually examined for mitotic delay.

1. Nonactivation Assay: Following exposure to the selected test doses (2.5 to 20 µg/mL), relative growth ranged from 4 to 24 percent at the five highest doses (20–10 µg/mL). At doses below 10 µg/mL, >50 percent of the cells were viable. Metaphases harvested from CHO cells treated with 5, 7.5, 10, 12.5, and 15.0 µg/mL were examined for mitotic delay and frequency of SCEs. At the three highest test material doses, the average percentage of first division metaphases (92 percent at 15.0 µg/mL, 75 percent at 12.5 µg/mL and 82 percent at 10.0 µg/mL) indicated that progression through the mitotic cell cycle was delayed. Cell cycle kinetics were not severely affected by 7.5 and 5 µg/mL. Due to extreme cytotoxicity at 15.0 µg/mL, only 38 metaphases were scored; however, a statistically significant increase in SCEs/cell (1.9 times higher than control) was reported. Significant and dose-related increases were also observed at doses of 12.5, 10.0, and 7.5 µg/mL. An elevated but not significant increase was reported at the lowest dose, 5.0 µg/mL. Results from analyzed nonactivated doses are shown in Table 2.

TABLE 1. Representative Results of the Relative Growth (Suspension) and Relative Clonal Survival Cytotoxicity Assays with Dithane M-45 (Chinese Hamster Ovary Cells)

Substance	Dose Unit/mL)	Activation Condition: -S9 % RS[a]	-S9 % CS[b]	+ Mouse S9 % RS[a]	+ Mouse S9 % CS[b]	+ Rat S9 % RS[a]	+ Rat S9 % CS[b]
Negative Control							
Culture Media		100	100	100	100	100	100
Test Material							
Dithane M-45	1	100	76.0	100	111.1	100	100.4
	2	100	43.7	100	72.2	100	82.2
	4	104	8.3	-[c]	0	92	7.9
	6	94	0	74	0	85	0
	8	84	0	78	0	90	0
	10	65	0	39	0	67	0
	20	10	0	12	0	71	0
	40	4	0	11	0	77	0
	60	2	0	9	0	40	0
	100	4	0	8	0	20	0

[a]% RS--Relative survival derived from mean value of negative control and 1 and 2 µg/mL of the test material.

[b]% CS--Clonal survival; % CS relative to negative control.

[c]No value, culture contaminated.

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TABLE 2. Representative Results from the Nonactivated Sister Chromatid Exchange Assay in Chinese Hamster Ovary Cells Treated with Dithane M-45

Substance	Dose (unit mL)	% Relative Survival	No. Metaphases Scored	Average % M_1 Cells[a]	Average % M_2 Cells[a]	No. SCEs/cell ±SD	Fold Increase in in SCEs[b]
Negative Control							
Culture Media	—	100	50	10	90	8.7±0.4	—
	—[c]	ND[d]	50	14	86	7.5±0.4	—
Positive Control							
Mitomycin C	10 ng	ND	25	8	92	13.1±0.9*	1.5
	10 ng[c]	ND	25	17	83	10.0±0.6*	1.3
Test Material							
Dithane M-45	5.0 µg	108	50	28.3	71.7	10.5±0.5	1.2
	7.5	58	50	55.0	45.0	13.0±0.7*	1.5
	10.0	24	50	82.0[e]	18.0	14.2±0.8*	1.6
	12.5	16	50	75.0[e]	25.0	14.3±1.0*	1.6
	15.0	11	38	92.0[e]	8.0	16.5±1.0*	1.9
	10.0[c]	ND	50	42.0	58.0	8.4±0.4	1.0
	12.5	ND	50	41.5	58.5	8.7±0.5	1.2
	15.0	ND	50	35.0	65.0	10.0±0.6*	1.3
	17.5	ND	50	43.5	56.5	10.0±0.6*	1.3

[a]Averaged by our reviewers; M_1=first division metaphases and M_2=second division metaphases.

[b] Fold Increase in SCEs = $\frac{\text{No. of SCEs/cell (treatment group)}}{\text{No. of SCEs/cell (negative control)}}$; calculated by our reviewers.

[c]Repeat study.

[d]ND—Not determined.

[e]Marked increase in M_1 cells indicative of mitotic delay (cytotoxicity).

*Significant increase ($p < 0.05$) by t-test.

