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

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~~APR - 1 1986~~    ~~MAR 21 1986~~

OFFICE OF  
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Mutagenicity Studies with Zineb

TO: J. Dizikes  
Special Review Branch (TS-767C)

FROM: Byron T. Backus  
Toxicologist  
Toxicology Branch

THROUGH: Clint Skinner, Ph.D. *[Signature]*  
Head, Section III *3/24/86*  
and  
Theodore Farber, Ph.D. *[Signature]*  
Chief, Toxicology Branch  
Hazard Evaluation Division (TS-769) *[Signature]*

Chemical no. 930

Project No. 614

Action Requested:

The Special Review Branch has requested a review of these five mutagenicity studies in order to determine whether they are the ones requested by EPA, and (presumably) whether they are acceptable.

Comments and Conclusions:

1. The following studies have been classified as acceptable:

- i) Edgar, D. H., Ransome, S. J., Banks, S. J., and Bosworth, H. J. An assessment of the mutagenic potential of Zineb using an in vitro mammalian cell test system. (Unpublished study No. FMT6/85539 prepared by Huntingdon Research Centre, Ltd., Huntingdon, England; dated July 23, 1985. Acc. no. 259476).
- ii) Allen, J. A., Proudlock, R. J., and Pugh, L. C. Micronucleus test on Zineb. (Unpublished study No. FMI 7/85518 prepared by Huntingdon Research Centre, Huntingdon, England; dated July 29, 1985. Acc. no. 259479).

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- iii) Richold, M., Jones, E., and Fenner, L. Ames metabolic activation test to assess the potential mutagenic effect of Zineb. (Unpublished report No. FMT4/85394 prepared by Huntingdon Research Centre, Cambridgeshire, England; dated August 23, 1985. Acc. no. 259477).

It should be noted that although this particular study was acceptable, it was only marginally so. The incidence of revertant colonies in all tester strains treated with the solvent control, despite being within published ranges, appeared low for a 72-hour incubation. The lower than expected background counts may have resulted from using a bactericidal agent, sodium lauryl sulfate, as the test material solvent. Since spontaneous reversion frequencies for untreated cultures were not reported, we were unable to assess if the test strains were slightly inhibited by sodium lauryl sulfate.

Additionally, the reason for using 10% S9 in the S9 mix was not given. The use of 10% S9 mix as the primary screening concentration may be required for some tests; however, the standard S9 mix usually contains 1% S9. The lower than expected revertant counts induced by the S9-activated positive control (2-AA, 2 µg/plate) might have been due to the high S9 content within the mix.

2. The following studies have been classified as unacceptable. A summary of the reasons for this classification follow after each citation:

- i) Allen, J. A. and Proudlock, R. J. Assessment of unscheduled DNA repair synthesis in mammalian cells after exposure to Zineb. (Unpublished study No. FMT 2/85519 prepared by Huntingdon Research Centre, Ltd., Huntingdon, Cambridgeshire, England; dated July 19, 1985. Acc. no. 259480).
- a) The test material precipitated at all doses; therefore, a valid interpretation of the data was not possible.
- b) A cell line (HeLa S3) lacking an appropriate level of sensitivity (the following reference is given: Mitchell, A. D., Casciano, D. A., Meltz, M. L., Robinson, D. E., San, R. H. C., William, G. M., and Von Halle, E. S. Unscheduled DNA Synthesis tests, a report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutat. Res. 123(1983):363-410) was used for the assays; therefore, the response cannot be considered a

reliable measure of genotoxic activity.

- c) Cytoplasmic background counts were not performed; hence, the net increase in nuclear grains cannot be assessed.
- ii) Allen, J. A., Brooker, P. C., and Howell, A. Analysis of metaphase chromosomes obtained from CHO cells cultured in vitro and treated with Zineb. (Unpublished study No. FMT5/85621 prepared by Huntingdon Research Centre, Ltd., Huntingdon, Cambridgeshire, England; dated July 18, 1985. Acc. no. 259478).
- a) Under the conditions of this in vitro Chinese Hamster ovary (CHO) cell cytogenetics study, the clastogenic potential of Zineb cannot be assessed because the majority of the cells analyzed for chromosomal aberrations were probably from second division metaphases (cell harvest was at 20 hours postexposure).
3. On page 5 of the data call-in notice of 17 January 1983 there is a listing of required mutagenicity study testing for Zineb. The following gives this listing, along with comments after each of the specified studies as to whether or not that particular data requirement was met:
- a. Assays for gene mutation in Salmonella typhimurium (TA strains) or Escherichia coli WP2, performed with and without the use of mammalian metabolic activation (S9) from induced (Aroclor 1254) liver microsomes from both mouse (B6C3F1) and rat (Fisher 344) strains.
- Comment:
- Although the study titled "Ames metabolic activation test to assess the potential mutagenic effect of Zineb" (Acc. 259477) has been classified as acceptable it involved S9 activation only from the rat (although the data call-in notice specified the Fisher 344 strain and the strain used was CD this is not considered a major deviation) and not from the mouse. The submitted study then only partially satisfies the data requirement specified in the data call-in notice; what is still required is a study with activation utilizing mouse S9.
- b. Host-Mediated Assays in the same strain of mice or rats as specified in (a.) above, by the oral route of administration, with appropriate microbial or mammalian indicator cells.

Comment:

None of the five studies in this group is of this type.

- c. In vitro Mammalian Cell gene mutation assays, using the mouse lymphoma cell line L5178Y (for TK), or Chinese Hamster Ovary cells (CHO, for HGPRT), performed with and without the use of mammalian metabolic activation systems (S-9) derived from Aroclor 1254 induced liver microsomes from the same strains of mice and rats specified in (a.) above.

Comment:

This requirement was satisfied by the study titled "An assessment of the mutagenic potential of Zineb using an in vitro mammalian cell test system" in Acc. no. 259476.

- d. In vitro or in vivo Cytogenetic Assays, for both gross chromosome aberrations (clastogenesis) and for sister-chromatid exchanges (SCE's); in vitro assays may be performed with any recognized, established mammalian cell line or primary cell line, but must be performed with and without the use of metabolic activation (S-9) from induced strains of mice and rats, as specified in (a.) above.

Comment:

The study titled: "Analysis of metaphase chromosomes obtained from CHO cells cultured in vitro and treated with Zineb" in acc. 259478 was classified as not acceptable. However, the study titled "Micronucleus test on Zineb" in acc. 259479 was classified as acceptable. Although the data call-in notice specified a gross chromosome aberration study, the micronucleus test is acceptable in lieu of such a study. However, no sister-chromatid exchange study was present in this group of five reports, and so this part of the data requirement remains unsatisfied.

- e. Primary Hepatocyte Repair Test for unscheduled DNA synthesis (UDS), performed with the same strains of rats or mice as specified in (a.) above, employing in vivo or in vitro exposure to test compounds.

Comment:

The study titled: "Assessment of unscheduled DNA repair synthesis in mammalian cells after exposure to Zineb" in Acc. 259480 was classified as not acceptable. One of the reasons given for this classification is that the cell line used (HeLa S3) lacks an appropriate

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level of sensitivity. Note that the Agency specified a primary hepatocyte repair test in the data call-in notice of 17 January 1983.

4. Copies of the individual DER's are attached.

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DOES NOT CONTAIN  
NATIONAL SECURITY INFORMATION (EO 12065)

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EPA: 60-02-4225  
DYNAMAC No. 1-20A2  
March 12, 1986

DATA EVALUATION RECORD

ZINEB

Mutagenicity--Micronucleus Test in Mice

STUDY IDENTIFICATION: Allen, J. A., Proudlock, R. J., and Pugh, L. C.  
Micronucleus test on Zineb. (Unpublished study No. FMT 7/85518 prepared  
by Huntingdon Research Center, Huntingdon, England, for Farmoplant, Milan,  
Italy; dated July 29, 1985.) Accession No. 259479.

APPROVED BY:

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

Signature:

*I. Cecil Felkner*

Date:

3-12-86

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- 1. CHEMICAL: Zineb; zinc ethylenebisdithiocarbamate.
- 2. TEST MATERIAL: Zineb, technical grade, was described as a creamy white powder with a reported purity of 91.5%.
- 3. STUDY/ACTION TYPE: Mutagenicity--micronucleus test in mice.
- 4. STUDY IDENTIFICATION: Allen, J. A., Proudlock, R. J., and Pugh, L. C. Micronucleus test on Zineb. (Unpublished study No. FMT 7/85518 prepared by Huntingdon Research Center, Huntingdon, England, for Farmoplant, Milan, Italy; dated July 29, 1985.) Accession No. 259479.

