



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

005084

4/27/86

MEMORANDUM

OFFICE OF  
PESTICIDES AND TOXIC SUBSTANCES

SUBJECT: Review of Mutagenicity Studies on Endosulfan  
as follow-up to the 1982 Registration Standard

TO: George LaRocca  
PM 15  
Registration Division (TS-767)

FROM: Margaret L. Jones *M.L. Jones 4/18/86*  
Review Section III  
Toxicology Branch

THRU: William Burnam  
Acting Head  
Review Section III  
Toxicology Branch

*W. Burnam 4/24/86*  
*W. Burnam 4/27/86*

and

Theodore M. Farber, Ph.D., D.A.B.T.  
Chief  
Toxicology Branch

Chemical: Endosulfan (Thionex®)

Record No.: 154115

Caswell No.: 420

Tox. Project No.: 485

Accession No.: 256128

Registrant: American Hoechst

Action Requested: Review of 5 mutagenicity studies submitted  
as IBT replacement studies following the 1982 Registration  
Standard. The following studies were reviewed:

1. Mitotic gene conversion in Saccharomyces cerevisiae D4
2. Mouse lymphoma mutation assay
3. Rat primary hepatocyte unscheduled DNA synthesis assay
4. Forward mutation in Schizosaccharomyces pombe assay
5. Micronucleus test in mice

Background: The 1982 Pesticide Assessment Guidelines:  
Subdivision F require mutagenicity testing from three general  
categories: gene mutation, structural chromosome aberration, and  
other genotoxic effects.

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The 1982 Endosulfan Registration Standard reviewed the current mutagenicity data and concluded the category of structural chromosome aberration was satisfied. The gene mutation category contained several supplementary studies. The attached pages 48-49 describe the mutagenicity data reviewed for the Endosulfan registration standard. The standard required further testing to determine whether Endosulfan could induce point mutations and chromosomal mutations directly or indirectly.

Results: The following are brief results of the five mutagenicity studies. The data evaluation reports are attached and should be consulted for further details.

1. Mitotic gene conversion in Saccharomyces cerevisiae D<sub>4</sub> - The assay is unacceptable since it was performed with stationary phase cultures which may have compromised the sensitivity of the assay.
2. Mouse lymphoma mutation - acceptable  
Endosulfan was nonmutagenic in this assay.
3. Rat primary hepatocyte unscheduled DNA synthesis - acceptable  
Endosulfan was inactive in this assay.
4. Forward mutation in Schizosaccharomyces pombe - The assay is unacceptable since mutation frequencies were lower than published values and incubation took place at the wrong temperature for expression of mutation.
5. Micronucleus test in mice - The assay is unacceptable since toxic effects were not demonstrated at the highest dose tested and the sampling intervals did not cover the entire hematopoietic cycle.

Conclusions: With the newly submitted studies, at least one valid test now exists for each of the mutagenicity categories. No further mutagenicity testing is required at this time.

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DOES NOT CONTAIN  
NATIONAL SECURITY INFORMATION (EO 12065)

005084

EPA: 68-02-4225  
DYNAMAC No. 050-A4  
April 16, 1986

DATA EVALUATION RECORD

ENDOSULFAN

Rat Primary Hepatocyte Unscheduled DNA Synthesis Assay

STUDY IDENTIFICATION: Cifone, M. A., and Myhr, B. C. Evaluation of HOE 002671-substance technical in the rat primary hepatocyte unscheduled DNA synthesis assay. (Unpublished study No. 20991 prepared by Litton Bionetics, Inc., Kensington, MD, for Hoechst Aktiengesellschaft, Frankfurt, West Germany; dated November 1984.) Accession No. 256128.

APPROVED BY:

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

Signature: I. Cecil Felkner

Date: 4-16-86

005084

1. CHEMICAL: Endosulfan; Thiodan; HOE 002671
2. TEST MATERIAL: HOE 002671-substance technical, code No. HOE 002671-01 ZD97 0003, was a caramel-colored solid with a purity of 97.2 percent.
3. STUDY/ACTION TYPE: Rat primary hepatocyte, unscheduled DNA synthesis assay.
4. STUDY IDENTIFICATION: Cifone, M. A., and Myhr, B. C. Evaluation of HOE 002671-substance technical in the rat primary hepatocyte unscheduled DNA synthesis assay. (Unpublished study No. 20991 prepared by Litton Bionetics, Inc., Kensington, MD; for Hoechst Aktiengesellschaft, Frankfurt, West Germany; dated November 1984.) Accession No. 256128.

5. REVIEWED BY:

Barry R. Scott, Ph.D.  
Principal Reviewer  
Dynamac Corporation

Signature: Ira Cecil Felkner for  
Date: 4-15-86

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

Signature: Nancy E. McCarroll  
Date: 4-15-86

6. APPROVED BY:

I. Cecil Felkner, Ph.D.  
Genetic Toxicology  
Technical Quality Control  
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Signature: Ira Cecil Felkner  
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Margaret Jones  
EPA Reviewer

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Date: 4-16-86

Clint Skinner, Ph.D.  
EPA Section Head

Signature: W2/Skinner  
Date: 4-24-86

7. CONCLUSIONS:

- A. Under the conditions of the assay, doses of HOE 002671-substance technical, ranging from 25.5 to 0.102  $\mu\text{g/mL}$ , did not induce an appreciable change in the pattern of nuclear labeling of rat hepatocytes. These doses resulted in a cell survival range of 31.5 to 105.5 percent. HOE 002671-substance technical is, therefore, considered inactive in the primary rat hepatocyte unscheduled DNA synthesis (UDS) assay.
- 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: HOE 002671-substance technical was described as a caramel-colored solid; the purity was listed as 97.2 percent. Storage conditions of the test material were unspecified. A clear, amber solution of the test chemical (100 mg/mL) in dimethylsulfoxide (DMSO) was prepared on the day of use by sonication for 45 seconds and incubation at 37°C for 10 minutes. Just prior to each assay, stock solutions were prepared by serial dilutions in DMSO.
2. Indicator Cells: Hepatocytes were obtained from a male Fischer 344 rat (150-300 g) quarantined for a minimum of 5 days. This animal was identified by cage card and was fed Purina Certified Chow (formula 5002) and water ad libitum.
3. Cell Preparation:
  - a. Perfusion Technique: The liver was perfused with Hanks' balanced salts containing 0.5 mM EGTA and HEPES pH 7.0 buffer for 4 minutes and with 100  $\mu\text{g/mL}$  Type I collagenase for 10 minutes. The liver was excised and mechanically dispersed in incomplete WME and collagenase to release the hepatocytes.
  - b. Hepatocyte Harvest/Culture Preparation: Recovered cells were centrifuged, resuspended in complete WME, counted, and aliquoted ( $0.5 \times 10^6$  cells/3 mL WME) onto plastic coverslips. The cultures were placed in a humidified, 37°C, 5 percent  $\text{CO}_2$  incubator\* for a 1.5- to 2-hour attachment period. Unattached cells were removed; viable cells were refed and established as monolayer cultures.

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

4. Dose Selection: Initially, 15 doses of the test material were applied (1000-0.025  $\mu\text{g/mL}$  in dilutions of approximately twofold steps). When the viability estimate was obtained (20-24 hours after treatment initiation), seven of these doses were chosen for analyzing nuclear labeling, starting with the highest dose that resulted in a sufficient number of survivors with intact morphologies and proceeding to successively lower doses.
5. UDS Assay:
  - a. Treatment: At least seven doses were tested in the UDS assay. Five replicate, monolayer cultures were exposed to the selected doses of the test material and negative (DMSO) or positive controls (2-acetylaminofluorene (2-AAF, 0.1  $\mu\text{g/mL}$ ) for 18-19 hours in WME containing 1  $\mu\text{Ci/mL}$  [ $^3\text{H}$ ]-thymidine. Treated monolayers were washed twice with WME; two of the five replicates for each treatment group were used to determine cytotoxicity. These cultures were refed, reincubated, and monitored for cytotoxicity at 20-24 hours posttreatment.
  - b. UDS Slide Preparation: The remaining cultures (3 replicate coverslips/treatment group) were washed with media containing 1 mM thymidine. Treated hepatocytes attached to coverslips were exposed to 1 percent sodium citrate for 10 minutes, fixed in acetic acid/ethanol (1:3), dried, and mounted.
  - c. Preparation of Autoradiographs/Grain Development: Slides were coated with Kodak NTB2 emulsion, dried for 7-10 days at 4°C in light-tight dessicated boxes, developed in Kodak D-19, fixed, stained with Williams' modified hematoxylin and eosin, coded, and counted.
  - d. Grain Counting: The nuclear grains of 150 morphologically normal cells for each test dose and negative and positive controls were counted microscopically. Net nuclear grain counts were determined by subtracting the nuclear grain counts of each cell from the mean cytoplasmic grain count of three nuclear-sized areas adjacent to each nucleus.
6. Evaluation Criteria: The assay was considered positive if a) an increase in the mean nuclear grain count was  $\geq 6$  grains/nucleus over the negative control value, or b) the percent of nuclei with  $\geq 6$  grains exceeded 10 percent of the negative control population, or c) the percent of nuclei with  $\geq 20$  grains was  $\geq 2$  percent of the examined population.