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Based upon the evidence of a significant and dose-related positive effect and at the request of the sponsor, the non-activated SCE assay was repeated with 5, 10, 12.5, 15, and 17.5 µg/mL. A relative growth assay was not performed; the integrity of the monolayers and the degree of cell replication were determined qualitatively. Although there was no reduction in monolayer confluency, cell cycle delay was observed at test material doses of 15 and 17.5 µg/mL. Accordingly, cells treated with 12.5, 15, and 17.5 µg/mL were permitted a longer expression in the presence of BrdU. Following the additional incubation, metaphases were scored for all doses except the low dose (5 µg/mL). Slight mitotic delay was still apparent at all doses. Significant increases in the frequency of SCEs were reported for the 15.0 and 17.5 µg/mL dose levels. From the initial findings and the confirming results of the second nonactivated SCE assay, the authors concluded that Dithane M-45 was positive in the nonactivated assay. Representative results from all analyzed doses in the repeat study are presented in Table 2.

2. Activated Assays:

   a. Mouse S9 Activation: In the presence of mouse liver microsomes, relative survival ranged from 4 to 92 percent over concentrations spanning 20.0 to 2.5 µg/mL. Due to the extreme cytotoxicity at 20.0 µg/mL, no metaphases were obtained. The four intermediate doses that were scored (10.0, 12.5, 15.0, and 17.5 µg/mL) showed no definitive evidence of cell cycle delay. At these levels, statistically significant increases in SCEs per cell were observed. However, they were not dose related. The assay was repeated with 12.5, 15, 17.5, and 20.0 µg/mL. Confluency of the monolayers and cell replication were determined qualitatively. At 15 and 20 µg/mL, monolayer confluency was reduced and mitotic delay was observed. Expression time for these cultures in the presence of BrdU was extended and metaphases were scored at 12.5 through 20.0 µg/mL. The significant, but not dose-related, increases in SCEs per cell reported in the initial mouse S9-activated test were not reproduced. The authors concluded that the test material under mouse S9-activated conditions was not genotoxic. However, the comparative response of CHO cells to the activated positive control, cyclophosphamide (1.5 µg/mL), indicated that in the repeat study the number of SCEs induced by cyclophosphamide was approximately 50 percent lower than in the initial trial. Representative results from all analyzed doses in the initial and repeat studies are shown in Table 3.

TABLE 3. Representative Results from the Mouse S9-activated Sister Chromatid Exchange Assay in Chinese Hamster Ovary Cells Treated with Dithane M-45

Substance	Dose (µg/mL)	% Relative Survival	No. Metaphases Scored	Average % M_1 Cells[a]	Average % M_2 Cells[a]	No. SCEs/cell ±SD	Fold Increase in SCEs[b]
Negative Control							
Culture Media	—	100	50	3.0	97.0	9.0±0.5	—
	—[c]	ND[d]	50	16.5	83.5	8.8±0.4	—
Positive Control							
Cyclophosphamide	1.5	ND	25	ND	ND	60.2±2.0*	6.7
	1.5[c]	ND	25	14.0	86.0	25.2±1.0*	2.9
Test Material							
Dithane M-45	10.0	32	50	27.5	72.5	12.8±0.6*	1.4
	12.5	22	50	40.5	59.5	14.5±0.8*	1.6
	15.0	18	50	32.0	68.0	11.0±0.5*	1.2
	17.5	16	50	36.0	64.0	13.6±0.6*	1.5
	12.5[c]	ND	50	73.5	26.5	9.2±0.4	1.0
	15.0	ND	50	44.5	50.5	9.6±0.5	1.1
	17.5	ND	50	43.5	56.5	8.3±0.4	<1.0
	20.0	ND	50	43.5	56.5	9.3±0.5	1.0

[a]Averaged by our reviewers.

[b] Fold Increase in SCEs = $\frac{\text{No. of SCEs/cell (treatment group)}}{\text{No. of SCEs/cell (negative control)}}$; calculated by our reviewers.

[c]Repeat study.

[d]ND - Not determined.

*Significant increase ($p < 0.05$) by t-test.

b. Rat S9 Activation: Results from the rat liver S9-activated assay with eight doses (2.5-20 µg/mL) indicated that Dithane M-45 was less toxic in the presence of rat S9. At 20.0 µg/mL, 36 percent of the CHO cells survived; percent survival for the remaining doses ranged from 34 percent at 17.5 µg/mL to 91 percent at 2.5 µg/mL.

Metaphases from cells exposed to 12.5, 15, 17.5, and 20 µg/mL were analyzed for SCE frequencies. An evaluation of cell cycle kinetics showed that a slight depression of second division metaphase cells occurred at all dose levels; the reduction was not sufficient to extend the expression period. A statistically significant but less than doubling of the SCE frequency was noted at 17.5 µg/mL. Although the remaining doses had elevated counts, no significant increases were observed. Representative results are presented in Table 4.