5. REVIEWED BY:  
 Nancy E. McCarroll, B.S.  
 Principal Reviewer  
 Dynamac Corporation

Signature: Nancy E. McCarroll  
 Date: March 11, 1986

Brenda Worthy, M.T.  
 Independent Reviewer  
 Dynamac Corporation

Signature: Brenda Worthy  
 Date: 3-11-86

6. APPROVED BY:  
 I. Cecil Felkner, Ph.D.  
 Genetic Toxicology  
 Technical Quality Control  
 Dynamac Corporation

Signature: Ira Cecil Felkner  
 Date: 3-12-86

Byron T. Backus, M.S.  
 EPA Reviewer

Signature: Byron T. Backus  
 Date: 3-17-86

Clint Skinner, Ph.D.  
 EPA Section Head

Signature: Clint Skinner  
 Date: 3/25/86



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7. CONCLUSIONS:

- A. Under the conditions of this study, the acute oral exposure of male and female mice to 10,000 mg/kg Zineb did not significantly increase micronuclei in the polychromatic erythrocytes (PCE) of bone marrow cells sampled over the entire hematopoietic cycle. The positive control, 4 mg/kg mitomycin C dosed orally, clearly demonstrated the sensitivity of the assay to detect a genotoxic effect.
- 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. Zineb, technical grade, was described as a creamy white powder with a purity of 91.5%, its chemical structure was provided. The authors stated that the test material was insoluble in water and that suspensions of Zineb were prepared in aqueous 1% methylcellulose (MC) using a Silverson high-speed mixer.
2. Test Materials: Male and female specific pathogen-free CD-1 outbred mice of Swiss origin were obtained from Charles River U.K., Ltd., Kent, England. The weight range for all animals upon receipt was 18-21 g.

Animal Maintenance: Animals were weighed upon arrival, randomly assigned to groups, ear marked, gang caged (2-5/cage), and acclimated to laboratory conditions for 10 days. Throughout the acclimation and study periods, animals were maintained in an environment controlled for air (30 changes/hour), temperature (22°C), and light (12 hours); Labure LAD rodent diet and tapwater were provided ad libitum. Animals were observed daily for toxic signs and weighed prior to dosing.

3. Compound Administration: Suspensions of the test material and negative (1% MC) and positive controls (mitomycin C, 4.0 mg/kg in 0.9% saline) were administered by intragastric gavage at a dosing volume of 20 mL/kg.
4. Preliminary Toxicity Test: Four animals (2 males and 2 females) were orally administered single test doses of 500, 1,000, 2,000, 4,000, and 8,000 mg/kg. A second toxicity test was performed with 8,000 and 10,000 mg/kg administered to five males and five females per group. Toxic effects were observed periodically over a 72-hour period.

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Nondose-related and slight toxic effects (pilo-erection and hunched posture) were reported for all dose groups. With the exception of the 10,000-mg/kg animals, toxic reactions subsided approximately 4 hours after treatment; 6.5 hours following administration of the highest test dose, no toxic signs were reported.

5. Micronucleus Test:

a. Test Animals and Compound Administration: Based on the lack of a definitive toxic effect in the preliminary studies, 10,000 mg/kg (maximum dosable amount) was selected as the test dose for the micronucleus assay. Thirty mice (15 males and 15 females) were administered 10,000 mg/kg of the test material or vehicle; mitomycin C was administered to five males and five females. Animals were periodically observed for mortalities or clinical signs of reaction over the 72-hour exposure period.

b. Animals Sacrifice/Bone Marrow Harvest: Five males and five females from the test dose or vehicle control group were sacrificed by cervical dislocation 24, 48, and 72 hours after dosing. The positive control group was sacrificed 24 hours postexposure. Bone marrow smears were made directly from both femurs; slides were air dried, fixed in methanol, stained using Giemsa technique, mounted, and coded.

c. Slide Analysis: One thousand PCE per animal were scored for the incidence of micronucleated cells; the ratio of PCE to normochromatic erythrocyte (NCE) per animal was determined from the examination of at least 1,000 erythrocytes.

6. Evaluation Criteria: An assay was considered positive if the test material caused a statistically significant increase ( $p < 0.05$ ) in the number of micronucleated polychromatic erythrocytes (MPE) at any of the three sacrifice times.

7. The data from each sacrifice interval were analyzed statistically by the Wilcoxon's sum of ranks test.

B. Protocol: A protocol was not provided.

12. REPORTED RESULTS:

At the selected dose (10,000 mg/kg) no deaths or definitive evidence of a toxic effect were observed. No significant increase in the incidence of MPE or significant shift in the PCE:NCE ratio was reported following exposure of male and female mice to 10,000 mg/kg Zineb over a 72-hour exposure period. Representative data are presented in Table 1.

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TABLE I. Representative Results of the Micronucleus Assay in Mice with Zineb

Substance	Dose (mg/kg)	Exposure Time	Sex	No. of Animals Analyzed	No. of PCE <sup>a</sup> Analyzed Per Group	Total No. of MPE <sup>b</sup>	Percent MPE Per Group <sup>b</sup>	Average PCE:NCE <sup>b</sup>
<u>Vehicle Control</u> 1% Methylcellulose	-	24 h	M	5	5,000	6	0.12	0.8:1
			F	5	5,000	4	0.80	0.7:1
	48 h	M	5	5,000	2	0.04	0.7:1	
		F	5	5,000	3	0.06	0.7:1	
	72 h	M	5	5,000	3	0.06	0.8:1	
		F	5	5,000	1	0.02	0.8:1	
<u>Positive Control</u> Mitomycin C	4	24 h	M	5	5,000	233*	4.7	0.5:1
			F	5	5,000	197*	3.9	0.6:1
<u>Test Material</u> Zineb	10,000	24 h	M	5	5,000	2	0.04	0.9:1
			F	5	5,000	2	0.04	0.6:1
	48 h	M	5	5,000	2	0.04	0.8:1	
		F	5	5,000	5	0.10	0.7:1	
	72 h	M	5	5,000	2	0.04	0.9:1	
		F	5	5,000	4	0.08	0.9:1	

<sup>a</sup>PCE: Polychromatic erythrocytes  
<sup>b</sup>MPE: Micronucleated polychromatic erythrocytes  
NCE: Normochromatic erythrocytes.

<sup>b</sup>Calculated by our reviewers.

\*Significant at  $p < 0.001$  by Wilcoxon's sum of ranks test; results from both sexes combined for statistical analysis.

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13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

The authors concluded, "It is concluded from the results obtained that Zineb shows no evidence of mutagenic potential or bone marrow cell toxicity when administered orally in this in vivo test procedure."

A quality assurance statement was signed and dated July 26, 1985.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

It is our assessment that the study was well conducted and the authors' interpretation of their data was correct. Although the authors were unable to show definitive toxicity within 72 hours and a 7-day mortality/toxicity study should have been performed,<sup>2</sup> the dose selected, 10,000 mg/kg, was considered sufficient for the evaluation of a nontoxic chemical in this test system.

The significant increase in micronuclei induction elicited by 4 mg/kg mitomycin C dosed by the same route as the test material ( $p < 0.001$ ) adequately demonstrated the sensitivity of the assay to detect a genotoxic effect.

Item 15--see footnote 1.

16. CBI APPENDIX:

Appendix A, Materials and Methods, CBI pp. 2-5.

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<sup>2</sup> Heddle, J. A., Hite, M., Kirkhart, B., Mavournin, K., MacGregor, J. T., Newell, G. W., and Salamone, M. F. The Induction of Micronuclei as a Measure of Genotoxicity. A Report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutat. Res. 123(1983): 61-118.

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**Appendix A**  
**Materials and Methods**

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Pages 13 through 16 are not included.

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  - Identity of product impurities.
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  - Description of quality control procedures.
  - Identity of the source of product ingredients.
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  - A draft product label.
  - The product confidential statement of formula.
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  - FIFRA registration data.
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EPA: 68-02-4225  
DYNAMAC No. 1-020-A4  
March 14, 1986

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DATA EVALUATION RECORD

ZINEB

Mutagenicity--Reverse Mutation in Salmonella

STUDY IDENTIFICATION: Richold, M., Jones, E., and Fenner, L. Ames metabolic activation test to assess the potential mutagenic effect of Zineb. (Unpublished report No. FMT4/85394 prepared by Huntingdon Research Centre, Cambridgeshire, England; dated August 23, 1985.) Accession No. 259477.

