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

006358

MEMORANDUM

JUL 30 1987

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

SUBJECT: MANEB
Special Review; Mutagenicity Data
EPA ID#014505; Action Code 870
Record #194660; Caswell #539
Tox Project #7-0615

TO: Joan Warshawsky, PM #77
Registration Division (TS-767C)

FROM: Alan C. Katz, M.S., D.A.B.T. 7/20/87
Toxicology Branch, Section 3
Hazard Evaluation Division (TS-769C)

THROUGH: Marcia vanGemert, Ph.D. 7/23/87
Head, Section 3
Toxicology Branch/HED (TS-769C)

and

Theodore M. Farber, Ph.D. 7/24/87
Chief, Toxicology Branch (TS-769C)

Attached are Data Evaluation Records for the following studies:

1) In Vitro Sister Chromatid Exchange Assay in Cultured Chinese Hamster Ovary (CHO) Cells Treated With Technical Grade Maneb

2) [Ames Test] Salmonella/Microsome Mutagenesis Assay on Technical Grade Maneb

3) CHO/HGPRT In Vitro Mammalian Cell Mutation Assay on Technical Grade Maneb

4) In Vitro Unscheduled DNA Synthesis Assay in Rat Hepatocytes: The Effect of Technical Grade Maneb

Results of these evaluations may be summarized as follows:

TEST	RESULTS	CLASSIFICATION
1) Sister Chromatid Exchange		acceptable
-without activation	negative	
-with activation	positive	
2) Ames Test		acceptable
-without activation	negative	
-with activation	negative	

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TEST	RESULTS	CLASSIFICATION
3) CHO/HGPRT		Provisionally acceptable*
-without activation	negative	
-with activation	negative	
4) Unscheduled DNA synthesis (Rat Hepatocytes)	inconclusive	Unacceptable*

*- Additional data required (see DER)

DATA EVALUATION REPORT

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A. Study Type: Mutagenicity

B. Compound: MANEB Technical, 88.1% a.i.

C. Study Report Citation:

Title: In Vitro Unscheduled DNA Synthesis Assay In Rat Hepatocytes: The Effect of Technical Grade Maneb

Author: Kenneth Loveday, Ph.D.

Laboratory: American Biogenics Corporation, Woburn, MA

Project Number: 850047-20

Date: 11/6/86

D. Reviewed By: Alan C. Katz, M.S., D.A.B.T.
Toxicologist
Toxicology Branch
Hazard Evaluation Division (TS-769C)

(Signature) Alan C Katz
(Date) 7/20/87

E. Secondary Review: Marcia vanGemert, Ph.D.
Head, Section III
Toxicology Branch (TS-769C)

(Signature) Byron T. Backus
(Date) 07/23/87
M. vanGemert 7/23/87

F. Classification:

Unacceptable. Individual data are required for a more complete evaluation.

G. Conclusion:

Although Maneb did not appear to induce unscheduled DNA synthesis (UDS) under the conditions of this assay, sufficient evidence of the validity of the experiment was not provided.

H. Materials and Methods:

See Appendix for details, as excerpted from the study report. Maneb was evaluated for UDS induction in rat hepatocytes in vitro at concentrations of 0.5, 1, 5, 10 and 100 ug/ml.

The test compound was identified as technical grade Maneb (ABC No. 85-11A; Lot/Batch No. MT01, 88.1% a.i.). The hepatocytes were isolated from a male Fischer rat (Charles River Breeding Laboratories, Wilmington, MA). The culture medium served as a negative (vehicle) control; 9,10-dimethyl-1,2-benzanthracene (DMBA) in dimethylsulfoxide (DMSO), 0.0625 mg/ml, was used as a positive control. ^{3}H-thymidine (4 uCi/ml) was added to the medium.

An electronic counter (Biotran III automatic colony counter with microscope and TV monitor) was used to count nuclear grains. One hundred cells (fifty cells per slide x 2 slides) in each group were scored. Background

H. Materials and Methods (Cont'd):

levels of cytoplasmic incorporation of radioactivity were evaluated by counting the silver grains in 2 nuclear-equivalent areas adjacent to each nucleus.