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. The authors concluded, "Given the repeatable positive response under -S9 conditions, the test article is considered positive for inducing sister chromatid exchange under the conditions of this assay."

B. A quality assurance statement was signed and dated September 4, 1984.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

It is our assessment that the study was conducted properly and that the authors' interpretation of the data was correct. Statistically significant, dose-related, and reproducible increases in SCE frequency were demonstrated under nonactivated conditions. The lack of reproducible, significant increases in the mouse S9-activated assays may have been related to reduced sensitivity of the CHO cells in the repeat test. As shown in Table 3, the increase in SCEs from the cyclophosphamide treatment in the repeat assay (2.9 times) was approximately half of the response reported in the initial assay (6.7 times). Similarly, the percent second division metaphase cells for the negative control in the second assay was lower (83.5%) than in the first assay (97.0%). These two factors may have contributed to reduced cell sensitivity and, therefore, could have obliterated subtle effects induced by mouse-activated Dithane M-45. However, had the authors been able to reproduce the significant increases observed in the first trial, it is doubtful that the effect could be considered a definitive positive response. The increases for both the initial and repeat

TABLE 4. Representative Results from the Rat S9-activated Sister Chromatid Exchange Assay in Chinese Hamster Ovary Cells Treated with Dithane M-45

Substance	Dose (µg/mL)	% Relative Survival	No. Metaphases Scored	Average % M_1 Cells[a]	Average % M_2 Cells[a]	No. SCEs/cell ±SD	Fold Increase in SCEs[b]
Negative Control							
Culture Media	—	100	50	1	99	9.6±0.5	—
Positive Control							
Cyclophosphamide	1.5	ND[c]	25	3	97	29.9±1.4*	3.1
Test Material							
Dithane M-45	12.5	51	50	50.0[d]	51.0[d]	10.2±0.5	1.1
	15.0	42	50	33.5	66.5	10.8±0.5	1.1
	17.5	34	50	45.0	55.0	11.6±0.4*	1.2
	20.0	36	50	42.0	58.0	10.3±0.5	1.1

[a]Averaged by our reviewers.

[b] Fold Increase in SCEs = $\frac{\text{No. of SCEs/cell (treatment group)}}{\text{No. of SCEs/cell (negative control)}}$; calculated by our reviewers.

[c]ND—Not determined.

[d]Reported values $M_1 + M_2 > 100\%$.

*Significant increase ($p < 0.05$) by t-test.

mouse-activated assays satisfied neither the reporting laboratory's nor the USEPA Gene-Tox Program's[2] criteria for a positive response in this assay. Criteria established by the USEPA Gene-Tox Program for evaluating a positive effect require:

A. The ability to induce at least a twofold increase over baseline SCE frequencies or

B. The demonstration of a three-point, dose-response curve showing a progressive increase over baseline SCE frequencies with at least one SCE value at $p < 0.001$ level.

It is our assessment, therefore, that the test material is genotoxic in the absence of S9 activation; tne inability to reproduce significant responses induced by mouse S9 activation did not alter the overall conclusions presented by the authors.

The significant increases elicited by the positive controls (Mitomycin C, 10 ng/mL -S9; cyclophosphamide, 1.5 µg/mL + mouse or rat S9) adequately demonstrated the sensitivity of the te' system to detect genotoxic activity. Although a reduction in assay sensitivity was observed in the repeat mouse S9-activated test, the effect induced by cyclophosphamide was significant.

Item 15--see footnote 1.

16. CBI APPENDIX: Appendix A, Materials and Methods (Protocol), CBI pp. 26-32.

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[2] Latt, S. A., Allen, J., Bloom, S. E., Carrano, A., Falke, E., Kram, D., Schneider, E., Schreck, R., Tice, R., Whitfield, B., and Wolff, S. Sister-Chromatid Exchanges: A Report of the Gene-Tox Program, Mutation Research 87(1981): 17-62.

APPENDIX A

Materials and Methods (Protocol)

14504 MANCOZEB RIN 2617-93

Page_____ is not included in this copy.

Pages 238 through 244 are not included.

The material not included contains the following type of information:

____ Identity of product inert ingredients.

____ Identity of product impurities.

____ Description of the product manufacturing process.

____ Description of quality control procedures.

____ Identity of the source of product ingredients.

____ Sales or other commercial/financial information.

____ A draft product label.

____ The product confidential statement of formula.

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(14)

EPA: 68-02-4225
DYNAMAC No. 009-[illegible]
April 3, 1986

005418

DATA EVALUATION RECORD

MANCOZEB

Mutagenicity--In vitro Transformation Assay
in C3H/10T 1/2 Mouse Fibroblasts

STUDY IDENTIFICATION: McGlynn-Kreft, A. M., and McCarthy, K. L. Dithane M-45 mammalian cell transformation test. (Unpublished study No. 84R-055 prepared and submitted by Rohm and Haas Co., Spring House, PA; dated November 19, 1984.) Accession No. 259044.