APPROVED BY:

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

Signature: I. Cecil Felkner

Date: 3-14-86

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1. CHEMICAL: Zineb; Zinc ethylenebisdithiocarbamate.
2. TEST MATERIAL: Zineb is an off-white powder with a purity of 91.5%.
3. STUDY/ACTION TYPE: Mutagenicity--reverse mutation in Salmonella typhimurium.
4. STUDY IDENTIFICATION: Richold, M., Jones, E., and Fenner, L. Ames metabolic activation test to assess the potential mutagenic effect of Zineb. (Unpublished report No. FMT4/85394 prepared by Huntingdon Research Centre, Cambridgeshire, England; dated August 23, 1985.) Accession No. 259477.

5. REVIEWED BY:

Christine Sexsmith, B.S.  
Principal Reviewer  
Dynamac Corporation

Signature: Ira Cecil Felkner for

Date: 3-14-86

Nancy McCarroll, B.S.  
Independent Reviewer  
Dynamac Corporation

Signature: Nancy L. McCarroll

Date: 3-14-86

6. APPROVED BY:

I. Cecil Felkner, Ph.D.  
Genetic Toxicology  
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EPA Reviewer

Signature: Byron T. Backus

Date: 3-17-86

Clint Skinner, Ph.D.  
EPA Section Head

Signature: \_\_\_\_\_

Date: \_\_\_\_\_



7. CONCLUSIONS:

- A. Under the conditions of two independent Salmonella typhimurium reverse mutation assays, 15 to 5000 µg/plate Zineb did not induce a mutagenic response in S. typhimurium strains TA1535, TA1537, TA1538, TA98, or TA100 with or without S9 activation. It should be noted however that the S9-activated test was conducted with a high level (10%) rather than the recommended 4% level of S9 in the S9 mix. Additionally, the background counts for all strains were relatively low but within acceptable ranges<sup>1</sup> for a 72-hour incubation. The reduced number of spontaneous revertants may have been related to the solvent, sodium lauryl sulfate, a known bactericidal agent.
- B. The study is acceptable.

8. RECOMMENDATIONS: Future studies should be conducted with the recommended screening concentration of S9 in the S9 mix (4%)<sup>2</sup> or the authors should justify the use of 10% S9.

Items 9 and 10--see footnote 3.

11. MATERIALS AND METHODS (PROTOCOLS):

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

1. Test material: Zineb was described as an off-white powder with a purity of 91.5%. The test material was dissolved in water containing 1 mg/mL sodium lauryl sulfate.
2. S. typhimurium strains TA1535, TA1537, TA1538, TA98, and TA100 were used for the assay. No source or storage conditions were given. Prior to use, subcultures from an unspecified source were grown in nutrient broth at 37°C for 18 hours.

<sup>1</sup> de Serres, F. J. and Shelby, M. D. Recommendations on data production and analysis using Salmonella/microsome mutagenicity assay, Mutat. Res. 64(1979): 159-165.

<sup>2</sup> Maron, M. and Ames, B. Revised methods for the Salmonella mutagenicity test, Mutat. Res. 113(1983): 173-215.

<sup>3</sup> Only items appropriate to this DER have been included.

3. S9 Fraction: The S9 fraction was made from the livers of male Sprague-Dawley rats (180-200 g) induced by a single ip injection of 500 mg/kg Aroclor 1254. The S9 mix was prepared according to the method of Ames et al.;<sup>4</sup> however, the S9 mix contained 10% S9 fraction.

4. Positive Controls: The following controls were used:

2-Aminoanthracene (2-AA)--2 µg/plate  
2-Nitrofluorene (2-NF)--10 µg/plate  
9-Aminoacridine (9-AA)--20 µg/plate  
Sodium azide (SA)--5 µg/plate.

The solvent(s) used to dissolve the positive controls was (were) not reported.

5. Cytotoxicity Assessment: Four concentrations (5, 50, 500, and 5000 µg/plate) of the test material and the solvent control were tested with the five bacterial strains to assess cytotoxicity during a preliminary study. To glass bijou bottles containing 0.1 mL of an overnight broth culture of each bacterial strain, 0.5 mL of 0.1 M sodium phosphate buffer, pH 7.4, or 0.5 mL of the S9 mix and 0.1 mL of the appropriate test material concentration or the solvent were added. The reaction components were mixed with 2 mL of histidine-deficient agar, poured over the surface of single minimal agar plates, and incubated at 37°C for 72 hours. Revertant colonies were counted, and the appearance of the background lawn growth was assessed. Cytotoxic effects were indicated by a substantial reduction in revertant colony counts or by the absence of a complete background bacterial lawn. Media components of the histidine-deficient and minimal agar were not specified.

6. Mutagenicity Assay: Two independent mutagenicity assays were conducted with each of the selected test doses as described for the cytotoxicity assay. Positive controls were included and triplicate plates were prepared for each test dose and solvent or positive controls. Additionally, sterility checks of the highest test dose and the S9 mix were performed.

7. Evaluation Criteria: The compound was considered to be potentially mutagenic if a statistically significant (unspecified method of analysis) dose-related increase in the number of revertant colonies was observed in two separate experiments and if the increase in the number of revertant colonies was at least twice the concurrent solvent control value.

<sup>4</sup> Ames, B. N., McCann, J., and Yamasaki, E. Methods for detecting carcinogens and mutagens with *Salmonella*/mammalian-microsome mutagenicity test. Mutat. Res. 31(1975): 347-364.

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8. Means and standard deviations of the counts of triplicate plates for each test dose, solvent, and positive controls were presented for each independent study. The methods used to assess the significance of a dose-related effect were not described.

B. Protocol: See Appendix B.

12. REPORTED RESULTS:

Cytotoxicity Assay: In the nonactivated assay, Zineb was cytotoxic to all tester strains at the highest dose level (5000 µg/plate); in the presence of 10% S9 there was no clear cytotoxic effect. When S9 activated, 5000 µg/plate was slightly cytotoxic for strains TA1537, TA1538, and TA100. The highest dose selected for the first mutation assay was 5000 µg/plate.

Mutagenicity Assay: In the first mutagenicity test, doses of 5000 and 1500 µg/plate of nonactivated test material were cytotoxic to all five tester strains. A slight reduction in the number of revertants of strains TA1535, TA1537, TA1538, and TA100 was noted at 5000 µg/plate with 10% S9 activation; at 1500 µg/plate with S9 activation no appreciable cytotoxic effect was seen; however, a slight reduction in revertant colonies of strain TA100 was recorded. Decreased revertant counts were also reported for all strains at 500 µg/plate non-activated; below this concentration little or no evidence of a cytotoxic effect was noted.

No appreciable increase in revertant colonies was observed in S. typhimurium strains TA1535, TA1537, TA1538, TA98, or TA100 when treated with Zineb at doses of 15 to 5000 µg/plate with or without S9 activation. Representative data for the first assay are shown in Table 1.

In the second assay, the highest dose was 1500 µg/plate. Cytotoxicity was noted at this level in strains TA1535, TA1537, TA98, and TA100 without activation; reduced revertant counts were reported for all strains at 500 µg/plate without activation. The reduced count for TA1538 was questionable since the high dose was not cytotoxic for this strain. In the presence of 10% S9, a 30% or greater reduction in His<sup>+</sup> colonies was reported for all strains except TA1538 at 1500 µg/plate.

The response elicited by the promutagen (2-AA) in both assays was lower than expected.<sup>5</sup> Representative data from the second assay are presented in Table 2.

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<sup>5</sup> McCann, J., Choi, E., Yamasaki, E., and Ames, B. N. Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72(1975): 5135-5139.