B. Protocol: See Appendix B.

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12. REPORTED RESULTS:

UDS Assay: The cytotoxicity and UDS assay were performed in parallel. Fifteen test concentrations ranging from 1020 to 0.102  $\mu\text{g/mL}$  were used. The authors stated that the test material appeared soluble in media up to 51  $\mu\text{g/mL}$ , but higher concentrations were increasingly cloudy. The test material was toxic at 51  $\mu\text{g/mL}$ . Survival decreased from 102.8 percent at 0.255  $\mu\text{g/mL}$  to 31.5 percent at 25.5  $\mu\text{g/mL}$ . Survival was not determined for the lowest dose. Based on these observations seven treatments (25.5-0.102  $\mu\text{g/mL}$ ) were selected for analysis of nuclear labeling. The positive control (0.10  $\mu\text{g/mL}$  2-AAF) was weakly toxic (85.7%) and induced a significant increase in UDS. No appreciable increase in nuclear grain counts of cells exposed to graded doses of the test material were observed when compared to the solvent control. Representative results are presented in Table 1.

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

- A. The authors concluded that "The test material, HOE 002671 - substance technical, did not induce significant change in the nuclear labeling of Primary Rat Hepatocytes" and "therefore, the test material was evaluated as inactive in the Primary Rat Hepatocyte UDS assay."
- B. A quality assurance statement was signed and dated November 9, 1984.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

It is our assessment that the study was conducted properly and the authors' interpretation of the data was correct.

None of the doses induced an appreciable increase in UDS grains/nuclei. The data demonstrated a satisfactory reduction in the viability of hepatocytes (31.5% at 25.5  $\mu\text{g/mL}$ ). This indicated that the test substance entered the hepatocytes and that the lack of response was not due to the inability of the test material to penetrate the cell wall. The study adequately demonstrated both the solubility limits and cytotoxicity of the test material. Similarly, the ability of the test system to detect UDS was clearly shown by the findings with the positive control (2-AAF, 0.1  $\mu\text{g/mL}$ ).

Item 15--see footnote 1.

16. CBI APPENDIX:

Appendix A, Materials and Methods, CBI pp. 1-2. Appendix B, Protocol, CBI pp. 6-12.

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TABLE 1. Representative Results of the Unscheduled DNA Synthesis Rat Hepatocyte Assay with HOE 002671-Substance Technical

Treatment	Dose	Cells Scored	Percent Survival <sup>a</sup> (22 Hours)	Average Nuclear Grain Count	Average Percent Nuclei w/ ≥6 Grains	Average Percent Nuclei w/ ≥20 Grains
<u>Solvent Control</u>						
Dimethylsulfoxide	1%	150	100	0.69	0.0	0.0
<u>Positive Control</u>						
2-Acetylaminofluorene	0.1 µg/mL	100 <sup>b</sup>	85.7	8.31 <sup>c</sup>	57.0 <sup>c</sup>	10.0 <sup>c</sup>
<u>Test Material</u>						
HOE 002671	25.5 µg/mL <sup>d</sup>	150	31.5	0.61	0.0	0.0

% Survival  $\frac{\text{No. of viable cells/unit area test dose}}{\text{No. of viable cells/unit area solvent control}} \times 100$

Technical error, 100 cells counted.

Fulfills reporting laboratory's criteria for positive effect.

Highest, slightly cytotoxic dose; doses below this concentration (10.2, 5.1, 1.0, 0.5, 0.3, and 0.1) were comparable to the solvent control and, therefore, not presented.



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APPENDIX A  
Materials and Methods

Endosulfan toxicology review

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DOES NOT CONTAIN  
NATIONAL SECURITY INFORMATION (EO 12065)

005064

EPA: 68-02-4225  
DYNAMAC No. 1-050-A3  
April 16, 1986

DATA EVALUATION RECORD

ENDOSULFAN

Mutagenicity--Forward Mutation in Schizosaccharomyces pombe Assay

STUDY IDENTIFICATION: Mellano, D., and Berruto, G. Study of the mutagenic activity in vitro of the compound Endosulfan - technical (code HOE 002671 01 Z097 0003) with Schizosaccharomyces pombe. (Unpublished study No. A29312 prepared by Istituto di Ricerche Biomediche, "Antoine Marxer," Ivrea, Italy, for American Hoechst Corp., Somerville, NJ; dated June 18, 1984.) Accession No. 256128.

APPROVED BY:

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

Signature: I. Cecil Felkner

Date: 4-16-86

005064

1. CHEMICAL: Endosulfan; Thiodan.
2. TEST MATERIAL: Endosulfan technical, code No. HOE 002671 01 Z097 0003, was described by the authors as a "beige, dark brown powder" with a purity of 97.2%.
3. STUDY/ACTION TYPE: Mutagenicity--Forward mutation in Schizosaccharomyces pombe assay.
4. STUDY IDENTIFICATION: Mellano, D., and Berruto, G. Study of the mutagenic activity in vitro of the compound Endosulfan - Technical (code HOE 002671 01 Z097 0003) with Schizosaccharomyces pombe. (Unpublished study No. A29312 prepared by Istituto di Ricerche Biomediche, "Antoine Marxer," Ivrea, Italy, for American Hoechst Corp., Somerville, NJ; dated June 18, 1984.) Accession No. 256128.

5. REVIEWED BY:

Barry R. Scott, Ph.D.  
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Clint Skinner, Ph.D.  
EPA Section Head

Signature: Clint Skinner  
Date: 4-24-86

7. CONCLUSIONS:

A. Under the conditions of this assay, doses of Endosulfan ranging from 62.5 to 500 µg/mL, both in the presence and absence of S9 activation, did not induce a mutagenic effect in Schizosaccharomyces pombe; however, the study cannot be assessed for the following reasons:

1. The mutation frequencies (MFs) of S. pombe with the solvent control both in the presence and absence of S9 activation were lower than published values.<sup>1</sup>
2. Optimal conditions for expression of mutation were not provided. The stationary phase culture was incubated at 32°C for 48 hours; this is in contrast to the recommended incubation at 30°C for 36 hours required to achieve optimum sensitivity.<sup>2</sup>
3. No indication of variability in mutant and survivor plate counts was reported.

B. The study is unacceptable.

8. RECOMMENDATIONS: It is recommended that the repeat assay be performed in a manner consistent with established procedures.<sup>3</sup> Given the biological similarities between S. pombe and Saccharomyces cerevisiae, it is further suggested, in accordance with Zimmermann's recommendations for S. cerevisiae mutation studies,<sup>4</sup> that overall assay sensitivity may be improved if exponentially grown S. pombe cells are used.

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<sup>1</sup> Loprieno, N. Screening of coded carcinogenic/noncarcinogenic chemicals by a forward-mutation system with the yeast Schizosaccharomyces pombe, in: Evaluation of Short-Term Tests for Carcinogens, ed. de Serres, F. J. and Ashby, J., 1981, Elsevier/North Holland, NY, pp. 424-433.

<sup>2</sup> Ibid.

<sup>3</sup> Ibid.

<sup>4</sup> Zimmerman, F. K., Procedures used in the induction of mitotic recombination and mutation in the yeast Saccharomyces cerevisiae, in: Handbook of Mutagenicity Test Procedures, ed. Kilbey, B. J., Legator, M., Nichols, W., and Ramel, C., 1979, Elsevier North-Holland Biomedical Press, NY, pp. 119-134.

Items 9 and 10--see footnote 5.