I. Results/Discussion:

The results of the UDS assay with Maneb are summarized in Table 1, as excerpted from the study report. These summary results do not provide a sufficient basis for evaluation of the results of the study. Individual scores were not presented. While the data presented provide no evidence of Maneb-induced UDS, values reported for background counts of negative control (100% medium) slides appear to be unacceptably high and at least one of the positive control (DMBA) slides was not clearly shown to have met appropriate criteria for a significant induction of UDS.

TABLE 1

The Effect of Technical Grade Maneb on the Induction of Unscheduled DNA Synthesis in Rat Hepatocytes

Concentration (ug/ml suspension)	Average Grains/Nucleus	Average Background[a]	Average[b] Net Grains/Nucleus
Technical Grade Maneb			
500 toxic, almost no cells; none with cytoplasm			
100	5.3	4.6	0.8
	3.5	2.6	0.9
50 toxic, no cells with cytoplasm			
10	4.2	3.2	1.0
	4.6	3.5	1.0
5	8.6	7.0	1.6
	4.9	2.5	2.4
1	11.3	10.0	1.3
	9.3	9.3	0.1
0.5	9.7	8.4	1.3
	7.2	8.2	-0.9
100% medium	20.7	25.5	-4.8
	18.7	17.5	1.2
DMBA 62.5	> 10.7[c]	NA	> 8.1
	> 5.9[d]	NA	> 4.1

DMBA - 9,10-Dimethy-1,2-benzanthracene

[a]Average Background is the average number of grains in an area equal to the size of a nucleus. Background counts were determined for each nucleus from 2 adjacent nucleus size areas.

[b]Rounded to nearest 0.1 based on raw data

[c]Includes 26 cells with greater than 10 net grains/nucleus

[d]Includes 10 cells with greater than 10 net grains/nucleus

NA, not applicable, background counts are not taken for cells with greater than 10 net grains/nucleus.

APPENDIX

2.0 MATERIALS

2.1 Test Sample Description

Identification:	Technical Grade Maneb
ABC No.:	85-11A
Lot/Batch No.:	MT 01
Physical State:	powder
Color:	yellow
Purity:	88.1% a.i. Technical 11.9% Inerts
Composition:	see purity
Stability of Test Article:	determined by sponsor
Stability of Formulations:	determined by sponsor
Solubility:	slightly soluble in water
Storage Conditions:	4°C
Safety Precautions:	avoid topical and respiratory contact.

The Sponsor has assumed responsibility for performing analysis to determine the characterization and stability of the test sample and concentration and stability analysis of the test sample mixture, as appropriate. Homogeneity analysis of the test sample as a suspension in water was not performed.

A 1 mg/ml suspension of the test sample was prepared in cell culture medium (Williams medium E) containing ^{3}HTdR. All dilutions were made in the same medium immediately prior to use. The initial stock solution and all dilutions were thoroughly agitated immediately prior to each use. The appropriate aliquot was added to the exposure medium.

2.2 Negative Control Description

Cells exposed to medium containing only ^{3}HTdR provided negative control data.

2.3 Positive Control Substance

Name:	9,10-Dimethyl-1,2-benzanthracene (DMBA)
Lot No.:	44F-0813
Supplier:	Sigma
Physical State:	solid
Color:	yellow
Purity:	at least 95%
Composition:	on file with manufacturer
Stability: (solid)	at least 1 year
(solution)	one month at 4°C
Solubility:	soluble in dimethylsulfoxide
Storage Conditions:	room temperature (solid)
Safety Precautions:	avoid topical and respiratory contact

A 25 mg/ml stock solution of the positive control was prepared in DMSO and aliquots added to the exposure medium. All dilutions were made in DMSO. Five concentrations of the positive control (0.25, 0.125, 0.0625, 0.025, and 0.0125 mg/ml) were tested to ensure a positive response. Only cells exposed to 0.0625 mg/ml were used in the UDS analysis.