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005016TABLE 1. Representative Results of the First Salmonella typhimurium Mutagenicity Assay with Zineb

Substance	Dose ( $\mu\text{g}/\text{plate}$ )	S9 Activa- tion	Bacterial Tester Strains <sup>a</sup>				
			TA98	TA100	TA1538	TA1537	TA1538
<u>Solvent Control</u>							
Sodium lauryl sulfate, 1 mg/mL	--	-	25 $\pm$ 3.6	91 $\pm$ 16.7	12 $\pm$ 3.0	15 $\pm$ 1.5	11 $\pm$ 0.6
		+	20 $\pm$ 0.6	111 $\pm$ 5.7	11 $\pm$ 1.0	12 $\pm$ 3.2	13 $\pm$ 1.2
<u>Positive Controls</u>							
Sodium Azide	5	-	--	456 $\pm$ 37.1	572 $\pm$ 100.3	--	--
9-Aminoacridine	20	-	--	--	--	70 $\pm$ 8.7	--
2-Nitrofluorene	10	-	236 $\pm$ 21.5	--	--	--	180 $\pm$ 8.7
2-Aminoanthracene	2	+	265 $\pm$ 9.5	263 $\pm$ 52.2	85 $\pm$ 9.3	56 $\pm$ 12.0	211 $\pm$ 35.6
<u>Test Material</u>							
Zineb	500 <sup>b</sup>	-	20 $\pm$ 4.0	69 $\pm$ 18.6	9 $\pm$ 2.5	10 $\pm$ 2.0	6 $\pm$ 3.1
	5000 <sup>b</sup>	+	22 $\pm$ 4.9	86 $\pm$ 14.4	4 $\pm$ 0.6	7 $\pm$ 1.7	6 $\pm$ 2.0

<sup>a</sup> Means and standard deviations of the counts of triplicate plates.<sup>b</sup> Highest marginally cytotoxic dose; not cytotoxic for strain TA98; response at lower doses were comparable to the solvent counts.

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

Substance	Dose ( $\mu\text{g}/\text{plate}$ )	S9 Activa- tion	Bacterial Tester Strains <sup>a</sup>				
			TA98	TA100	TA1535	TA1537	TA1538
<u>Solvent Control</u>							
Sodium lauryl sulfate, 1 mg/mL	--	-	19 $\pm$ 1.2	81 $\pm$ 8.6	11 $\pm$ 3.1	13 $\pm$ 2.1	7 $\pm$ 4.5
		+	18 $\pm$ 2.6	80 $\pm$ 10.1	13 $\pm$ 4.7	14 $\pm$ 2.5	11 $\pm$ 1.5
<u>Positive Controls</u>							
Sodium Azide	5	-	--	542 $\pm$ 27.1	596 $\pm$ 31.0	--	--
9-Aminoacridine	20	-	--	--	--	151 $\pm$ 48.0	--
2-Nitrofluorene	10	-	191 $\pm$ 16.3	--	--	--	158 $\pm$ 5.1
2-Aminoanthracene	2	+	332 $\pm$ 28.1	590 $\pm$ 8.6	91 $\pm$ 3.5	69 $\pm$ 11.7	432 $\pm$ 32.2
<u>Test Material</u>							
Zineb	500 <sup>b</sup>	-	12 $\pm$ 3.6	52 $\pm$ 9.5	3 $\pm$ 2.3	6 $\pm$ 2.6	3 $\pm$ 1.0 <sup>c</sup>
	5000 <sup>b</sup>	+	12 $\pm$ 2.0	53 $\pm$ 9.0	7 $\pm$ 4.0	9 $\pm$ 2.3	11 $\pm$ 1.5

<sup>a</sup> Means and standard deviations of the counts of triplicate plates.

<sup>b</sup> Highest marginally cytotoxic dose; lower doses were, in general, comparable to the solvent counts.

<sup>c</sup> Questionably cytotoxic; at higher levels (1500  $\mu\text{g}/\text{plate}$ ) revertant count was similar to solvent control.

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

- A. The authors concluded that "no evidence of mutagenic potential of Zineb was obtained in this bacterial test system at the dose levels used."
- B. A quality assurance statement was signed and dated May 1, 1985, by the Director of Quality Assurance; it also indicated that the study was conducted in compliance with FDA GLP standards.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

It is our assessment that the authors interpreted the data correctly and that Zineb did not induce a mutagenic response in S. typhimurium strains TA1535, TA1537, TA1538, TA98, and TA100 at doses of 15, 50, 150, 500, 1500, or 5000 µg/plate in the presence or absence of metabolic activation in two independent assays.

The incidence of revertant colonies in all tester strains treated with the solvent control, although within published ranges, appeared low for a 72-hour incubation. The lower than expected background counts may have resulted from using a bactericidal agent, sodium lauryl sulfate, as the test material solvent. Since spontaneous reversion frequencies for untreated cultures were not reported, we were unable to assess if the test strains were slightly inhibited by sodium lauryl sulfate.

The reason for using 10% S9 in the S9 mix was not given. The use of 10% S9 mix as the primary screening concentration may be required for some tests (Maron and Ames);<sup>7</sup> however, the standard S9 mix should contain 4% S9. The lower than expected revertant counts induced by the S9-activated positive control (2-AA, 2 µg/plate) might have been due to the high S9 content in the S9 mix.

All five strains, when treated with the positive control substances, however, produced greater than a twofold increase in revertant colonies over the negative control in the presence and absence of S9 activation.

We concluded that while the assays did have some problems, the overall study was marginally acceptable.

Item 15--see footnote 3.

16. CBI APPENDIX: Appendix A, Materials and Methods, CBI p. 1. and Appendix B, Protocol, CBI pp. 10-13.

<sup>6</sup> de Serres and Shelby, pp. 159-165.

<sup>7</sup> Maron and Ames, pp. 173-215. 8

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APPENDIX A  
Materials and Methods

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METIRAM

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DOCUMENT SUMMARY

005016

Document Id: 1570T  
Document Name: ZINEB #3  
Operator: GC/DA/MR  
Author: SEXSMITH[95020-A-4]

Comments: MUTA./S.typhimurium

STATISTICS

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Last Revised	12/05/85	15:06	:17	319
Last Printed	12/05/85	15:25		
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005016  
EPA: 68-02-4225  
DYNAMAC No. 20-A3  
March 12, 1986

005016

DATA EVALUATION RECORD

ZINEB

Mutagenicity--In vitro Cytogenetics Study  
with Chinese Hamster Ovary Cells

STUDY IDENTIFICATION: Allen, J. A., Brooker, P. C., and Howell, A. Analysis of metaphase chromosomes obtained from CHO cells cultured in vitro and treated with Zineb. (Unpublished study No. FMT5/B5621 prepared by Huntingdon Research Centre, Ltd., Huntingdon, Cambridgeshire, England, for Farmopiant SpA, Milan, Italy; dated July 18, 1985.) Accession No. 259478.

APPROVED BY:

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

Signature: I. Cecil Felkner  
Date: 3-12-86

005016

1. CHEMICAL: Zineb.

2. TEST MATERIAL: Zineb was described as a creamy white powder; the purity was not reported.

3. STUDY/ACTION TYPE: Mutagenicity--In vitro cytogenetics study with Chinese hamster ovary cells.

4. STUDY IDENTIFICATION: Allen, J. A., Brooker, P. C., and Howell, A. Analysis of metaphase chromosomes obtained from CHO cells cultured in vitro and treated with Zineb. (Unpublished study No. FMT5/85621 prepared by Huntingdon Research Centre, Ltd., Huntingdon, Cambridgeshire, England, for Farmoplant SpA, Milan, Italy; dated July 18, 1985.) Accession No. 259478.

5. REVIEWED BY:

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Principal Reviewer  
Dynamac Corporation

Signature: Nancy E. McCarroll  
Date: March 11, 1986

Brenda Worthy, M.T.  
Independent Reviewer  
Dynamac Corporation

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Date: 3-11-86

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Byron T. Backus, M.S.  
EPA Reviewer

Signature: Byron T. Backus  
Date: 3-17-86

Clint Skinner, Ph.D.  
EPA Section Head

Signature: \_\_\_\_\_  
Date: \_\_\_\_\_

## 7. CONCLUSIONS:

- A. Under the conditions of this in vitro Chinese hamster ovary (CHO) cell cytogenetics study, the clastogenic potential of Zineb cannot be assessed because the majority of the cells analyzed for chromosomal aberrations were probably from second division metaphases (cell harvest was at 20 hours postexposure).
- B. The study is unacceptable.

## 8. RECOMMENDATIONS:

The following recommendations are made to upgrade a repeat study:

- a. The assay should be conducted in a manner that ensures that first posttreatment mitoses are analyzed (i.e., cell harvest 12 hours after continuous compound exposure).
- b. To maximize assay sensitivity, the inclusion of multiple harvests both for continuously exposed cells and cells exposed for shorter intervals (S9-activated assays) should be performed; this will ensure that late, middle, and early stages of the cell cycle are analyzed for possible cell cycle-specific clastogenic activity.<sup>1</sup>

Items 9 through 10--see footnote 2.