APPROVED BY:

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

Signature: I. Cecil Felkner

Date: 4-3-86

1. CHEMICAL: Mancozeb; Dithane M-45, coordination product of zinc and manganese ethylene bis-dithiocarbamate.

2. TEST MATERIAL: Dithane M-45, sample TD 83-224, lot No. 0842, prepared by Rohm and Haas Co. had a purity of 88 percent; its physical description was not reported.

3. STUDY/ACTION TYPE: Mutagenicity--In vitro transformation assay in C3H/10T 1/2 mouse fibroblasts.

4. STUDY IDENTIFICATION: McGlynn-Kreft, A. M., and McCarthy, K. L. Dithane M-45 mammalian cell transformation test. (Unpublished study No. 84R-055 prepared and submitted by Rohm and Haas Co., Spring House, PA; dated November 19, 1984.) Accession No. 259044.

5. REVIEWED BY:

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

Signature: Nancy E. McCarroll
Date: 4-3-86

Brenda Worthy, M.T.
Independent Reviewer
Dynamac Corporation

Signature: Brenda Worthy
Date: 4-3-86

6. APPROVED BY:

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

Signature: I. Cecil Felkner
Date: 4-3-86

Irving Mauer, Ph.D.
EPA Reviewer

Signature: Irving Mauer
Date: 4-4-86

Jane Harris, Ph.D.
EPA Section Head

Signature: Jane Harris
Date: 04-04-86

7. CONCLUSIONS:

A. Under the conditions of this assay and in the absence of S9 activation, Dithane M-45 at doses of 0.05, 0.15, 0.25, 0.4, and 0.5 µg/mL did not cause an increase in the number of transformed foci in C3H/10T 1/2 cells. The performance of the assay without an exogenous metabolic activation system is an acceptable practice because this cell line can metabolize certain chemicals to active carcinogens; the presence of this metabolic system was adequately demonstrated by the positive response with the known procarcinogen/promutagen, 7,12-dimethylbenzanthracene (DMBA).

B. The study is acceptable.

Items 8 through 10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):

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

1. Test Material: Dithane M-45, sample TD83-224, lot No. 0842, was described as a fungicide with a purity of 88 percent; it was suspended in water. The stock solution and required dilutions were prepared on the day of treatment.

2. Cell Line: Clone 8 of C3H/10T 1/2 mouse embryo fibroblast cells were obtained from Dr. Charles Heidelberger, University of Southern California, Los Angeles. Logarithmic phase cultures, grown from frozen stock cultures, were used as the target cells.

3. Cytotoxicity Assay: Prepared cells, seeded at a density of 200 cells per plate, were exposed in triplicate to an unspecified number of test material concentrations spanning at least a 4-log dose range. After a 24-hour exposure to the test material, the medium was removed and cells were incubated with growth medium for 9-10 days. Surviving colonies were fixed, stained, counted, and compared to the number of colonies in the solvent control.

4. Cell Transformation Assay: Based on the results of the cytotoxicity assay, five doses of test material estimated to yield growth in a survival range of > 90 percent to < 50 percent were selected for the cell transformation assay.

a. Exposure: Prepared cultures, seeded with 200 or 2,000 cells/plate, were treated with the five selected doses of

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

the test material, solvent, or positive control. After a 24-hour exposure, the medium was removed and cultures were refed with fresh growth medium. Cells seeded at the lower density were used to determine the plating efficiency. Surviving colonies in these plates were fixed, stained, and counted after 9-10 days of incubation. The remaining cultures, seeded at 2,000 cells/plate, were periodically refed with growth medium throughout the 6-week incubation period. At the conclusion of incubation, cells in the transformation plates were fixed, stained, and scored for transformed foci.

b. Scoring Transformed Foci: Foci were scored by the criteria of Reznikoff et al.[2] as follows:

Type I--Densely stained areas, composed of tightly packed cells.

Type II--More densely stained areas than Type I, with piling up of cells and overlapping nuclei.

Type III--Highly polar, piling up of cells, composed of areas exhibiting crisscrossing at the interface of the focus and monolayer.

5. Evaluation Criteria: The assay was considered positive if a dose-response relationship was apparent or the incidence of plates with Type III foci at one dose level was significantly higher than the historical untreated and solvent controls.