2.4 Solutions for Liver Perfusion

Solution I

Hanks' Balance Salt Solution without calcium, magnesium and phenol red containing;

1. 0.5 mM Ethyleneglycol-bis (B-aminoethylether) N,N-tetraacetic acid (EGTA) (0.1902 mg/ml)

2. 10.0 mM Hepes Buffer (2.383 mg/ml)

pH adjusted to 7.35

Solution II

Williams' Medium (WME) containing:

1. 2 mM L-glutamine (292 mg/L)

2. Gentamycin Sulfate (50 ug/ml)

3. Type IV Collagenase (100 units/ml)

4. 10.0 mM Hepes Buffer (2.383 mg/ml)

pH adjusted to 7.3

2.5 Radioactive Material Characterization

The following radioactive material was used in this study:

Name:	Thymidine, [Methyl-^{3}H]
Lot No.:	2257-229
Source:	New England Nuclear, Boston, MA
Storage Conditions:	4°C

2.6 Cell Line

Primary cultures of rat hepatocytes were prepared fresh prior to the assay.

2.7 Animals

Species:	Rattus norvegicus
Strain:	Fischer
Supplier:	Charles River Breeding Laboratories, Wilmington, MA
Weight at Start of Study:	249 g
Number and Sex:	one male per assay

2.8 Identification of Test System

All the experimental vessels were labeled with the last five digits of the project number and a code number. The key to both the assay and the code numbers is given in the raw data sheets.

3.0 EXPERIMENTAL DESIGN

3.1 Preparation of Hepatocyte Cultures

A male Fischer rat was anesthetized with Metofane[R] prior to surgery. A ventral midline incision was made through the skin and abdominal muscle layer. A loose tie was placed around the infrahepatic inferior vena cava. A large gauge butterfly needle was inserted in the hepatic portal vein and clamped in place. Perfusion was initiated with sterile Soln. I (37°C) at a rate of approximately 8 ml/min for 1.5 min. Immediately following the initiation of the perfusion, the vena cava was ligated and severed distal to the tie which was tightened. The thoracic cavity was opened and the thoracic inferior vena cava was cannulated by puncturing the right atrium allowing the perfusate to drain. The perfusion speed with Soln. I was increased to approximately 40 ml/min until most of the blood had been removed from the liver. The liver was then perfused with Soln. II (37°C) for 10 min. at approximately 10 ml/min. The liver was covered with sterile gauze and kept warm by placing a light bulb approximately 6 cm above it.

When the perfusion was completed, the liver was placed in a petri dish containing WME, trimmed of fat and connective tissue and transferred to a petri dish containing 50 ml Soln. II. The capsule was opened and peeled away. The hepatocytes were combed into the solution with a stainless steel comb. Using a wide bore pipet the hepatocyte suspension was aliquoted evenly into two 50 ml centrifuge tubes and the volume adjusted to 50 ml using WME supplemented with 10% fetal bovine serum (WMES). The suspensions were centrifuged for 5 min. and resuspended in WMES. A viable count was taken using trypan blue; 5×10^5 viable cells were seeded onto 25 mm round plastic coverslips in WMES and incubated at 37°C.

3.2 Exposure to the Test Sample

Two hours after seeding the cells were washed leaving only attached viable cells on the coverslips. The exposure medium, WME containing 0.292 mg/ml L-glutamine, 4 uCi/ml ^{3}HTdR and 50 ug/ml gentamycin sulfate was then added. Cells were exposed to Technical Grade Maneb (concentrations ranging from 0.05 to 500 ug/ml) for 18 hours.

Cells exposed to medium for 18 hours served as the negative control. Cells exposed to DMBA served as the positive control. A range of DMBA concentrations was used to ensure a positive response (12.5 to 250 ug/ml). Six coverslips were treated for each test sample concentration and positive control while 12 coverslips were treated for the negative control. The temperature during the exposure was maintained at 37°C. At the end of the exposure period the cells were washed 3 times with WME and incubated at room temperature for 15 minutes in 1% Sodium citrate. The cells were fixed 3 times in 3:1 ethanol-glacial acetic acid and the coverslips were mounted on glass slides.

3.3 Autoradiography

For each test sample concentration and positive control three of the six slides were dipped in Kodak NTB-2 emulsion, air dried and stored at 4°C in a light-proof slide box containing Drierite. Six slides were dipped for the negative control. After 7 days, the slides were developed and fixed using Kodak D-19 developer and Kodak Fixer. The slides were stained for 7 min. in Harris hematoxylin.