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11. MATERIALS AND METHODS (PROTOCOLS):

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

1. The test material, Endosulfan-technical, was described by the authors as a "beige, dark brown powder"; the purity was listed as 97.2%. Storage conditions of the test material were not specified.
2. S. pombe double mutant haploid P<sub>1</sub> strain (SP ade 6-60/rad 10-198, h<sup>-</sup>) was obtained from Laboratorio di Mutagenesi e Differenziamento, Pisa, Italy, and was maintained as a permanent stock on silica gel. Cultures for the assay were grown from this stock in 100 mL of liquid yeast media (3% glucose, 0.5% yeast extract, and 0.0075% adenine sulfate), shaken for 48 hours at 32°C, washed, and resuspended in physiological saline.
3. The S9 fraction used for metabolic activation was prepared from the livers of Aroclor-1254-induced male Sprague-Dawley rats. Each batch was assayed for protein content and activity by the Ames test.
4. Cytotoxicity Test: Dose levels were chosen on the basis of a preliminary toxicity test; the procedure was not described.
5. Mutagenicity Test: The maximum dose used for the mutation assay was selected to achieve a survival rate of at least 30%. Four doses of the test material, the negative control, or the positive control (methyl methanesulfonate (MMS) for the non-activated and dimethylnitrosamine (DMNA) for the activated exposure) were added in 0.1-mL volumes to tubes containing 1 mL of  $5.5 \times 10^8$  cells and 2.9 mL phosphate buffer, pH 7.4. Tubes used for activation contained 1.9 mL phosphate buffer and 1 mL of the S9 mix. Tubes containing the reaction components were incubated at 35°C with shaking for 4 hours. The mixtures were diluted and plated for survival (4 plates) and mutation (14 plates). Plates were incubated at 32°C for 4 days. The numbers of white (mutant) and red (wild type) colonies were counted; relative survival and MFs were determined.
6. Evaluation Criteria: No criteria for a positive response, the validity of the assay, or the biological significance of the findings were presented.
7. The data were analyzed by the Chi-square test at  $p < 0.05$ ,  $< 0.01$ , and  $< 0.001$ .

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<sup>5</sup> Only items appropriate to this DER have been included.

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B. Protocol: A protocol was not provided.

12. REPORTED RESULTS:

A. Cytotoxicity Assay: No details were given for the preliminary range-finding experiment.

B. Mutation Assay: Based on the findings of the unreported cytotoxicity assay, the doses selected for the mutagenicity assay were 62.5, 125, 250, and 500 µg/mL for both the assay with and without S9 activation. Without activation these doses resulted in a survival range of 93 to 46%, with activation the survival range was 87 to 74%. Under both conditions of activation, no significant increase in mutation of S. pombe was observed. However, at the highest nonactivated dose a doubling of the MF was reported. Representative results are presented in Table 1.

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. The authors concluded that "up to the concentration of 500 µg/mL the test article Endosulfan-technical did not induce significant increases in gene mutation of Schizosaccharomyces pombe in vitro either in the presence or in the absence of hepatic microsomal enzymes."

B. A quality assurance study was signed and dated April 16, 1984.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

It is our assessment that the study as presented cannot be evaluated for the following reasons:

1. The solvent control MFs in this study ( $0.5 \times 10^{-4}$ , -S9;  $0.3 \times 10^{-4}$ , +S9) were lower than the expected rate.
2. The working stock suspension of S. pombe was incubated for a longer period (48 hours) and at a higher temperature (32°C) than is currently recommended by Loprieno,<sup>6</sup> who incubates working

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<sup>6</sup> Loprieno, pp. 424-433.

<sup>7</sup> Loprieno, pp. 424-433.

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TABLE 1. Representative Results of the *S. pombe* Forward Mutation Assay with Endosulfan

Substance	S9 Activation	Dose (µg/mL)	% Survival <sup>a</sup>	No. of Mutant Colonies <sup>b</sup>	Total Population <sup>b</sup> x 10 <sup>4</sup>	Mutation Frequency <sup>c</sup> x 10 <sup>-4</sup>
<u>Solvent Control</u>						
Dimethylsulfoxide	-		100	2	4.46	0.45
	+		100	2	6.46	0.33
<u>Positive Control</u>						
Methyl methane-sulfonate	-	84.5	57	45	2.54	17.68*
Dimethylnitrosamine	+	375.0	78	16	5.03	3.18*
<u>Test Material</u>						
Endosulfan	-	250 <sup>d</sup>	70	1	3.13	0.32
	+	250	75	2	4.83	0.41
	-	500 <sup>e</sup>	46	2	2.07	0.97
	+	500	75	0	4.87	--

$$\% \text{ Survival} = \frac{\text{Total Population of Test Dose}}{\text{Total Population of Solvent Control}} \times 100$$

Average of 14 replicates for mutant counts; four replicates for total population.

$$\text{Mutation Frequency} = \frac{\text{No. of Mutant Colonies}}{\text{Total Population}}$$

Highest marginally cytotoxic dose; mutation frequencies for 62.5 and 125 µg/mL were similarly not significantly higher than the solvent control.

Highest dose tested; mutation frequency was doubled in the nonactivated assay.

Significant increase in mutation frequency by Chi-square test; p value was not specified.



stock suspensions at 30°C for 36 hours. Therefore, the sensitivity of the cells to detect weak mutagenic activity was questionable. Although it may not be pertinent to S. pombe, Sharp and Perry, in agreement with Zimmerman, have demonstrated that exponential Saccharomyces cerevisiae cells are more responsive to certain mutagens (safrole, auramine, diethylstilbestrol, and n-nitrosomorpholine) than are stationary phase cultures. We are not aware of any studies exploring this possibility in S. pombe; however, the similarities between the two yeast cells suggested to us that increased sensitivity could be achieved if actively growing cultures were used.

3. Loprieno<sup>10</sup> has also shown that allowing colonies to grow 5 days at 32°C and 1 to 2 days at 4°C enhanced the accumulation of red pigment by ade6 colonies, which facilitates the differentiation of complete white and complete red from white and red sector colonies. This approach has to an extent improved assay sensitivity by reducing the fraction of false positives.
4. The presentation of mean values without some indication of the variability (standard deviation) is not an acceptable practice.

The doubling of MF at 500 µg/plate (-S9) must be interpreted with caution. Test material cytotoxicity can cause selective survival, hence it is possible for the mutant population to remain relatively stable and the total population to decrease, which results in test material MFs that are higher than the control MF. It is, therefore, necessary to view increased MF relative to both the mutant population and the percent survival. As shown in Table 1, the increased MF calculated for the 500-µg/mL nonactivated dose was accompanied by a 46% survival; however, the number of mutant colonies (2) was comparable to the solvent control. It appears that this increased MF is not the result of genetic activity, but rather the consequence of selective cytotoxicity. It is our assessment, therefore, that this effect had no genetic significance. However, because of the study deficiencies outlined above, the potential, if any, of Endosulfan to induce a mutagenic effect in this assay was not demonstrated.

Item 15--see footnote 5.

16. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 5-9.

\* Sharp, D. C. and Parry, J. M. Induction of mitotic gene conversion by 41 coded compounds in yeast strain JD1, in: Evaluation of Short-Term Tests for Carcinogens, Elsevier/North Holland, NY, pp. 491-502.

\* Zimmerman, pp. 119-134.

<sup>10</sup> Ibid.

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APPENDIX A  
Materials and Methods

Endosulfan toxicology review

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005084

EPA: 68-02-4225  
DYNAMAC No. 1-50-A2  
April 15, 1986

DATA EVALUATION RECORD

ENDOSULFAN

Mutagenicity--Mitotic Gene Conversion in Saccharomyces cerevisiae D<sub>4</sub>

STUDY IDENTIFICATION: Mellano, D. and Berruto, G. Study of the mutagenic activity of the compound Endosulfan-technical (code HOE 002671 01 ZD97 0003) with Saccharomyces cerevisiae. (Unpublished study No. A29313 prepared by Istituto Di Ricerche Biomediche, "Antoine Marxer," Ivrea, Italy; dated June 18, 1984.) Accession No. 256128, experiment No. M707.