6. The incidence of plates with Type III foci was statistically compared to the historical untreated and solvent controls by the Fisher Exact test.

7. Evaluation Criteria for Postive Control: Results for the positive control were not analyzed by statistics; the protocol stated that the positive control group must yield an incidence of at least 15 percent of the plates with Type III foci for the positive control to be considered acceptable.

B. Protocol: See Appendix B.

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[2] C. A. Reznikoff, D. W. Brankow, and C. Heidelberger. Establishment and characterization of a cloned cell line C3H mouse embryo cells sensitive to postconfluence inhibition of division. Cancer Res. 33(1973): 3231-38.

12. REPORTED RESULTS:

Cytotoxicity Assay: The preliminary cytotoxicity assay was conducted with 14 concentrations of the test material ranging from 0.1 to 1000 µg/mL, two doses of the positive control, DMBA, and the solvent control. Precipitation of Dithane M-45 occurred at all doses above 5 µg/mL, and no cells survived doses ranging from 2.5 to 1,000 µg/mL. Increasing survival (3-81 percent) was observed in a descending dose-related manner for the remaining test concentrations (1.0-0.1 µg/mL). Percent survival for 1.0 and 2.5 µg/mL DMBA, the positive control, was 59 and 56 percent, respectively.

Transformation Assay: Based on the preliminary cytotoxicity findings, doses selected for the transformation assay were 0.05, 0.15, 0.25, 0.4, and 0.5 µg/mL. The concentration of DMBA used was 0.5 µg/mL. Twenty replicates were plated for the five selected doses of the test material and untreated and positive controls; 30 replicates were used for the solvent control. Plating efficiency was determined from the counts of triplicate plates for all test doses and controls. Following exposure, survival ranged from 14 to 96 percent for doses spanning a 0.5 to 0.05 µg/mL concentration range. No Type III foci were found following exposure of the cells to five doses of the test material; statistics were, therefore, not performed. Representative data from this assay are presented in Table 1.

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. The authors concluded, "This study demonstrates that Dithane M-45 produces no adverse effects in the Mammalian Cell Transformation Test under the conditions specified."

B. A quality assurance statement was signed and dated November 14, 1984.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

It is our assessment that the study was properly conducted and the authors' interpretation of the data was correct. There was a dose-related increase in cytotoxicity that accompanied exposure to increased doses of the test material. Since no validated exogenous metabolic activation system currently exists for this test, using the C3H/10T 1/2 transformation assay in the absence of S9 activation is acceptable.[3] The sensitivity of the test system to detect the

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[3] C. Heidelberger, A. E. Freeman, R. J. Pienta, A. Sivak, J. S. Bertram, B. C. Casto, V. C. Dunkel, M. W. Francis, T. Kakunaga, J. B. Little, and L. Schechtman. Cell transformation by chemical agents--a review and analysis of the literature. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutat. Res. 114(1983): 283-385.

TABLE 1. Representative Results of the C3H/10T 1/2 Transformation Assay with Dithane M-45

Substance	Dose (µg/mL)	% Survival[a]	No. Plates w/Type II Foci/ Total No. Replicates	% Replicates w/Type II Foci	No. Plates w/Type III Foci/ Total No. Replicates	% Replicat w/Type I Foci
Negative Control						
Culture Media	--	99	0/20	0	0/20	0
Solvent Control						
Water	--	100	0/30	0	0/30	0
Positive Control						
7,12-dimethyl-benzanthracene	0.5	78	5/20	25	9/20	45[c]
Test Material						
Dithane M-45	0.5[b]	14	1/20	5	0/20	0

[a] $\frac{\text{No. of colonies with test dose}}{\text{No. of colonies with solvent control}} \times 100$

[b] Highest dose tested; values for transformation assay at lower doses (0.4, 0.25, 0.1 and 0.05 µg/mL) were comparable to the solvent control. Percent survival at the doses ranged from 26 percent at 0.4 µg/mL to 96 percent at 0.05 µg/mL. These da were, therefore, not selected as representative.

[c] Positive by the authors' criterion; ≥ 15% increase in plates with Type III foci.

induction of transformants was adequately demonstrated by the positive control, 0.5 µg/mL DMBA, although this dose was equal to the highest dose of Dithane M-45 assayed. Hence, the assay system appeared to have the appropriate enzymes to metabolize DMBA to a form that is active for inducing cell transformation.

Item 15--see footnote 1.

16. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 1-7; Appendix B, Protocol, CBI pp. 15-22.

APPENDIX A
Materials and Methods

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Pages 253 through 269 are not included.