## 11. MATERIALS AND METHODS (PROTOCOLS):

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

1. Test Material: Zineb was described as a creamy white powder. The test material was not characterized as to purity, stability, or storage conditions. At the request of the sponsor, the test material was suspended in the aqueous anionic surfactant, 0.1% sodium dodecylsulfate (SDS); suspensions were prepared immediately prior to use.
2. Cell Line: Chinese hamster ovary (CHO) cells, strain K<sub>1</sub>-BH<sub>4</sub>, were obtained from BIBRA and were maintained frozen at -196°C in 90% fetal calf serum-10% dimethylsulfoxide. Cells used in this assay were harvested from routinely

<sup>1</sup> Preston, R. J., Au, W., Bender, M. A., Brewen, J. G., Carrano, A. V., Heddle, J. A., McFee, A. I., Wolf, S., and Wassom, J. S. Mammalian in vivo and in vitro cytogenetic assays: a report of the Gene-Tox program. Mutat. Res. 87(1981): 143-188.

<sup>2</sup> Only items appropriate to this DER have been included.

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grown monolayers, trypsinized, resuspended in Hams F12 + fetal calf serum, and dispensed at a density of  $1 \times 10^5$  cells/25 cm<sup>2</sup> tissue culture flasks, and the cultures were incubated for 24 hours at 37°C.

3. Metabolic Activation: The S9 fraction used for metabolic activation was derived from the livers of 10 adult male Sprague-Dawley rats induced with Aroclor 1254. The S9 mix contained 10% S9.
4. Preliminary Cytotoxicity Assay: Prepared cell cultures were exposed to seven doses of the test material in the presence or absence of S9 mix. Duplicate cultures, with or without S9 activation, were prepared for the solvent control. Cultures were exposed to the nonactivated test compound for 20 hours. The 2-hour exposure of cells to S9-activated test doses was terminated by removing the media, refeeding the cells with fresh Ham's F12 + 15% fetal calf serum, and reincubating the cultures for an additional 18 hours in the absence of the test material. Two hours prior to the conclusion of the 20-hour incubation, colchicine (final concentration of 0.25 µg/mL) was added to each culture, and the cultures were returned to the incubator. Cells were trypsinized, resuspended once in Hams F12 and in 2.5 mL 0.07 M KCL, recentrifuged, and fixed twice in methanol:glacial acetic acid fixative (3:1). Fixed cells were dropped onto slides, air dried, stained in 10% Giemsa, and mounted. The proportion of metaphase plates in 2000 cells/treatment group was recorded, mitotic indices were calculated, and the concentration at which a 50% reduction in the mitotic index (EC<sub>50</sub>) occurred was estimated. Based on the results of the preliminary cytotoxicity assay, the doses selected for the cytogenetics test included the EC<sub>50</sub> or the maximum soluble concentration, and doses of 50 and 10% of the highest test material dose.
5. Cytogenetics Assay:
  - a. Dosing: Four concentrations of the test material were assayed in the absence of S9 activation and three doses were assayed in the presence of the S9 mix. Duplicate prepared cultures, with or without S9 activation, were exposed to the appropriate test doses, the positive controls (mitomycin C, 0.4 µg/mL/-S9; cyclophosphamide, 20 µg/mL/+S9), or distilled water, the positive control solvent. Quadruplicate cultures were prepared for the nonactivated and S9-activated test material solvent (0.1% SDS). Cell preparation and exposure conditions were similar to those described under section 11.A.4 (Preliminary Cytotoxicity Assay).

- b. Harvest and Slide Preparation: All cultures were treated with colchicine prior to harvest, fixed, dropped onto slides, stained, and mounted as described in section 11.A.4. All slides were coded prior to analysis.
- c. Metaphase Analysis: At least 100 metaphases per culture were scored for cytogenetic abnormalities. Although not stated in the materials and methods, the authors indicated in the tabular results that chromosomal aberrations were characterized as chromatid breaks, interchanges, complex rearrangements, rings, single minutes, acentric fragments, cells with >10 aberrations, and gaps. Gaps were counted, but were not included in the final analysis.

6. Evaluation Criteria: The data were evaluated for statistical significance at p values of <0.05, <0.01, and <0.001 by Fisher's test.

B. Protocol: A protocol was not presented.

## 12. REPORTED RESULTS:

- A. Preliminary Cytotoxicity Assay: Seven concentrations of the test material, 0.16, 0.31, 0.63, 1.25, 2.5, 5, and 10  $\mu\text{g}/\text{mL}$ , were assayed in the presence or absence of S9 activation in the preliminary cytotoxicity test. The authors stated that 10  $\mu\text{g}/\text{mL}$  of Zineb was the highest possible dose level with the given solvent. In the absence of S9 activation, the highest test dose, 10  $\mu\text{g}/\text{mL}$ , caused a 62% reduction in the number of metaphase cells when compared to the solvent control. The average mitotic index at 5  $\mu\text{g}/\text{mL}$  was slightly higher than the control. Mitotic indices were not affected by the seven S9-activated doses of the test material.
- B. Cytogenetics Assay: Based on the preliminary cytotoxicity findings, 1, 5, 7.5, and 10  $\mu\text{g}/\text{mL}$  of the test material were evaluated in the nonactivated assay and 1, 5, and 10  $\mu\text{g}/\text{mL}$  were tested in the presence of S9 activation. No statistically significant increase in chromosomal aberrations was seen in CHO cells harvested 20 hours following exposure to the selected nonactivated or S9-activated doses of the test material. Although the authors reported a significant increase in the number of cells with gaps at the 10  $\mu\text{g}/\text{mL}$  S9-activated dose, this increase was not considered sufficient evidence of a clastogenic effect. We agree with the authors' conclusions that gaps do not constitute definitive evidence of clastogenicity, since the biological relevance, if any, of this type of chromosomal aberration is unknown. Representative data are presented in Table 1.

TABLE 1. Representative Results of the Chinese Hamster Ovary Cell  
in vitro Cytogenetic Study with Zineb; 20 Hours Postexposure

Substance	Dose ( $\mu\text{g/mL}$ )	S9 Activa- tion	No. of Metaphases Examined	No. of Cells with Aberra- tions <sup>a</sup>	Percent Aberrant Cells <sup>b</sup>	Total No. of Aberra- tions <sup>a</sup>	Percent Aber- rations <sup>c</sup>
<u>Test Material</u>							
<u>Solvent Control</u>							
0.1% Sodium dodecylsulfate	--	--	400	3	0.75	6	1.5
	--	+	400	2	0.5	2	0.5
<u>Positive Control</u>							
<u>Solvent</u>							
Distilled Water	--	--	200	1	0.5	1	0.5
	--	+	200	1	0.5	2	1.0
<u>Positive Controls</u>							
Mitomycin C	0.4	--	200	31	15.5*	62	31.0
Cyclophosphamide	20.0	+	200	29	14.5*	41	20.5
<u>Test Material</u>							
Zineb	10 <sup>d</sup>	--	200	4	2.0	4	2.0
		+	200	1	0.5	1	0.5

<sup>a</sup> Gaps not included.

<sup>b</sup> Percent aberrant cells =  $\frac{\text{No. of cells with aberrations}}{\text{No. of metaphases examined}} \times 100$

<sup>c</sup> Percent aberrations =  $\frac{\text{Total No. of aberrations}}{\text{No. of metaphases examined}} \times 100$

<sup>d</sup> Highest possible dose with the given solvent; % aberrant cells for lower doses (7.5, 5, and 1  $\mu\text{g/mL}$ -S9 and 5 and 1  $\mu\text{g/mL}$ +S9) were comparable to the test material solvent control and were, therefore, not presented.

\* Significantly higher than the appropriate controls,  $p < 0.001$  by Fisher's test.

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

- A. The authors concluded, "Zineb has shown no evidence of mutagenic potential in this in vitro cytogenetic assay."
- B. A quality assurance statement was signed and dated July 15, 1985.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

It is our assessment that the findings of this report cannot be used to support a negative result in this in vitro cytogenetics assay. The majority of cells analyzed for chromosomal aberrations assayed either with or without S9 activation were probably second division metaphases since cell harvest was not performed until 20 hours after treatment with Zineb. The normal cycling time for CHO cells in culture is 10-14 hours. Since preparations were made from cells cultured for a longer period, an increasing proportion of the cells were probably in second division and cells with Zineb-induced chromosomal damage would have failed to undergo division because of genetic imbalances. Thus, to maximize the detection of aberrations, first posttreatment mitoses should have been analyzed.