APPROVED BY:

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

Signature:

I. Cecil Felkner

Date:

4-16-86

005084

1. CHEMICAL: Endosulfan; Thiodan.
2. TEST MATERIAL: Endosulfan technical, code No. Hoe 002671 01 Z097 0003, was described as a "beige dark brown powder" with a purity of 97.2%, certificate of analysis No. 02184.
3. STUDY/ACTION TYPE: Mutagenicity--Mitotic gene conversion in Saccharomyces cerevisiae D4.
4. STUDY IDENTIFICATION: Mellano, D. and Berruto, G. Study of the mutagenic activity of the compound Endosulfan-technical (code HOE 002671 01 Z097 0003) with Saccharomyces cerevisiae. (Unpublished study No. A29313 prepared by Istituto Di Ricerche Biomediche, "Antoine Marxer," Ivrea, Italy; dated June 18, 1984.) Accession No. 256128, experiment No. M707.

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EPA Reviewer

Signature: M. L. Jones

Date: 4-18-86

Clint Skinner, Ph.D.  
EPA Section Head

Signature: W. L. Skinner

Date: 4-24-86

7. CONCLUSIONS:

A. Under the conditions of the Saccharomyces cerevisiae D<sub>4</sub> mitotic gene conversion assay, Endosulfan at 100, 500, 1000, and 5000 µg/mL, both in the presence and absence of S9 activation, did not cause an appreciable increase in tryptophan or adenine convertants. However, the assay was performed with stationary phase cultures; hence, this physiological state may have compromised the sensitivity of the assay.

B. The assay is unacceptable.

8. RECOMMENDATIONS: It is recommended that the assay be repeated using logarithmic phase cells.

Items 9 and 10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):

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

1. Test Material: Endosulfan-technical was described by the authors as "beige dark brown powder"; the purity was listed as 97.2%. Storage conditions of the test material were not specified. The test material (400 mg) was dissolved in dimethylsulfoxide (DMSO).

2. Microbial Strain:

a. Strain Description/Source: S. cerevisiae diploid strain D<sub>4</sub> was obtained from Laboratorio di Mutagenesi e Differenziamento, Pisa, Italy, and was maintained as permanent silica gel stocks.

b. Stock Culture Preparation/Maintenance: Stock cultures of S. cerevisiae were generated from silica gel stocks and prescreened for low background and for gene conversion frequencies. Silica granules were inoculated into 100 mL of liquid growth media (2% glucose, 1% yeast extract, 2% peptone, 0.004% adenine sulphate, and 0.004% tryptophan) and shaken for 16 hours at 32°C.

Following incubation, 0.05 mL of a 10<sup>-5</sup> dilution of the preculture(s) was inoculated into 10 mL of fresh growth medium and incubated for 48 hours in a 32°C, shaking water bath.

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

The viable cell population was determined by plating a  $10^{-5}$  dilution of the resulting culture(s) onto complete medium (2% glucose, 1% yeast extract, 2% peptone, and 1.5% agar). The number of background convertants was assessed by plating a  $10^{-4}$  dilution onto selective medium (2.22% MM82, Biolife, and 1.5% agar supplemented with either 0.004% adenine sulphate or tryptophan). After 3 to 4 days of incubation at 32°C, the ratio of total colonies to adenine and tryptophan convertants was determined. The culture yielding the lowest convertant frequency (CF) for both genes was selected as the stock culture, and it was stored at 4°C until use in the assay.

- c. Cell Preparation for Gene Conversion Assay: A 0.3-ml aliquot of the refrigerated stock suspension was added to 100 mL of liquid growth medium, incubated for 16 hours in a shaking, 32°C water bath, centrifuged, and resuspended in saline. Cell density was determined with a hemocytometer, and the suspension was adjusted to contain  $5 \times 10^8$  cells/mL.
3. The S9 fraction used for metabolic activation was prepared from the livers of Aroclor 1254-induced male Sprague-Dawley rats. Each batch was assayed for protein content and activity by the Ames test using 2-aminofluorene and Salmonella typhimurium strains TA1538, TA98, and TA100.
4. Cytotoxicity Test: Dose levels were chosen on the basis of a preliminary cytotoxicity test; the procedure was not described.
5. Mutagenicity Test: The maximum dose was chosen on the basis of achieving a survival rate of at least 50%. Four doses of the test material, the negative control (DMSO), or the positive controls (84.5 µg/mL methyl methanesulfonate (MMS) for the nonactivated exposure and 259 µg/mL cyclophosphamide (CP) for the S9-activated exposure) in 0.1-ml volumes were added to tubes containing 1 mL of  $5 \times 10^8$  cells and 2.9 mL phosphate buffer, pH 7.4. Tubes used for metabolic activation contained 1.0 mL S9 mix and 1.9 mL phosphate buffer. These tubes were incubated at 35°C with shaking. After 4 hours, the mixtures were diluted as described in Section 11.A.2.b and plated for viability (four plates) and convertants (four selective medium plates containing adenine sulphate and four selective medium plates containing tryptophan). Plates were incubated at 32°C for 4 days. The number of survivors, tryptophan convertants, and adenine convertants were counted; relative survival and CFs were determined.

6. Statistical Methods: The data were analyzed by the Chi-square test at  $p < 0.05$ ,  $< 0.01$ , and  $< 0.001$ .

7. Evaluation Criteria: No criteria for a positive response, the validity of the assay, or the biological significance of the findings were presented.

B. Protocol: A protocol was not provided.

## 12. REPORTED RESULTS:

A. Cytotoxicity Assay: No details were reported for the preliminary range-finding experiments.

B. Mitotic Gene Conversion Assay: Based on the findings of the unreported cytotoxicity assay, the doses selected for the S9-activated and nonactivated mitotic gene conversion assay were 100, 500, 1000, and 5000  $\mu\text{g/mL}$ . Without activation, 52% of the yeast cells survived treatment with 5000  $\mu\text{g/mL}$  of the test material. Below this concentration >80% of the cells survived and survival increased as the concentration range of test doses decreased. No appreciable increase in mutation at either the ade2 or trp5 loci resulted from exposure to the four nonactivated doses, and no significant increase in CF was observed.

In the presence of S9 activation, no cytotoxicity was apparent at any dose; tryptophan and adenine revertants were comparable to the solvent control, and no statistically significant increases in CFs were calculated. Representative results from the non-activated and S9-activated mitotic gene conversion assay are presented in Table 1. It should be noted in Table 1 that a discrepancy between the reported dose of the S9-activated positive control, CP (375  $\mu\text{g/mL}$ ) used in the assay and the level stated in the Materials and Methods section of this report (259  $\mu\text{g/mL}$ ) was uncovered.

## 13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. The authors concluded that, "Up to the concentration of 5000  $\mu\text{g/mL}$  the test article Endosulfan - Technical did not induce a significant increase in gene conversion in the Saccharomyces cerevisiae strain in vitro either in the presence or in the absence of hepatic microsomal enzymes."

B. A quality assurance statement was signed and dated June 15, 1984.

## 14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

A. We assess that the authors interpreted the data correctly. However, the assay was performed with working stock suspensions of stationary phase cultures. To improve assay sensitivity,



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TABLE 1. Representative Results of the *S. cerevisiae* D<sub>4</sub> Mitotic Gene Conversion Assay with Endosulfan

Substance	S9 Activation	Dose (µg/mL)	Total Survivors x10 <sup>6</sup> <sup>a</sup>	% Survival <sup>b</sup>	Convertants of <i>S. cerevisiae</i> (x10 <sup>1</sup> ) <sup>a</sup>		Convertant Frequency (x10 <sup>-5</sup> ) <sup>c</sup>	
					Trp <sup>+</sup>	Ade <sup>+</sup>	Trp <sup>+</sup>	Ade <sup>+</sup>
<u>Negative Control</u>								
Dimethylsulfoxide	-	27,500	9.4	100	13.5	16.5	1.4	1.8
	+	27,500	11.5	100	13.5	16.0	1.2	1.4
<u>Positive Control</u>								
Methyl methane-sulfonate	-	84.5	11.1	>100	98.8	87.8	8.9***	7.9***
Cyclophosphamide	+	259.0 (375) <sup>d</sup>	11.2	97.4	45.0	49.8	4.0***	4.4***
<u>Test Material</u>								
Endosulfan	-	5000 <sup>e</sup>	4.9	52.1	5.3	5.3	1.1	1.1
	+		12.5	>100	15.0	17.8	1.2	1.4

Average of quadruplicate plates.

$$\text{Survival} = \frac{\text{Total survivors with test dose}}{\text{Total survivors with solvent control}} \times 100$$

$$\text{Convertant Frequency} = \frac{\text{Total number of convertants (tryptophan or adenine)}}{\text{Total number of survivors}}$$

Notes: Materials and Methods 1.535 75.0 µg/mL as positive control dose, however tabular presentation of data indicates a dose of 375 µg/mL.

greatest dose assayed; values for lower doses (1000, 500, and 100) were comparable to the control results and were, therefore, not selected as representative.