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____ Identity of product impurities.

____ Description of the product manufacturing process.

____ Description of quality control procedures.

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005418

(15)

EPA: 68-02-4225
DYNAMAC No. 009-A3
April 3, 1986

DATA EVALUATION RECORD

MANCOZEB

Mutagenicity--*In vitro* Transformation Assay for Promotion in C3H/10T 1/2 Mouse Fibroblasts

STUDY IDENTIFICATION: McLeod, P. L. and Doolittle, D. J. Dithane M-45 mammalian cell transformation test for promotion. (Unpublished study No. 84R-297 prepared and submitted by Rohm and Haas Co., Spring House, PA; dated May 29, 1985.) Accession No. 259044.

APPROVED BY:

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

Signature: I. Cecil Felkner

Date: 4-3-86

270

1. CHEMICAL: Mancozeb; Dithane M-45; coordination product of zinc and manganese ethylene bis-dithiocarbamate.

2. TEST MATERIAL: Dithane M-45 prepared by Rohm and Haas Co. was from lot No. 0842, sample No. TD83-224; its purity was 88 percent and its physical description was not reported.

3. STUDY/ACTION TYPE: Mutagenicity--in vitro transformation assay for promotion in C3H/10T 1/2 mouse fibroblasts.

4. STUDY IDENTIFICATION: McLeod, P. L. and Doolittle, D. J. Dithane M-45 mammalian cell transformation test for promotion. (Unpublished study No. 84R-297 prepared and submitted by Rohm and Haas Co., Spring House, PA; dated May 29, 1985.) Accession No. 259044.

5. REVIEWED BY:

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

Signature: Nancy E. McCarroll
Date: 4-3-86

Brenda Worthy, M.T.
Independent Reviewer
Dynamac Corporation

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6. APPROVED BY:

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

Signature: I. Cecil Felkner
Date: 4-3-86

Irving Mauer, Ph.D.
EPA Reviewer

Signature:
Date: 04-24-86

Jane Harris, Ph.D.
EPA Acting Section Head

Signature:
Date: 04-24-86

7. CONCLUSIONS:

A. Under the conditions of the assay, Dithane M-45 at 0.1 µg/mL did not cause an increase in the number of transformed foci either in initiated or uninitiated C3H/10T 1/2 cells at a presumed maximum tolerated noncytotoxic dose. However, the assay was conducted with only one test dose, which may not be sufficient to conclude that the test material is not an in vitro tumor promoter.

B. The study is unacceptable because only a single dose was used; a single dose is insufficient to show that the test material did not have promoter activity.

8. RECOMMENDATIONS:

Dr. Craig J. Boreiko,[1] a noted expert on initiation/promotion assays, recommends the use of more than one dose level because promoters frequently induce erratic and nondose-related effects. In lieu of established guidelines for initiation/promotion assays, we feel his suggestions should be considered. We, therefore, recommend that the test material should be tested at more than one dose or the authors should justify the use of a single treatment level.

Items 9 and 10--see footnote 2.

11. MATERIALS AND METHODS (PROTOCOLS):

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

1. Test Material: Dithane M-45 (ethylene-bis-dithiocarbamate), lot No. 0842, sample No. TD83-224, had a purity of 88.0 percent and was dissolved in culture medium (Basal Medium Eagles).

2. Cell System: Clone 8 of C3H/10T 1/2 mouse embryo fibroblast cells were obtained from Dr. Charles Heidelberger, University of Southern California, Los Angeles. Logarithmic phase cultures, grown from frozen stock cultures, were used as the target cells.

3. Cytotoxicity Assays:

a. Range-Finding Cytotoxicity Test: Prepared cells, seeded at a density of 200 cells per plate, were exposed in triplicate to six concentrations of the test material.

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[1] Boreiko, C. J., Chemical Institute of Toxicology, Research Triangle Park, NC, personal communications.

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

Cells were exposed either for 24 hours or continuously throughout a 9-day incubation period. After the 24-hour exposure, the medium was removed and cells were incubated with growth medium for 8 days. Surviving colonies from both exposures were fixed, stained, and counted.

b. Cytotoxicity Assay with the Initiating Agent: Cultures, seeded at a density of 2000 cells per plate, were exposed to the initiating agent, 0.5 µg/mL N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), for 4 hours. Five days after initiation, cell viability was determined from three control cultures. The remaining cultures were continuously refed with media containing the six selected test doses or the solvent control; viability was monitored daily for 4 days and on alternate days thereafter until termination of the cytotoxicity test, day 11 postinitial compound treatment. A growth curve was plotted (total number of cells vs. days posttreatment) to determine growth inhibition.