Preston et al.<sup>3</sup> recommend cell harvest at 3, 8, and 12 hours following compound administration to ensure that late, middle, and early stages of the cell cycle are scored for cell cycle-specific aberrations. OECD guidelines require only one sampling interval if the target cells is exposed to the test material for an entire cell cycle. However, a continuous one cell-cycle exposure is not possible under conventional S9-activated conditions because of S9 cytotoxicity. OECD guidelines, in agreement with Preston et al.,<sup>4</sup> require multiple fixation times for short term exposures, generally 1, 6, and 10 hours after removal of the test material.

Item 15--see footnote 1.

16. CBI Appendix

Appendix A, Materials and Methods, CBI pp. 2-4 and 10.

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<sup>3</sup>Preston et al., pp. 143-188.

<sup>4</sup>Ibid.



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APPENDIX A  
Materials and Methods

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APPENDIX A  
Materials and Methods

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March 13, 1986

DATA EVALUATION RECORD

ZINEB

Mutagenicity--In vitro Assay in Mouse Lymphoma Cells

STUDY IDENTIFICATION: Edgar, D. H., Ransome, S. J., Banks, S. J., and Bosworth, H. J. An assessment of the mutagenic potential of Zineb using an in vitro mammalian cell test system. (Unpublished study No. FMT6/85539 prepared by Huntingdon Research Centre, Ltd., Huntingdon, England, for Farmoplant SpA, Milan, Italy; dated July 23, 1985.) Accession No. 259476.

APPROVED BY:

I. Cecil Felkner, Ph.D.  
Program Director  
Dynamac Corporation

Signature: I. Cecil Felkner

Date: 3-13-86

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46

1. CHEMICAL: Zineb.
2. TEST MATERIAL: Zineb was described as an off-white powder of an unspecified purity.
3. STUDY/ACTION TYPE: Mutagenicity--in vitro assay in mouse lymphoma cells.
4. STUDY IDENTIFICATION: Edgar, D. H., Ransome, S. J., Banks, S. J., and Bosworth, H. J. An assessment of the mutagenic potential of Zineb using an in vitro mammalian cell test system. (Unpublished study No. FMT6/85539 prepared by Huntingdon Research Centre, Ltd., Huntingdon, England, for Farmoplant SpA, Milan, Italy; dated July 23, 1985.) Accession No. 259476.

5. REVIEWED BY:

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Independent Reviewer  
Dynamac Corporation

Signature: Nancy E. McCarroll  
Date: 3-13-86

6. APPROVED BY:

I. Cecil Felkner, Ph.D.  
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Date: 3-13-86

Byron T. Backus, M.S.  
EPA Reviewer

Signature: Byron T. Backus  
Date: 3-17-86

Clint Skinner, Ph.D.  
EPA Section Head

Signature: \_\_\_\_\_  
Date: \_\_\_\_\_

7. CONCLUSIONS:

- A. Under the conditions of the assay Zineb, at five nonactivated doses ranging from 0.313 to 5 µg/mL or at six S9-activated doses ranging from 0.625 to 8 µg/mL, did not induce a mutagenic response in two independent mouse lymphoma assays. The highest dose (10 µg/mL) tested was cytotoxic with and without S9 activation in each independent mutation assay. The positive controls ethyl methanesulfonate (EMS) and cyclophosphamide caused a significant increase in mutation frequency (MF) compared to the solvent control.
- B. The study is acceptable.

8. RECOMMENDATIONS:

It is strongly recommended that the positive controls be used at concentrations that not only demonstrate the capability of the assay to detect a mutagenic effect, but also at concentrations comparable to the dose range of the test material. The nonactivated positive control EMS at 500 µg/mL demonstrated the capability of the assay to detect a mutagenic effect; however, the concentration of EMS was 50 times greater than the highest dose (10 µg/mL) of the test material. Thus, the sensitivity of the assay to detect a mutagenic effect at test material doses (0.313 to 10 µg/mL) was not established.

Items 9 and 10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):A. Materials and Methods: (See Appendix A for details.)

1. Test Material: Zineb was described as an off-white powder. No further description or purity was reported. The test material was suspended in 0.1 percent sodium dodecyl sulphate (SDS), at the request of the sponsor.
2. Cell Line: L5178Y TK<sup>±</sup> mouse lymphoma cells, clone 3.7.2c, were obtained from Dr. J. Cole, Sussex University. The cells were maintained, grown, and cleansed as described by Clive and Spector.<sup>2</sup>

<sup>1</sup> Only items appropriate to this DER have been included.

<sup>2</sup> Clive, D. and Spector, J. Laboratory Procedures for Assessing Specific Locus Mutations at the TK Locus in Cultured L5178Y Mouse Lymphoma Cells. Mutation Research 31(1975): 17-29.

3. S9 Fraction: The S9 fraction was prepared from the livers of SPF/CD Sprague-Dawley rats induced ip with 500 mg/kg of Aroclor 1254.
4. Preliminary Cytotoxicity Study: Duplicate cultures seeded at a density of  $1 \times 10^6$ /mL were treated with an unspecified number of the test material doses or the vehicle control with and without S9 activation. After a 3-hour exposure at 37°C, cells were washed, resuspended, and incubated for 48 hours. Cells were counted at 24 and 48 hours to determine cytotoxicity (reduction in cell population).
5. Mutation Assay: Cells were seeded, and samples were treated with six doses of the test material, vehicle, or positive controls and exposed as described in the cytotoxicity study. During the 48-hour expression period, cells were adjusted to a density of  $2 \times 10^5$  cells/mL. After the expression period, four doses of the test material were selected for cloning. For cloning the dose groups,  $10^6$  cells were plated in three selective medium plates containing trifluorothymidine (TFT), and 200 cells were cloned in three nonselective plates for cell viability. Plates were incubated for 12 days at 37°C in a humidified atmosphere of 5 percent CO<sub>2</sub> in air. After incubation colonies were counted, and cloning efficiency, total survival, and MF were determined.
6. Evaluation Criteria: A test material was considered positive if 1) an induction of at least a twofold increase in MF occurred over the vehicle control at a dose that caused >10 percent cell survival and 2) a statistically significant, reproducible dose-related response was demonstrated.
7. Statistically Analysis: The test material results were transformed and analyzed by analysis of variance.

B. Protocol: No protocol was provided.

## 12. REPORTED RESULTS:

- A. Preliminary Cytotoxicity Study--Without S9 Activation: Cells were treated with six doses of Zineb ranging from 0.313 to 10 µg/mL, which resulted in a percent relative survival (RS) of 47 percent at the 0.625 µg/mL level. Based on this data six doses were selected for the mutation assay.

Mutation Assay--Without S9 Activation: Two independent assays were performed.

Assay 1: Duplicate cultures treated with six doses of the test material ranging from 0.313 to 10 µg/mL resulted in an average RS of 52.5 percent at 0.313 µg/mL and <3 percent at the 5- and

10- $\mu\text{g}/\text{mL}$  doses. Based on this cytotoxicity data, four doses (0.313, 0.625, 1.25, and 2.5  $\mu\text{g}/\text{mL}$ ) were selected for cloning and mutation assessment. The average RS for these doses ranged from 49 percent at 0.313  $\mu\text{g}/\text{mL}$  to 3.5 percent at 2.5  $\mu\text{g}/\text{mL}$ , and the average MF ranged from 54 mutants/ $10^6$  viable cells at the lowest dose to 99 mutants/ $10^6$  at the highest dose. The average MF of the vehicle control was 46 mutants/ $10^6$  viable cells.

Assay 2: Duplicate cultures were treated with six doses ranging from 0.156 to 5  $\mu\text{g}/\text{mL}$ , resulting in an average RS of 74.5 percent to 37 percent, respectively. Doses selected for cloning were 0.625, 1.25, 2.5, and 5  $\mu\text{g}/\text{mL}$ . The average RS ranged from 50 percent to 36.5 percent. The average MF for each test dose was 20, 49, 66, and 77 mutants/ $10^6$ , compared to a MF of 59.3 for the vehicle control.