Significantly higher than control value ( $p < 0.001$ ) by  $\chi^2$  test.

Zimmermann et al.<sup>2</sup> have recommended the use of actively growing phase cells. Although a different S. cerevisiae strain was used, Sharp and Parry,<sup>3</sup> in agreement with Zimmermann et al.,<sup>4</sup> have demonstrated that positive results with the known carcinogens/mutagens, safrole, auramine, diethylstilbestrol, and n-nitrosomorpholine, were only achieved after treatment of logarithmic phase cells.

Although the authors demonstrated the sensitivity of S. cerevisiae D<sub>4</sub> for detecting the mutagenic effect of the positive controls (84.5 µg/mL MMS/-S9 and 259 or 375 µg/mL CP/+S9), the use of stationary phase cultures compromised the overall sensitivity of the test system to detect weak mutagenic activity. This is of paramount concern in light of the statistically significant increase in gene conversion of S. cerevisiae reported by Yadav et al.<sup>5</sup>

Item 15--see footnote 1.

16. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 4-10.

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<sup>2</sup> Zimmermann, F. K., von Borstel, R. C., von Halle, E. S., Parry, J. M., Siebert, D., Zetterberg, G., Barale, R., and Loprieno, N. Testing of chemicals for genetic activity with Saccharomyces cerevisiae: A Report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutat. Res. 133(1984): 199-244.

<sup>3</sup> Sharp, D.C. and Parry, J. M. Induction of mitotic gene conversion by 41 coded compounds in yeast strain JDI, in: Evaluation of Short-Term Tests for Carcinogens (New York, NY: Elsevier/North Holland, Vol. 1, 1981), pp. 491-501.

<sup>4</sup> Zimmerman et al., pp. 199-244.

<sup>5</sup> Yadav et al. Mutat. Res. 105(1982): 403-407.

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APPENDIX A  
Materials and Methods

Endosulfan toxicology review

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Pages 42 through 48 are not included in this copy.

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TABLE 2. (Continued)

Test Species	Exposure Level	Test Duration	Route and Exposure Medium	Quality Assurance Specifications	Miscellaneous Observed Effects	Reference
House	NOEL 2.0 and 3.9 ppm	78 wks	Oral	NR(b)	No carcinogenic effects	NCI, 1978
Rabbit	LD50 147-359 mg/kg LD50 360 mg/kg	--	Dermal Percutaneous	NR	--	Gupta and Gupta, 1979
Guinea Pig	LD50 1,000 mg/kg	--	Dermal	NR	--	Gupta and Gupta, 1979
Dog	LD50 30 mg/kg	--	Oral	NR	--	Gupta and Gupta, 1979
Dog	NOEL(c) 3 ppm	2 yr	Oral in diet	NR	--	Goebel et al., 1982
Drosophila melanogaster	100 ppm	48 hr	Oral	NR	Increase in percent of sex-linked recessive lethal genes in animals dosed at first instar larval stage.	Velazquez et al., 1984
Drosophila melanogaster	200 ppm	48 hr	Oral	NR	Increased sex-linked recessive lethal genes in 2-day-old adult.	Velazquez et al., 1984
Salmonella sp. (bacteria)	--	--	Nutrient medium	NR	No mutagenicity	Quinto et al., 1981
Saccharomyces cerevisiae (fungus)	--	10-30 min	Solution	Dilution method; replicates	Significant mutation, gene conversion, and chromosome breakage at 1% endosulfan (weight/volume).	Yadav et al., 1982

(a) LD50 = Lethal dose for 50% of test organisms (unless otherwise noted).

(b) NR = Not reported in source document.

(c) NOEL = No observed effect level.

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EPA: 68-02-4225  
DYNAMAC No. 1-50-A1  
April 16, 1986

DATA EVALUATION RECORD

ENDOSULFAN

Mutagenicity--Micronucleus Test in Mice

STUDY IDENTIFICATION: Jung, Weigand, and Kramer. Micronucleus test in male and female NMRI mice following oral administration of HOE 002671, active ingredient technical (HOE 002671 OI ZD97 0003). (Unpublished study No. A29689 prepared by Hoechst Aktiengesellschaft Pharma Forschung Toxikologie, Frankfurt, Federal Republic of Germany, for American Hoechst Corp., Somerville, NJ; dated October 3, 1983.) Accession No. 256128.

APPROVED BY:

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

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

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

*I. Cecil Felkner*  
*4-16-86*

1. CHEMICAL: Endosulfan; HOE 002671; thiodan.
2. TEST MATERIAL: Hoe 002671 substance technical, code No. Hoe 002671 01 ZD97 0003, was described as light brown flakes with a purity of 97.2%.
3. STUDY/ACTION TYPE: Mutagenicity--Micronucleus test in mice.
4. STUDY IDENTIFICATION: Jung, Weigand, and Kramer. Micronucleus test in male and female NMRI mice following oral administration of HOE 002671, active ingredient technical (HOE 002671 01 ZD97 0003). (Unpublished study No. A29689 prepared by Hoechst Aktiengesellschaft Pharma Forschung Toxikologie, Frankfurt, Federal Republic of Germany, for American Hoechst Corp., Somerville, NJ; dated October 3, 1983.) Accession No. 256128.

5. REVIEWED BY:

Barry R. Scott, Ph.D.  
Principal Reviewer  
Dynamac Corporation

Signature: Barry R. Scott  
Date: 4-15-86

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

Signature: Nancy E. McCarroll  
Date: 4-15-86

6. APPROVED BY:

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

Signature: J. Cecil Felkner  
Date: 4-16-86

Margaret Jones  
EPA Reviewer

Signature: Margaret Jones  
Date: 4-18-86

Clint Skinner, Ph.D.  
EPA Section Head

Signature: Clint Skinner  
Date: 4-24-86

7. CONCLUSIONS:

A. Under the conditions of the assay, 0.2, 1.0, and 5.0 mg/kg of HOE 002671 substance technical, administered twice orally at an interval of 24 hours to male and female NMRI mice, did not significantly increase the frequency of micronuclei in polychromatic erythrocytes (PCE) collected 6 hours following the second test substance administration. However, no conclusion can be made regarding the clastogenic potential of the compound for the following reasons:

1. Neither a toxic effect in the animals or a cytotoxic effect on bone marrow cells was demonstrated at the highest dose tested.
2. The sampling intervals were insufficient to assess the effects, if any, of the test material on the entire hematopoietic cycle.

B. The study is unacceptable.

8. RECOMMENDATIONS:

It is suggested that the assay be repeated with a dose level that elicits a toxic response in both males and females and/or a cytotoxic effect in the target cell, a low nontoxic dose, and an intermediate dose. It is further recommended that the repeated assay be performed using multiple sampling intervals (24, 48, and 72 hours postexposure to a single administration of the three doses) to ensure that cells are exposed to the test material during the entire hematopoietic cycle and that compound effects related to mitotic delay are adequately assessed.

Items 9 through 10--see footnote 1.

11. MATERIALS AND METHODS (PROTOCOLS):

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

1. Test Material: HOE 002671, active ingredient technical, was described as light brown flakes; the purity was listed as 97.2%. The test material was stored in the dark at approximately 4°C, and thoroughly mixed suspensions were prepared in sesame oil on each day of use.
2. Test Animals: Male and female NMRI mice, of unknown weight and age, were used for this study. The mouse strain, Hoe: NMRKF (SPF71), was obtained from Hoechst AG, Kastengrund, SPF breeding colony.