3.4 Analysis

Cells from five test sample concentrations, the negative control and one of the positive controls (62.5 ug/ml DMBA) were analyzed in duplicate (fifty cells from each of two slides were analyzed). The nuclear grains were counted using a Biotran III automatic colony counter with a microscope and T.V. screen attachment. Background counts were obtained from two nucleus sized areas adjacent to each nucleus. The background count was not recorded for those cells with greater than 10 grains since the number of grains in the background was small and did not affect the characterization of the cell as having greater than 50 grains. Net grain counts were determined by subtracting the average background count from the nuclear count. A minimum increase of five grains/nucleus when compared to the negative control was needed for a positive response to be concluded.

DATA EVALUATION REPORT

A. Study Type: Mutagenicity

B. Compound: MANEB Technical, 81.1% a.i.

C. Study Report Citation:

Title: CHO/HGPRT In vitro Mammalian Cell Mutation Assay on Technical grade Maneb

Author: Marybeth Thomas, B.A. (Study Director)

Study Director: Kenneth S. Loveday, Ph.D.

Laboratory: American Biogenics Corporation, Woburn, MA

Project Number: 850047-10

Date: 5/16/86

D. Reviewed By: Alan C. Katz, M.S., D.A.B.T.
Toxicologist
Toxicology Branch
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(Signature)

7/20/87
(Date)

E. Secondary Review: Marcia vanGemert, Ph.D.
Head, Section III
Toxicology Branch (TS-769C)

(Signature)

7/23/87
(Date)

7/23/87

F. Classification:

"Conditionally Acceptable," pending submission and evaluation of analytical characterization of test substance.

G. Conclusion:

Under the conditions of these assays, Maneb was found to be negative for induction of mutagenicity with and without metabolic activation.

H. Materials and Methods:

The test substance was reported as Lot No. MT01, Technical grade Maneb, ABC No. 85-11A, "81.1% Technical", "11.9% Inerts".

Deionized water was used as the vehicle and negative control. The assays were conducted with and without metabolic activation. The activated systems utilized the S9 microsome fraction from the livers of Sprague Dawley rats and B6C3F$_1$ mice (in separate assays) induced with Aroclor 1254.

Preliminary cytotoxicity rangefinding assays were performed. These preliminary experiments were conducted with and without rat S9 mix. The range of concentrations used with mouse S9 was based on the results of the rangefinding studies with rat S9.

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H. Materials and Methods (Cont'd):

In the mutagenicity assays, 200 cells were plated for each of 2 experiments at each concentration level. Positive controls for the activated and non-activated assays were 9,10-dimethyl-1,2-benzanthracene (DMBA) and ethylmethanesulfonate (EMS), respectively.

Details of the materials and procedures used, excerpted from the study report, are presented in the Appendix.

I. Results/Discussion:

Results of the study are presented in Tables 1 through 6, as excerpted from the study report (Note: these data are designated as Tables 2,3,5,6, 7 and 8, in the same sequence, in the study report).

In the initial non-activated assays (Tables 1 and 2), the data reveal moderate variability with respect to cell survival and mutagenicity; nevertheless, it is apparent that the mutant frequency values found for cells exposed to Maneb did not demonstrate a dose-related increase over control values. Both of the experiments without activation showed clear, strongly positive results for mutagenic activity with EMS.

In the 2 experiments using the rat S9-activated systems, considerable background variability was found; for example, mutant frequency for the negative control in one experiment (Table 3) ranged from 1.8 to 41.1 per million. No compound-induced increase in mutation was evident. The positive control (DMBA) consistently demonstrated significant mutagenic activity.

In the assays using the mouse S9 mix for metabolic activation, substantial differences were seen between the results of the two experiments with respect to cytotoxicity. For example, in the initial experiment, mean cell survival was reported to be 21.5 and 31.9 percent (relative to controls) in replicate assays at a concentration of 3 ug of Maneb per ml, and essentially no survival at higher concentrations. In the second experiment, however, mean survival was assayed at 75.4 and 77.8 percent at a concentration of 10 ug per ml. In both experiments, the mutant frequencies of the MANEB-exposed plates were within the general range of the negative control values. Mutant frequencies were significantly increased in all assays with the DMBA positive control.