The increase observed in assay 1 at the 2.5- $\mu\text{g}/\text{mL}$  dose was considered to be the result of toxicity (4 percent survival), and therefore disregarded. Zineb did not induce a positive response in L5178Y cell without S9 activation. However, the positive control EMS at 500  $\mu\text{g}/\text{mL}$  induced a significant increase in MF in both assays.

Representative results are presented in Table 1.

- B. Preliminary Cytotoxicity Study--With S9 Activation: In the preliminary cytotoxicity study with S9 activation, treatment with six doses of the test material ranging from 0.313 to 10  $\mu\text{g}/\text{mL}$  resulted in an RS of 32 percent at the highest dose tested, which also was the maximum soluble dose. Based on these results doses for the mutation assay with S9 activation were selected.

Mutation Assay--With S9 Activation: Two independent assays were performed.

Assay 1: Duplicate cultures were treated with six doses of the test material ranging from 0.313 to 10  $\mu\text{g}/\text{mL}$ , resulting in an average RS of 76.5 percent at the lowest dose and <1 percent at the highest dose. Four doses (0.625, 1.25, 2.5, and 5.0  $\mu\text{g}/\text{mL}$ ) were selected for cloning and mutation assessment. The average RS for these doses was >79 percent. The average MF ranged from 54 mutants/ $10^6$  viable cells at 0.625  $\mu\text{g}/\text{mL}$  to 59 mutants at 5  $\mu\text{g}/\text{mL}$ . The average MF for the vehicle control was 60 mutants/ $10^6$  viable cells.



TABLE 1. Representative Results from the Nonactivated Mouse Lymphoma Mutagenicity Assays with Zineb

Substance	Dose ( $\mu\text{g/mL}$ )	Assay	% Relative Survival	Average Viability Counts <sup>a</sup>	Average Mutant Counts <sup>a</sup>	MF/10 <sup>6</sup> viable cells <sup>b</sup>
<u>Vehicle Control</u>						
0.1% SDS	--	1	100	660	147	45
		2	100	382	116	60
<u>Positive Control</u>						
EMS	500	1	34	378	914	484*
		2	28	156	597	765*
<u>Test Material</u>						
Zineb	2.5 <sup>c</sup>	1	4	323	160	99*
		2	32	382	129	68

<sup>a</sup> Averaged by the reviewers.

<sup>b</sup>  $\text{MF}/10^6 = \frac{\text{Total No. Cells Plated (600)}}{\text{Average Viability Count}} \times \text{Average Mutant Count} \div \text{Total No. Plates (3)}$ .

<sup>c</sup> Dose at which a positive response was observed in assay 1; however, the increase was due to the excessive cytotoxicity; all other dose results, in both assays, were comparable to their respective vehicle controls.

\*Significantly greater than the vehicle control.

Assay 2: Duplicate cultures were treated with six doses of Zineb ranging from 1 to 10  $\mu\text{g/mL}$ . The average RS ranged from 92 percent at 1  $\mu\text{g/mL}$  to 2.5 percent at 10  $\mu\text{g/mL}$ . The doses selected for cloning and mutation assessment were 2, 4, 6, and 8  $\mu\text{g/mL}$ . These doses resulted in an average RS ranging from 82.5 percent to 77.5 percent with an average MF of 68 mutants at the lowest dose and 100 mutants at the highest dose; the MF of the vehicle control was 81 mutants/ $10^6$  viable cells.

The positive control, cyclophosphamide at 5  $\mu\text{g/mL}$ , induced a significant increase in MF in both assays compared to the vehicle control. No significant increases in MF were observed in either assay with Zineb.

Representative results are presented in Table 2.

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

- A. The authors concluded that "None of the criteria used to determine a positive response were satisfied after treatment with Zineb either in the absence or the presence of S9 mix. Therefore it is concluded that Zineb does not demonstrate mutagenic potential in this in vitro test system."
- B. A quality assurance statement was signed and dated May 14, 1985.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

It is our assessment that the authors interpreted the data correctly and that Zineb did not induce a mutagenic effect in L5178Y mouse lymphoma cells with or without S9 activation.

The positive control, EMS at 500  $\mu\text{g/mL}$ , although 50 times greater than the highest test material dose (10  $\mu\text{g/mL}$ ), demonstrated that the assay was capable of detecting a mutagenic effect without S9 activation. Cyclophosphamide at 5  $\mu\text{g/mL}$ , the control used in the S9-activated system, demonstrated that the assay was adequately sensitive to detect a mutagenic response and at a concentration comparable to the dose range of the test material.

Item 15--see footnote 1.

16. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 2-6.

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TABLE 2. Representative Results from the S9-Activated Mouse Lymphoma Mutagenicity Assay with Zineb

Substance	Dose ( $\mu\text{g/mL}$ )	Assay	% Relative Survival	Average Viability Counts <sup>a</sup>	Average Mutant Counts <sup>a</sup>	MF/10 <sup>6</sup> viable cells <sup>b</sup>
<u>Vehicle Control</u>						
0.1% SDS	--	1	100	349	108	62
		2	100	340	137	81
<u>Positive Control</u>						
Cyclophosphamide	5	1	10	72	155	431*
		2	19	106	267	504*
<u>Test Material</u>						
Zineb	5 <sup>c</sup>	1	85	404	117	58
	8 <sup>c</sup>	2	76	364	182	100

<sup>a</sup> Averaged by the reviewers.

<sup>b</sup>  $\text{MF}/10^6 \text{ viable cells} = \frac{\text{Total No. Cells Plated (600)} \times \text{Average Mutant Count}}{\text{Average Viability Count}} \div \text{Total No. Plates (3)}$ .

<sup>c</sup> Highest dose tested in each assay; lower dose results were comparable to their respective vehicle control.

\*Significantly greater than the vehicle control.

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APPENDIX A  
Materials and Methods

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EPA: 68-02-4225  
DYNAMAC No. 20-A1  
March 18, 1985

DATA EVALUATION RECORD

ZINEB

Mutagenicity--Unscheduled DNA Synthesis  
Assay in HeLa Cells

STUDY IDENTIFICATION: Allen, J. A. and Proudlock, R. J. Assessment of unscheduled DNA repair synthesis in mammalian cells after exposure to Zineb. (Unpublished study No. FMT 8/85519 prepared by Huntingdon Research Centre, Ltd., Huntingdon, Cambridgeshire, England, for Farmoplant S.p.A., Milan, Italy; dated July 19, 1985.) Accession No. 259480.

APPROVED BY:

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

Signature: I. Cecil Felkner  
Date: 3-18-86

1. CHEMICAL: Zineb; zinc ethylenebisdithiocarbamate.
2. TEST MATERIAL: Zineb (Iso), technical grade, CAS No. 12122-67-7, was described as a creamy white powder with a purity of 91.5%.
3. STUDY/ACTION TYPE: Mutagenicity--Unscheduled DNA synthesis assay in HeLa cells.
4. STUDY IDENTIFICATION: Allen, J. A. and Proudlock, R. J. Assessment of unscheduled DNA repair synthesis in mammalian cells after exposure to Zineb. (Unpublished study No. FMT 8/85519 prepared by Huntingdon Research Centre, Ltd., Huntingdon, Cambridgeshire, England, for Farmoplant S.p.A., Milan, Italy; dated July 19, 1985.) Accession No. 259480.

5. REVIEWED BY:

Nancy E. McCarroll, B.S.  
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Signature: Nancy E. McCarroll  
Date: March 17, 1986

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I. Cecil Felkner, Ph.D.  
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Date: 3-18-86

Byron T. Backus, M.S.  
EPA Reviewer

Signature: Byron T. Backus  
Date: 3-19-86

Clint Skinner, Ph.D.  
EPA Section Head

Signature: \_\_\_\_\_  
Date: \_\_\_\_\_

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7. CONCLUSIONS:

- A. Two independent HeLa cell unscheduled DNA synthesis (UDS) assays conducted with 12 test doses of Zineb (2.5 to 5120 µg/mL), both in the presence and absence of S9 activation, could not be evaluated for the following reasons:
  - a. The test material precipitated at all doses; therefore, a valid interpretation of the data was not possible.
  - b. A cell line lacking an appropriate level of sensitivity<sup>1</sup> was used for the assays; therefore, the response cannot be considered a reliable measure of genotoxic activity.
  - c. Cytoplasmic background counts were not performed; hence, the net increase in nuclear grains cannot be assessed.
- B. The assay is unacceptable.