3. Animal Maintenance: The animals were acclimatized for at least 5 days prior to the start of the study and were housed five to a Makarolon cage (Type 3) in fully air-conditioned rooms. The light was maintained on a 12-hour diurnal cycle, the temperature was controlled at  $22 \pm 2^\circ\text{C}$ , and the relative humidity was kept at  $55 \pm 10\%$ . Throughout the study, food (Atromin GmbH, Lage/Lippe) and water were provided ad libitum.
4. Assignment to Groups: Following acclimatization, animals were randomized, assigned to groups, and identified by  $\text{KMnO}_4$  marking on fur and cage numbering.
5. Test Compound Administration: The selected doses of the test material, solvent, and positive controls were administered via oral gavage. The volume of each dose administered was 10 mL/kg of body weight.
6. Preliminary Toxicity Study: Based on the results of a preliminary study, 5 mg was established as the maximum tolerated dose (MTD); however, no data were reported.
7. Micronucleus Test:
  - a. Test Animals and Compound Administration: Ten mice (five males and five females) per dose were administered the selected concentrations of the test material (0.2, 1.0, and 5.0 mg/kg), vehicle (sesame oil), or positive control (cyclophosphamide, 100 mg/kg) in two single applications separated by a 24-hour interval.
  - b. Animal Sacrifice/Bone Marrow Harvest: Six hours after the second administration of the test chemical, vehicle, or positive control, the animals were sacrificed by  $\text{CO}_2$  asphyxiation. Bone marrow cells were collected from both femurs by aspiration into fetal bovine serum. The mixtures were centrifuged, and most of the supernatant was discarded. One drop of the thoroughly mixed sediment was spread onto a clean slide and fan dried. Prepared slides were stained (May-Grünwald and Giemsa), mounted, and coded.
  - c. Slide Analysis: Two thousand PCEs were counted per animal. The number of cells with micronuclei, not the number of micronuclei, was recorded. As a control measure, 1000 mature erythrocytes, in the same field, were also scored for micronuclei. The ratio of PCE to normochromatic erythrocytes (NCE) was determined.

8. Evaluation Criteria: No criteria for a positive response, the validity of the assay, or significance of the biological findings were presented.

9. Statistical Analysis: The number of PCEs and NCEs with micronuclei was evaluated statistically ( $p < 0.05$ ) using the methods of binomial increase; the ratio of PCEs to NCEs was evaluated by the methods of Neményi. A reference was not furnished for the Neményi method.

B. Protocol: A protocol was not presented.

12. REPORTED RESULTS:

Micronucleus Assay: Based on the unreported findings of the preliminary toxicology study, three doses were chosen (0.2, 1.0, and 5 mg/kg) for the micronucleus assay. No evidence of a toxic effect was observed at any dose level. No increase in the number of PCE or NCE with micronuclei, compared to the numbers observed in the controls, was detected for either sex. At all concentrations of the test material, the ratio of PCEs to NCEs was comparable to the controls. Cyclophosphamide induced a marked increase in the number of PCEs compared to the control values in both male and female mice; the ratio of NCE to PCE in both sexes was shifted in favor of the mature cells. Representative results are shown in Table 1.

13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

A. The authors concluded that "the results indicate that HOE 002671 OI ZD97 0003 produces no chromosome mutations under the condition of the present study."

B. An unsigned quality assurance statement was dated October 3, 1983.

14. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS:

It is our assessment that the authors' interpretation of the data was correct. However, the lack of a toxic effect at the reported MTD suggested that the MTD was not achieved. Without some evidence of animal toxicity or cytotoxicity (i.e. bone marrow depression), we are unable to determine if the dose range selected was adequate to evaluate the clastogenic potential of HOE 002671. Although the sampling rationale (30 hours after the first compound treatment) conforms with

TABLE 1. Representative Results of the Micronucleus Assay in Mice with HOE 002671

Substance	Total Dose (mg/kg)	No. of Animals Analyzed per Group <sup>a</sup>	No. of PCE <sup>b</sup> Analyzed per Group	Total No. of MPE per Group <sup>c</sup>	Percent MPE per Group <sup>c</sup>	Ave Gro PCE
<u>Vehicle Control</u>						
Sesame oil	--	10	20,000	62	0.3	859 (0)
<u>Positive Control</u>						
Cyclophosphamide	100	10	20,000	1134	5.7*	625 (0)
<u>Test Material<sup>d</sup></u>						
Hoe 33171	5	10	20,000	51	0.3	386 (0)

<sup>a</sup> Five males and five females per treatment; no sex-related effect, therefore, results both sexes were combined.

<sup>b</sup> PCE = Polychromatic erythrocytes.

MPE = Micronucleated polychromatic erythrocytes.

NCE = Normochromatic erythrocytes.

<sup>c</sup> Tabulated by our reviewers from individual animal data.

<sup>d</sup> Similar results were obtained at the low (0.2 mg/kg) and mid (1.0 mg/kg) doses; therefore, the data from high (5 mg/kg) dose was selected as representative.

\*Significantly different from control value by binomial increase.

the methods of Schmid,<sup>2</sup> Salamone et al.<sup>3</sup> have shown that for certain compounds, the maximum frequency of PCEs with micronuclei may occur much later than 30 hours. Since PCEs have a lifespan of approximately 24 hours, it is recommended that sampling at 24, 48, and 72 hours be performed to ensure that the intervals over which maximum frequencies of micronuclei are known to occur are evaluated.<sup>4</sup>

The ability of the assay to detect genotoxicity 30 hours following the first compound administration or 6 hours after the second exposure was demonstrated using 100 mg/kg cyclophosphamide, the positive control.

Item 15--see footnote 1.

16. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp 4-8.

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<sup>2</sup> Schmid, W. The micronucleus test, in: Handbook of Mutagenicity Test Procedures, ed. B. Kilbey (New York: Elsevier/North-Holland, Inc., 1979), pp. 235-242.

<sup>3</sup> Salamone, M. J., Heddle, J. A., Stuart E., and Kate, M. Towards an improved micronucleus test: Studies on three model agents, mitomycin C, cyclophosphamide and dimethylbenzanthracene, Mutation Research 74 (1980): 347-356.

<sup>4</sup> Heddle, J. A., Hite, M., Kirthart, 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, Mutation Research 123 (1983):61-118.

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APPENDIX A  
Materials and Methods

Endosulfan toxicology review

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EPA: 68-02-4225  
DYNAMAC No. 1-050-A5  
April 10, 1986

DATA EVALUATION RECORD

ENDOSULFAN

Mutagenicity--Mouse Lymphoma Mutation Assay

STUDY IDENTIFICATION: Cifone, M. A., and Fisher, J. A. Mutagenicity evaluation of HOE 002671-substance technical in the mouse lymphoma forward mutation assay. (Unpublished study No. A29801 prepared by Litton Bionetics, Inc., Kensington, MD, for American Hoechst Corporation, Somerville, NJ; dated November 1984.) Accession No. 256128.

APPROVED BY:

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

Signature: I. Cecil Felkner

Date: 4-16-86

005084

1. CHEMICAL: Endosulfan; HOE 002671.
2. TEST MATERIAL: HOE 002671-substance technical, code No. HOE 002671 01 ZD97 0003, was described as tan granules with a purity of 97.2%.
3. STUDY/ACTION TYPE: Mutagenicity--mouse lymphoma mutation assay.
4. STUDY IDENTIFICATION: Cifone, M. A., and Fisher, J. A. Mutagenicity evaluation of HOE 002671-substance technical in the mouse lymphoma forward mutation assay. (Unpublished study No. A29801 prepared by Litton Bionetics, Inc., Kensington, MD, for American Hoechst Corporation, Somerville, NJ; dated November 1984.), Accession No. 256128.

5. REVIEWED BY:

Barry R. Scott, Ph.D.  
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Dynamac Corporation

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Date: 4-11-86

Brenda Worthy, M.T.  
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Signature: Brenda Worthy

Date: 4-10-86

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Signature: I. Cecil Felkner

Date: 4-11-86

Margaret Jones  
EPA Reviewer

Signature: Margaret Jones

Date: 4/11/86

Alan Katz, Ph.D.  
EPA Section Head

Signature: Alan Katz

Date: 4-24-86



7. CONCLUSIONS:

A. Under the conditions of the assay, six doses of HOE 002671-substance technical ranging from 6.25 to 50 µg/mL without S9 activation and seven doses from 6.25 to 100 µg/mL with S9 activation induced no significant increases in mutations at the thymidine kinase (TK) locus in L5178Y mouse lymphoma. Although an increase was detected at a single dose of 100 µg/mL in the S9-activated system, the response was considered to be spurious because of a low cloning efficiency associated with a very high toxicity (8.6% relative growth). The positive control 3-methylcholanthrene (MCA) at doses of 2.5 and 4 µg/mL clearly demonstrated the sensitivity of the S9-activated system to detect a mutagenic response at doses comparable to those of the test material. The positive control ethylmethanesulfonate (EMS) at 0.25 and 0.4 µL/mL, although used at doses well above those of the test material, demonstrated the capability of the assay to detect a mutagenic response. The test material is, therefore, considered to be nonmutagenic in the mouse lymphoma forward mutation assay.