This reviewer questions the purity of the test compound, reported to be "81.1% Technical" and "11.9% Inerts". The Registr[illegible] must provide appropriate and specific analytical data on the composi[illegible] of the test sample.

TABLE 1

The Effect of Technical grade Maneb on the Induction of HGPRT Mutants in CHO Cells; Nonactivated Assay
Experiment No. 1

	Toxicity Data				Mutagenicity Data			
Conc. (ug/ml) susp.	No. of Cells Remaining in Flask[a]	No. of Plated cells	No. of colonies	Percent[b] Survivors %	Plating efficiency (out of 200)[c]	No. of plated cells (x 10^6)	No. of Mutants	Mutant Freq. (x 10[illegible])
Technical grade Maneb								
30	$3.2X10^5$	200	0/0	1.0		Lost To Toxicity		
	$2.4X10^5$	200	0/0	1.0				
10	$2.3X10^5$	200	0/0	1.0		Lost To Toxicity		
	$1.8X10^5$	200	0/0	1.0				
5	$2.5X10^5$	200	1/2	1.5	89/122	1.2	0	[illegible]
	$2.6X10^5$	200	1/1	1.0	97/131	1.2	0	<1.5
3	$2.9X10^5$	200	10/13	11.2	139/93	1.2	0	<1.4
	$2.9X10^5$	200	29/30	28.6	110/84	1.2	0	<1.7
1	$1.7X10^6$	200	104/107	102.4	112/70	1.2	7	[illegible]
	$1.6X10^6$	200	42/54	46.6	165/166	1.2	25	[illegible]
0.5	$1.8X10^6$	200	109/106	104.4	141/116	1.2	15	[illegible]
	$1.8X10^6$	200	90/102	93.2	126/137	1.2	7	[illegible]
0.3	$2.4X10^6$	200	94/115	101.5	108/132	1.2	15	[illegible]
	$2.3X10^6$	200	93/79	83.5	112/107	1.2	3	[illegible]
0.15	$3.4X10^6$	200	71/77	71.8	177/138	1.2	[illegible]	[illegible]
	$2.4X10^6$	200	100/73	84.0	120/135	1.2	13	[illegible]
0.1	$2.3X10^6$	200	82/80	78.6	131/136	1.2	20	[illegible]
	$2.3X10^6$	200	70/61	63.6	147/150	1.2	22	[illegible]
Media [illegible]	$2.0X10^6$	200	125/124		130/158	[illegible]	15	[illegible]
	$2.3X10^6$	200	80/78		110/108	1.2	7	[illegible]
				100				
	$1.9X10^6$	200	134/114		131/117	1.2	14	[illegible]
	$2.3X10^6$	200	76/93		146/129	1.2	4	[illegible]
EMS 234 ug/ml	$2.3X10^6$	200	13/24	18.0	95/79	1.2	215	412
	$2.0X10^6$	200	13/15	13.5	65/89	1.2	280	506

EMS - Ethylmethanesulfonate

[a]Counts taken at time of toxicity plating.
[b]Percent survivors was calculated using the average number of colonies exposed to 100% Media as 100% survivors.

TABLE 2

The Effect of Technical Grade Maneb on the Induction of HGPRT Mutants in CHO Cells; Nonactivated Assay Experiment No. 2