4. Cell Transformation Assay for Promotion: Based on the combined results of the cytotoxicity assays, a single test dose was selected for the cell transformation test with promotion.

a. Exposure: The appropriate number of prepared cultures, seeded with either 200 or 2000 cells/plate, was treated with the following agents: 0.5 µg/mL MNNG, 0.5 percent acetone (MNNG solvent), 0.5 µg/mL 7,12-dimethylbenzanthracene (DMBA), or 1.0 µg/mL 3-methylcholanthrene (MCA). Exposure to the initiating agents, MNNG and acetone, was terminated at 4 hours; the exposure period for DMBA and MCA was 24 hours. Five days after initiation, the cultures were refed with media containing the selected dose of the test material, acetone, or the known promoting agent, 0.25 µg/mL 12-o-tetradecanoylphorbol-13-acetate (TPA). This exposure was continued throughout the 6-week promotion phase. The single test material dose was exposed to 20 replicates of untreated cells, 20 replicates of cells preinitiated with MNNG, or 20 replicates of cells pretreated with acetone. The remaining control or TPA-treated groups were similarly added to 20 replicate untreated or preinitiated cultures. Cultures treated with DMBA or MCA were not exposed to promoting agents because the compounds are complete carcinogens. Plating efficiency was not determined for uninitiated or initiated cultures exposed to the test material but was determined for select controls. Surviving colonies on these plates were fixed, stained, and counted after 9-10 days of incubation. Throughout the approximately 6-week promotion phase, cells were continuously refed with media containing the test dose, solvent, or control promoters. At the conclusion of incubation, cells in the transformation plates were fixed, stained, and scored for transformed foci.

b. Scoring Transformed Foci: Foci were scored by the criteria of Reznikoff et al.[3] as follows:

Type I-- Densely stained areas, composed of tightly packed cells.
Type II-- More densely stained areas than Type I, composed of piling up of cells and overlapping nuclei.
Type III-- Highly polar, piling up of cells, composed of areas exhibiting crisscrossing at the interface of the focus and monolayer.

5. Evaluation Criteria: The assay was considered positive if an increase in Type III foci was observed. If an appreciable increase in Type II foci occurred, the test would be repeated with more replicates and/or more or different test concentrations.

6. Evaluation Criteria for Positive Controls: The positive control, DMBA, must yield an incidence of at least 15 percent of the plates with Type III foci to be considered valid evidence of test system sensitivity. Similarly, the promoter control, TPA, must increase the incidence of plates with Type III foci compared to that of the initiating agent alone to be considered acceptable.

7. The data were not statistically analyzed.

8. Protocol: See Appendix B.

12. REPORTED RESULTS:

Cytotoxicity Assays:

a. Range-Finding Cytotoxicity Test: The preliminary cytotoxicity assays were conducted with 0.01, 0.033, 0.066, 0.1, 0.25, and 0.5 µg/mL of the test material. Following the 24-hour exposure, cell survival at 0.5 and 0.25 µg/mL was 67 and 83 percent, respectively; below these doses, survival in test groups was comparable to the untreated control. The continuous 9-day exposure resulted in 42 and 76 percent survival at 5 and 0.25 µg/mL, respectively; the remaining doses were not cytotoxic.

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[3] Reznikoff, C. A., Brankow, D. W., and Heidelberger, C. Establishment and characterization of a cloned cell line C3H mouse embryo cells sensitive to postconfluence inhibition of division. Cancer Res. 33(1973): 3231-38.

b. Cytotoxicity Assay with Initiating Agent: Cultures preinitiated with 0.5 µg/mL MNNG were continuously exposed to 0.01, 0.033, 0.066, 0.1, 0.25, and 0.5 µg/mL of the test material. Based on the reported growth curve results, an approximately 30 percent inhibition of cells was plotted at day 4 for the highest dose. By day 7, growth at this level was equivalent to the untreated control. At all other concentrations, with the exception of 0.066 µg/mL, cell growth was comparable to the untreated control. The authors did not consider the growth inhibition plotted for 0.066 µg/mL (50 percent at day 4, 30 percent at day 7) treatment related.

Since the cytotoxicity assay with initiated cells showed no definitive cytotoxic effect, the results of the range-finding cytotoxicity tests were used to determine the appropriate dose for the transformation assay.

c. Transformation Assay with Promotion: The selected dose, 0.1 µg/mL, was continuously applied to untreated and acetone- or MNNG-pretreated cells. No foci were observed on untreated or acetone-initiated test plates. Plates of MNNG-initiated, test material-promoted cells contained 10.5 percent Type II foci and 5.3 percent Type III foci. However, the percent Type III foci on these plates was equivalent to the MNNG-initiated, untreated control plates. Representative results are shown in Table 1.