B. The study is acceptable.

8. RECOMMENDATIONS:

Although the nonactivated positive control EMS gave a positive response at 0.25 and 0.40 µL/mL, these concentrations are (approximately 250 and 400 µg/mL, respectively) well above the range used to assay the nonactivated test material, i.e., 6.25 to 75.0 µg/mL. We therefore strongly recommend the use of a positive control at a concentration comparable to that of the test material. For the mouse lymphoma or Chinese hamster ovary assays, either nitrosoguanidine or methylmethanesulfonate should be more suitable and would be expected to induce a significant mutagenic response well within the range used for the test material in this assay.

Items 9 and 10--see footnote 1.

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

## 11. MATERIALS AND METHODS (PROTOCOLS):

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### A. Materials and Methods:

1. The test material, HOE 002671-substance technical, was described as tan granules; the purity was listed as 97.2%. Storage conditions of the test material were unspecified. A clear, pale yellow solution of 100 mg/mL test material was prepared in dimethylsulfoxide (DMSO) on the day of use. Just prior to each assay, stock solutions were prepared by serial dilutions in DMSO. This test material was assayed at pH 7.0-7.4.
2. The L5178Y TK +/- mouse lymphoma cells, clone 3.7.2C, were derived from the Fischer L5178Y line of Dr. Donald Clive. The stocks were maintained, grown, and cleansed as described by Clive and Spector.<sup>2</sup>
3. The S9 homogenate used for the metabolic activation was prepared from the livers of adult male rats (strain not specified) treated with Aroclor 1254. Each batch was checked for sterility, aryl hydrocarbon hydroxylase (AHH) activity, and protein content.
4. Cytotoxicity: Cytotoxicity was determined from the reduction in cell population after dosing with the test material. The assay was performed with and without S9 activation. After 4 hours of exposure at 37°C the cells were washed, resuspended in growth medium, and incubated for 24 hours. Cells were then counted to determine reduction in growth relative to the solvent control.
5. Mutagenicity Assay: Logarithmically growing laboratory cultures were seeded into a series of tubes at a density of  $6 \times 10^6$  cells per tube. Thirteen tubes received various doses of the test material; an S9 mixture was added to six of these tubes. Additional tubes were also prepared; these were used for the solvent and positive control groups.

All tubes were incubated with shaking at 37°C for 4 hours. After exposure the cells were washed, resuspended in fresh growth medium, and incubated for 2 days. During this period, the cell density was adjusted to  $3 \times 10^5$  cells/mL to obtain optimal growth. Cells exposed to the various doses of test material, in the presence and absence of S9, were then selected for cloning. For estimation of the number of mutants, cells exposed to each dose of the chemical and solvent control exposures were plated in triplicate in selective

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<sup>2</sup> Clive, D., and J. Spector, Laboratory procedures for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells, Mutation Research 31(1975): 17-29.

medium containing 5-trifluorothymidine (TFT) to yield  $1 \times 10^6$  cells/plate. The cloning efficiency was determined by plating at a dilution estimated to contain 200 cells from each test dose and controls in nonselective medium.

After 10 to 14 days of incubation, the colonies were counted with an electronic colony counter. The mutant frequency was calculated by dividing the total number of colonies in each set of three mutant selection dishes by the corresponding viable counts and multiplying by  $2 \times 10^{-4}$ . The measurement of toxicity of each treatment is the relative suspension growth of the cells over the 2-day expression period multiplied by the cloning efficiency relative to the solvent control. This is not strictly a measure of cell survival, but provides a measure for the effectiveness of the treatment.

6. Assay Evaluation Criteria: A number of criteria were established so that an assay would be normally considered acceptable for evaluation (see Appendix A). These requirements include (a) the range of absolute cloning efficiency of the negative control (60-130%); (b) the suspension growth of the negative control should have a minimum value of 8 for the 2-day expression period; (c) the background mutation frequency for the negative control with and without activation should be within the range  $10 \times 10^{-6}$  to  $110 \times 10^{-6}$ ; (d) the minimum mutation frequencies for the positive controls, 0.25 and 0.4  $\mu\text{L/mL}$  EMS or 2.5 and 4  $\mu\text{g/mL}$  MCA, is  $200 \times 10^{-6}$ ; (e) the test material should reduce the relative growth 10-20% compared to the solvent control; (f) the experimental frequency of the cloning efficiency should exceed 10%; and (g) the total number of viable clones should exceed 60.
7. Evaluation Criteria: The minimum criteria necessary for demonstrating mutagenesis was a frequency of  $10^{-5}$  or more and at least a 1.5-fold increase in total mutants relative to the concurrent background. The background frequency was defined as the average mutagenic frequency of the solvent control. In addition, a dose-related or toxic-related increase in mutant frequency should be demonstrated.

For the assay to be regarded as negative a minimum increase in mutant frequency was not observed for (a) a range of concentrations that restricts the relative growth 10-20% and (b) a range of applied concentrations that extends to the maximum of 5 mg/mL (5  $\mu\text{L/mL}$ ) unless limited by solubility.

B. Protocol: See Appendix A.

## 12. REPORTED RESULTS:

- A. Cytotoxicity Assay: In the preliminary cytotoxicity assay the test material was lethal with or without metabolic activation at 250  $\mu\text{g/mL}$ .

- B. Mutagenicity Assay: Based on the cytotoxicity assay, 13 doses of test material were assayed for mutation induction. Under nonactivation conditions, six concentrations (6.25-50 µg/mL) were assayed in duplicate. Low to moderate toxicities were observed, 99.3-32.1% relative growth, which corresponds to induced mutation frequencies ranging from  $28.9 \times 10^{-6}$  to  $41.6 \times 10^{-6}$ . It was not possible with this test material to include highly toxic exposures as small increases in concentration (50-70 µg/mL) were lethal. Representative results are presented in Table 1.

In the presence of metabolic activation, exposure to concentrations ranging from 6.25-100 µg/mL induced a wide range of toxicities (relative growth, 85.4-8.6%). The range for the induced mutation frequency was  $60.3 \times 10^{-6}$  to  $38 \times 10^{-6}$ . Only one spurious exposure (100 µg/mL) induced a mutation frequency that exceeded the minimum criteria for mutagenesis. This was caused by a sharp decrease in cloning efficiency because there was a very high toxicity (8.6% relative growth) rather than an increase in total mutant colonies. Representative results are presented in Table 2.

- C. Cloning Efficiencies: In the assays conducted, the average cloning efficiencies for the solvent controls varied from 88.1% without activation to 97.2% with activation.

### 13. STUDY AUTHORS' CONCLUSIONS/QUALITY ASSURANCE MEASURES:

- A. The authors concluded that "The test material HOE 002671 - substance technical, did not induce significant increases in the mutation frequency at the TK locus in L5178Y TK +/- cells" and "therefore is considered inactive with and without metabolic activation in the Mouse Lymphoma Forward Mutation Assay."
- B. A quality assurance statement from the laboratory performing the study was signed and dated November 9, 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 presented was correct.

As shown in Tables 1 and 2, none of the doses employed induced a significant increase in the mutation frequency at the TK locus in the mouse lymphoma forward mutation assay. However, the data does demonstrate a satisfactory reduction in the relative growth rate in the absence (73.9-44.7%) and presence (75.8-18.8%) of metabolic activation. This may indicate that the test substance was entering the lymphoma cells and that the lack of response of this system was not due to the inability of HOE 002671 to penetrate the cell wall.