8. RECOMMENDATIONS:

The following recommendations are given to upgrade a repeat UDS assay(s):

- 1. The test material's solubility in nonaqueous solvents should be determined so that the exposure of cells and delivery of test material to the target site can be reasonably ensured.
- 2. The assay(s) should be repeated using three or more nonprecipitating dose levels to ensure that sufficient doses are available for analysis.
- 3. Rather than use a cell line lacking appropriate sensitivity, the repeat assay(s) should be performed with one of the recommended cell lines, i.e., WI38 diploid human fibroblasts and/or primary rat hepatocytes.<sup>2</sup>
- 4. The procedures used should be consistent with recognized and established protocols for the UDS assay.

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<sup>1</sup> Mitchell, A. D., Casciano, D. A., Meltz, M. L., Robinson, D. E., San, R. H. C., William, G. M., and Von Halle, E. S. Unscheduled DNA synthesis tests, a report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutat. Res. 123(1983):363-410.

<sup>2</sup> Ibid.



11. MATERIALS AND METHODS (PROTOCOLS):

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

1. Test Material: Zineb (Iso), technical grade, was described as a creamy white powder with a purity of 91.5%. The test material was stored in the dark at 4°C and was prepared as a suspension in aqueous 0.1% sodium dodecyl sulphate (SDS). The selection of an anionic surfactant as the solvent of choice was requested by the sponsor.
2. Cell Strain: HeLa epitheloid S3 cells were obtained from Flow Laboratories, Ltd., and were stored at -196°C in Eagle's Minimum Essential Medium (EMEM) containing 5% dimethylsulfoxide. Cells used for the assay were generated from frozen stocks and were resuspended and incubated in fresh EMEM.
3. Metabolic Activation: The S9 fraction used for metabolic activation was prepared from the liver of 6- to 8-week-old, specific pathogen-free CD Sprague-Dawley male rats induced with Aroclor 1254.
4. DNA Repair Assays:
  - a. Exposure: Aliquots of prepared cell suspensions ( $5 \times 10^4$ ) were dispensed into multi-well tissue culture dishes containing glass coverslips. Cultures were placed in a humid environment for 96 hours and refed with arginine-deficient medium over a 72-hour period. Tritiated thymidine (final concentration, 5  $\mu$ Ci/mL), S9 mix for the activated test, and the appropriate concentration of the test material and negative or positive controls were added. At the conclusion of a 180-minute exposure, coverslips were washed, fixed, stained, and mounted for autoradiography.
  - b. Preparation of Autoradiographs: Autoradiographs were prepared in a darkroom. Slides were coated with stripping film and dried sequentially at room temperature and at 4°C to allow exposure of the photographic emulsion to the radioactive isotope. Autoradiographs were developed, rinsed, fixed, air dried, and coded, and the labeled nuclei were scored.

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<sup>3</sup>Only items appropriate to this DER have been included.

- c. Grain Counting: The number of silver grains overlying the nuclei of 100 non-S-phase cells was counted for each treatment and control culture. The number of S-phase nuclei having >5 grains/nucleus was also counted.
5. Evaluation Criteria: The assay was considered positive if a reproducible and statistically significant increase in the number of nuclear grains of compound-treated cells was observed when compared to the concurrent controls.
6. The results were analyzed for significance by the Student's t test and one-way analysis of variance (ANOVA).
- B. Protocol: A protocol was not presented.

12. REPORTED RESULTS:

DNA Repair Assays: The maximum starting concentration of the test material, 5120 µg/mL, and 11 twofold serial dilutions were assayed in the presence and absence of S9 activation in two separate experiments. Five dose levels of each positive control, 0.05, 0.1, 0.2, 0.4, and 0.8 µg/mL 4-nitroquinoline-1-oxide (4-NQO) without S9 activation and 2.5, 5, 10, 20, and 40 µg/mL 2-aminoanthracene (2-AA) with S9 activation, were included in both experiments. Compound precipitation, described as an insoluble residue, was reported at all test doses either with or without S9 activation in both the initial and independent UDS assays. Excessive compound precipitation at doses ranging from 5120 to 160 µg/mL interfered with slide analysis; hence, slides from these doses were not scored. Although compound precipitation was observed on slides prepared for the remaining six doses, no appreciable increase in nuclear grain counts was noted in either study under S9-activated or nonactivated conditions following exposure of HeLa cells to 2.5, 5, 10, 20, 40, and 80 µg/mL of the test material.

Representative results for the initial and independent assays are shown in Table 1.

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

- A. The authors concluded, "Zineb has failed to show any evidence of mutagenic potential when tested as a suspension in this in vitro test for unscheduled DNA synthesis."
- B. A quality assurance statement was signed and dated July 16, 1985.

TABLE 1. Representative Results of the HeLa Cell Unscheduled DNA Repair Synthesis Assays with Zineb

Substance	Dose	S9 Activation	No. of Scored Nuclei	Average Nuclear Grain Count <sup>a</sup>
<u>Solvent Control</u>				
Sodium dodecyl sulfate	0.1%	-	600	293
		+	1000	202
		- <sup>b</sup>	1000	242
		+ <sup>b</sup>	1000	233
<u>Positive Controls<sup>c</sup></u>				
4-Nitroquinoline-1-oxide	0.2 µg/mL	-	200	3093*
		- <sup>b</sup>	200	5161*
2-Aminoanthracene	10.0 µg/mL	+	200	473*
		+ <sup>b</sup>	200	744*
<u>Test Material</u>				
Zineb	80 µg/mL <sup>d</sup>	-	200	212
		+	200	162
	80 µg/mL	- <sup>b</sup>	200	261
		+ <sup>b</sup>	200	252

<sup>a</sup> Average value of duplicate cultures for the positive controls and test material doses; average value of six to ten replicate cultures for the solvent control.

<sup>b</sup> Results from independent trial.

<sup>c</sup> Five levels of each positive control were assayed, the mid dose for each mutagen was selected as representative; all other doses induced significant increases in UDS.

<sup>d</sup> Highest dose scored; compound precipitation was apparent at all doses (2.5, 5, 10, 20, 40, and 80 µg/mL). Values for doses <80 µg/mL were comparable to control values and, therefore, not selected as representative.

\* Significantly increased over control ( $p < 0.001$ ) by Student's *t* and ANOVA.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

It is our assessment that the results of the study, as performed, cannot be evaluated for the following reasons:

1. The dose range selected for the UDS assays was inappropriate to assess the genotoxic potential of Zineb; compound precipitation occurred at all 12 doses. The highest assayed dose of a noncytotoxic insoluble material should be selected to show the solubility limit; however, a minimum of three nonprecipitating concentrations are necessary to draw conclusions relative to a dose-response relationship.
2. The transformed HeLa cell line is not recommended for use in this assay since entry of HeLa cells into scheduled DNA synthesis is not sufficiently suppressed by arginine deprivation.<sup>4</sup> Although S-phase cells can be visually excluded from the analysis, the resulting high background levels render the assay insensitive to detect the subtle increases in grain counts due to UDS. The reported findings with the positive controls (4-NQO/-S9 and 2AA/+S9) illustrate the problem resulting from high background levels. At all nonactivated 4-NQO doses 100% of the S-phase nuclei had >5 grains. It was unclear to us how the investigators differentiated cells undergoing scheduled from unscheduled DNA synthesis since the average grain count/cell for the 0.2- $\mu$ g/mL dose of 4-NQO was approximately 31 grains/nucleus in the first experiment and 52 grains/nucleus in the second assay. Similarly for the activated control series the percent S-phase nuclei with >5 grains was high and the average grain count/cell at 10.0  $\mu$ g/mL was 4.7 in the first experiment and 7.4 in the second experiment. To avoid excessive background levels, the investigators should have used one of the preferred cell lines recommended for the UDS assay (WI38 diploid human fibroblasts or primary rat hepatocytes).<sup>5</sup>
3. Sensitivity of the assay was further compromised because the cytoplasmic grain counts were not determined. Counting three adjacent nuclear-sized cytoplasmic areas and subtracting this average value from the nuclear grain count is the recommended procedure.<sup>6</sup> Without this adjustment, no distinction can be made between [<sup>3</sup>H]thymidine incorporation into mitochondrial DNA or into molecules in the cytoplasm and nuclear incorporation.

Item 15--see footnote 3.

16. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 1-6.

<sup>4</sup>Mitchell et al. Mutat. Res. 123 (1983): 363-410.

<sup>5</sup>Ibid.

<sup>6</sup>Ibid.

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APPENDIX A  
Materials and Methods

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