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. The authors concluded, "This study demonstrates that Dithane M-45 does not promote morphological transformation in the Mammalian Cell Transformation Test for Promotion under the conditions specified."

B. A quality assurance statement was signed and dated May 7, 1985.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

It is our assessment that the study was properly conducted and the authors' interpretation of the data was correct. No *in vitro* assays to detect tumor promoters have been validated and probably must await the clearer understanding of tumor promotion mechanisms before their use in screening programs can be fully realized.

The authors stated, in accordance with Frazelle et al.,[4] "a nontoxic concentration of test compound is the maximum tolerated dose for assessing promoting activity in this assay."

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[4] Frazelle, J. H., Abernethy, D. J., and Boreiko, C. J. Determination of cell culture conditions optimal for the study of initiation and promotion in C3H 10T 1/2 cells. Environmental Mutagenesis 4(1982): 331-332.

TABLE 1. Representative Results of the C3H/10T 1/2 Transformation Assay for Promotion with Dithane M-45

Substance[a]		No. Plates w/ Type II Foci/ Total No. Replicates	% Replicates with Type II Foci	No. Plates w/ Type III Foci/ Total No. Replicates	% Replicates with Type III Foci
Initiator	Promoter				
Negative Control					
Media	--	0/20	0	0/20	0
Solvent Control					
Acetone (0.5%)	--	0/20	0	0/20	0
MNNG (0.5 µg/mL)	--	0/20	0	1/20	5
Positive Control					
DMBA (0.5 µg/mL)	--	8/20	40	14/20	70[b]
MCA (1.0 µg/mL)	--	4/20	20	8/20	40[b]
MNNG (0.5 µg/mL)	TPA (0.25 µg/mL)	8/20	40	10/20	50[c]
Test Substance					
Media	Dithane M-45 (0.1 µg/mL)	0/20	0	0/20	0
Acetone (0.5%)	Dithane M-45 (0.1 µg/mL)	0/20	0	0/20	0
MNNG (0.5 µg/mL)	Dithane M-45 (0.1 µg/mL)	2/19[b]	10.5	1/19[d]	5.3

[a]MNNG = N-methyl-N'-nitro-N-nitrosoguanidine
TPA = 12-o-Tetradecanoyl-phorbol-13-acetate
DMBA = 7,12-Dimethylbenzanthracene
MCA = 3-Methylcholanthrene

[b]Positive by the authors' criterion (≥ 15% increase in plates with Type III foci).

[c]Positive by the authors' criterion (increased incidence of plates with Type III foci as compared to initiating agents alone).

[d]One plate not scored; monolayer was not intact.

We confirmed this statement with Frazelle and Boreiko[5] who indicated that the majority of promoters are noncytotoxic. Boreiko[6] recommended, however, that more than a single dose should be assayed (five doses of an unknown agent are routinely evaluated in his laboratory) since promoters frequently induce erratic and nondose-related effects. Although Dithane M-45 was negative at the selected concentration, it is possible that 0.1 µg/mL was not the effective level and tumor promotion could have been detected if more doses were evaluated.

No established guidelines exist for initiation and promotion assays. Since Dr. Boreiko is a recognized expert in this area, his recommendation should be considered in lieu of published guidelines. It is our assessment, therefore, that the results reported by the authors are insufficient to support the conclusion that Dithane M-45 does not promote neoplastic transformation in this assay.

The ability of the known tumor promoter, TPA (0.25 µg/mL), to induce neoplastic transformation in initiated cells was demonstrated. Similarly, the direct induction of transformed clones by DMBA (0.5 µg/mL) and MCA (1.0 µg/mL) was adequately shown. Although no criteria were presented to evaluate a positive effect with MCA, we assumed that the criterion reported for DMBA (≥15% increase in plates with Type III foci) applied to both polycyclic aromatic hydrocarbons.

Item 15—see footnote 2.

16. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 1-7; Appendix B, Protocol, CBI pp. 16-28.

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[5] Sanchez, J. H. (nee Frazelle) and Boreiko, C. J., Chemical Institute of Toxicology, Research Triangle Park, NC, personal communications.

[6] Ibid.

APPENDIX A

Materials and Methods

14504 MANCOZEB RIN 2017-93

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Pages 279 through 299 are not included.

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____ Identity of product inert ingredients.

____ Identity of product impurities.

____ Description of the product manufacturing process.

____ Description of quality control procedures.

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