TABLE 1. Representative Results from the Mouse Lymphoma Forward Mutation Assay  
Treated with HOE 002671 in the Absence of S9 Activation

Substance	Dose	Total Mutant Counts	Total Viable Colonies	Cloning Efficiency (%)	Relative Growth (%)	Mutant Frequency $\times 10^{-6}$	Fc Inc
<u>Solvent Control</u>							
Dimethylsulfoxide	--	89	567	94.5	100	31.4	
<u>Positive Control</u>							
Ethylmethanesul- fonate ( $\mu$ L/mL)	0.25	813	233	38.8	20.2	697.8	
	0.40	739	234	39.0	20.4	631.6	
<u>Test Material</u>							
HOE 002671 ( $\mu$ g/mL)	6.25	82	471	89.1	73.9	34.8	
	12.50	73	505	95.5	99.3	28.9	
	18.80	84	557	105.4	69.8	30.2	
	25.0	92	564	106.7	86.9	32.6	
	37.5	65	418	79.1	44.7	31.1	
	50.0	112	538	101.8	32.1	41.6	
	75.0	Excessive Toxicity					

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TABLE 2. Representative Results from the Mouse Lymphoma Forward Mutation Assay Treated with HOE 002671 in the Presence of S9 Activation

Substance	Dose ( $\mu\text{g/mL}$ )	Total Mutant Counts	Total Viable Colonies	Cloning Efficiency (%)	Relative Growth (%)	Mutant Frequency $\times 10^{-6}$	Fold Increase
<u>Solvent Control</u>							
Dimethyl- sulfoxide	--	123	609	101.5	100	40.4	--
<u>Positive Control</u>							
3-Methyl- cholanthrene	2.5	668	432	72.0	51.9	309.2	7.7
	4.0	664	343	57.2	17.5	387.2	9.6
<u>Test Compound</u>							
HOE 002671	6.25	101	335	57.4	50.3	60.3	1.49
	12.50	69	363	62.2	60.6	38.0	0.94
	25.0	143	493	84.5	75.8	58.0	1.44
	50.0	121	459	78.7	67.4	52.7	1.30
	75.0	118	396	67.9	35.7	59.6	1.48
	100.0 <sup>a</sup>	113/131	408/274	70/47	18.8/8.6	55.4/95.6 <sup>b</sup>	1.37/2.40

<sup>a</sup>n/n = Results presented from duplicate cultures for the 100- $\mu\text{g/mL}$  dose only.

<sup>b</sup>The apparent increase in mutation frequency was caused by a decrease in the cloning efficiency due to cytotoxicity (8.6% relative growth). This increase was not confirmed in the duplicate culture.

The positive response from the 100- $\mu$ g/mL dose with activation was thought by the study authors to be spurious for two reasons: (1) the duplicate culture at this dose gave half the number of mutants and a mutation frequency that was negative by the evaluation criteria and (2) the response could be explained by the low cloning efficiency. Moreover, we assess that this exposure, unlike all others in the study, should be rejected on the basis that it did not meet the assay evaluation criteria (see section 11A6).

Data presented in Tables 1 and 2 also show that the response to the positive controls, EMS and MCA, as well as the spontaneous mutation frequency of the solvent control (negative control) are within acceptable ranges. However, EMS at the doses used in the nonactivated assay was well above the dose range of the test material. We assess, therefore, that although the study authors were able to produce mutagenicity with EMS, they did not demonstrate the sensitivity of the assay to detect a mutagenic response at doses comparable to that of the test material.

Item 15--see footnote 1.

16. CBI APPENDIX: Appendix A, Protocol, CBI pp. 6-16.

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APPENDIX A  
Protocol



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APPENDIX A  
Protocol

Endosulfan toxicology review

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percent in the other group. There was also a ten-fold increase in large atria in one group not reported.

As a result of these discrepancies and problems, this study is considered invalid and cannot be used to support the safety of endosulfan with respect to the teratogenic potential.

Gupta et al. (1978, MRID 05003227) investigated the teratogenic and embryotoxic effects of endosulfan in rats. One female rat died in the 5 mg/kg dose group and five females died in the 10 mg/kg group. At the high dose (10 mg/kg), there was a significant increase in the number of litters with resorptions and in litters with skeletal abnormalities. Since no raw data were presented in this study, it cannot be considered a reliable assessment for the teratogenic potential of endosulfan.

In a teratogenic study in the rat, a number of skeletal, visceral, and external anomalies as well as significant reductions in size and weight were reported in fetuses of the high (6 mg/kg) treatment group (Raltech Scientific Service, 1981, MRID GS014008). However, at this dose level, maternal toxicity was evident as manifested by decreased body weight and decreased body weight gain, and clinical observations indicating central nervous system stimulation. The NOEL for fetotoxicity is considered by the authors to be 2 mg/kg.

In another study (Raltech Scientific Services, 1981, MRID GS014023) endosulfan was orally administered to groups of pregnant rabbits at the rate of 0.3, 0.7, or 1.8 mg/kg/day on days 6 to 28 of gestation. Animals were sacrificed on day 29 of gestation. Maternal toxicity was evident in the 1.8 mg/kg group as manifested by noisy and rapid breathing, hyperactivity, convulsions and death. There were no significant differences in the mean number of corpora lutea, implantation efficiency, litter size, sex ratio, mean fetal length and weight or in the number and percent of live and resorbed fetuses.

Gross and histopathological examinations of the fetuses did not reveal any treatment related effects. However, common skeletal variations and anomalies were present in all groups. The NOEL for maternal toxicity is considered to be 0.7 mg/kg/day.

The above studies satisfy the Agency's requirements for teratology data.

#### 6. Mutagenicity

A dominant lethal study in the mouse indicates that the number of implantations, resorptions, and embryos were not affected by endosulfan treatment (Arnold, 1972, MRID 00003711). The results did not indicate a dominant lethal response at 5 and 10 mg/kg.

In another study by Dikshith and Datta (1978, MRID 05003502), endosulfan was administered orally to rats at 0, 11.0, 22.0, 36.0, and 55 mg/kg daily for five days. The rats were injected with 4 mg/kg of colchicine four hours before they were killed by decapitation.

Seminiferous tubules and bone marrows from the femurs were examined. There were no major chromosomal aberrations either in the bone marrow cells or

spermatogonial cells. An unspecified number of chromatid breaks with one or two exchange figures were found in the bone marrow cells but not in the spermatogonial cells. There was no chromosomal deletion nor formation of large numbers of fragments. No significant mitotic inhibition were reported in any of the treated groups. No details or quantitative effects data were reported, therefore, no reliable conclusions can be drawn from this study.

In a recent study by Dorrough et al. (1978, MRID 05003703), endosulfan and its major metabolites were tested in Salmonella typhimurium mutagenicity test using tester strains TA98, TA100, TA1535, and TA1978. The chemicals were tested at concentrations of 10, 100, 500, and 1000 ug/plate in duplicates in the presence and absence of an activating system. Acetoaminoflourine was included as a positive control. Neither endosulfan I or II, nor any of the metabolites tested showed any increase in the reversion rates beyond the controls, both in the presence or absence of the activating systems. The diol, alpha hydroxy ether, and the lactone metabolites severely inhibited bacterial growth even at the lowest concentration used.

In this experiment it was obvious that only one S-9 concentration and insufficient duplication were used. Furthermore, no raw data were provided. For these reasons this study cannot provide reliable assessment for the mutagenic potential of endosulfan.

In a supplementary study (Fahrig, 1974, MRID 05014009), endosulfan did not exhibit any positive response when tested for mutagenic potential in Saccaromyces cervisia (mitotic gene conversion), Escherichia coli (forward mutation), and Serratia marcescens (reverse mutation).

The Agency requires a battery of valid mutagenicity tests which determine the potency of the chemical to induce point mutations and chromosomal mutations either directly or indirectly. The submitted studies do not adequately define the mutagenic potential of endosulfan, and therefore additional testing will be required.

## 7. Metabolism

The metabolism of endosulfan has been adequately delineated in a number of different mammalian species. In some studies conducted on rats (Dorrough et al., 1978, MRID 05003703) it was found that endosulfan metabolites accumulated in tissues, especially in the kidney and liver. Metabolites of endosulfan in the rat include endosulfan sulfate, endosulfan diol, endosulfan ether, endosulfan alpha-hydroxy ether, and endosulfan lactone. The sulfate and alpha-hydroxy ether are the principal metabolites accumulated in tissues. Animals administered endosulfan I eliminated 74.8 percent and 13.2 percent in the feces and urine respectively, while those administered endosulfan II eliminated 68.3 percent and 18.5 percent in the feces and urine respectively in a period of 120 hours. Up to 47 percent of the administered dose was eliminated via the bile. Enterhepatic circulation was not apparent.

In another study (Deema et al., 1966, MRID 00004257) when mice were fed endosulfan, large amounts of endosulfan sulfate were recovered in the liver, small intestine and visceral fat with a trace of this metabolite in the muscle