	Toxicity Data				Mutagenicity Data			
Conc. (ug/ml) susp.	No. of Cells Remaining in Flask[a]	No. of Plated cells	No. of colonies	Percent Survivors[b] %	Plating efficiency (out of 200)	No. of plated cells (x 10^6)	No. of Mutants	Mutant Freq. (x 10^{-6})
Technical Grade Maneb								
5	$5.0X10^5$	200	33/36	27.4	139/146	1.2	0	<1.2
	$7.7X10^5$	200	52/64	46.0	135/115	1.2	1	1.3
3	$6.0X10^6$	200	127/116	96.3	231/196	1.2	21	16.4
	$2.8X10^6$	200	157/172	130.4	107/112	1.2	13	19.9
1	$7.1X10^6$	200	100/91	75.7	99/96	1.2	1	1.7
	$9.9X10^5$	200	152/143	116.9	113/116	1.2	0	<1.5
0.5	$1.1X10^7$	200	99/110	82.9	103/116	1.2	6	3.1
	$7.2X10^6$	200	159/175	132.4	107/137	1.2	9	12.3
0.3	$8.9X10^6$	200	160/130	115.0	109/102	1.2	1	1.6
	$7.0X10^6$	200	171/171	135.6	128/115	1.2	0	<1.4
0.15	$5.9X10^6$	200	190/160	138.8	110/124	1.2	7	10.0
	$5.9X10^6$	200	155/161	125.3	113/113	1.2	4	5.9
0.1	$6.8X10^6$	200	146/139	113.0	101/118	1.2	6	9.1
	$5.7X10^6$	200	129/146	109.0	104/112	1.2	6	9.3
Media 100%	$1.0X10^7$	200	79/80	100	83/81	1.2	0	<2.0
	$6.3X10^5$	200	83/76		97/93	1.2	3	5.3
	$5.0X10^6$	200	154/122		103/92	1.2	3	5.1
	$5.7X10^5$	200	205/210		68/56	1.2	0	<2.7
EMS								
234 ug/ml	$7.1X10^5$	200	53/56	43.2	103/110	1.2	435	681
	$4.3X10^6$	200	58/74	52.3	75/85	1.2	279	581

EMS - Ethylmethanesulfonate

[a]Counts taken at time of toxicity plating.
[b]Percent survivors was calculated using the average number of colonies exposed to 100% Media as 100% survivors.

TABLE 3

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The Effect of Technical Grade Maneb on the Induction of HGPRT Mutants in CHO Cells; Activated Assay (Rat S9)

Experiment No. 1

Conc. (ug/ml) susp.	No. of Cells Remaining in Flask[a]	No. of Plated cells	No. of colonies	Percent[b] Survivors %	Plating efficiency (out of 200)	No. of plated cells ($x 10^6$)	No. of Mutants	Mutant Freq. ($x 10^{-6}$)
	Toxicity Data				Mutagenicity Data			
Technical Grade Maneb +S9								
	$3.2X10^5$	200	0/0	1.1		Lost To Toxicity		
50	$3.9X10^5$	200	0/0	1.1		Lost To Toxicity		
	$6.1X10^5$	200	5/6	6.1	93/106	1.2	16	26.8
30	$6.0X10^5$	200	0/0	1.1		Lost To Toxicity		
	$5.9X10^5$	200	38/52	50.0	103/94	1.2	20	30.8
10	$5.3X10^5$	200	54/40	52.2	113/121	1.2	20	28.5
	$3.4X10^6$	200	98/71	93.9	87/97	1.2	4	7.2
3	$4.9X10^6$	200	66/66	73.3	88/100	1.2	12	21.[illegible]
	$8.9X10^6$	200	52/51	57.2	99/91	1.2	5	10.5
1	$5.5X10^6$	200	71/60	72.8	118/120	1.2	2	7.6
	$9.6X10^6$	200	76/77	85.0	101/82	1.2	1	5.5
0.3	$1.0X10^7$	200	63/56	66.1	69/76	1.2	6	13.8
	$9.8X10^6$	200	70/59	71.7	83/87	1.2	3	5.9
0.1	$9.3X10^6$	200	90/95	102.8	133/135	1.2	4	5.0
Media + S9 100%	$8.6X10^7$	200	83/78		93/106	1.2	5	8.4
	$7.9X10^6$	200	100/97	100	156/118	1.2	2	2.4
	$8.0X10^6$	200	91/91		77/107	1.2	1	1.8
	$8.1X10^6$	200	81/99		115/104	1.2	27	41.1
DMBA +S9								
15 ug/ml	$4.2X10^6$	200	31/24	30.6	91/100	1.2	338	590
	$4.6X10^6$	200	45/30	41.7	75/77	1.2	209	458

DMBA - 9,10-Dimethyl-1,2-benzanthracene

[a] Counts taken at time of toxicity plating.
[b] Percent survivors was calculated using the average number of colonies exposed to 100% Media + S9 as 100% survival.

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TABLE 4

The Effect of Technical Grade Maneb on the Induction of HGPRT Mutants in CHO Cells; Activated Assay (Rat S9)
Experiment No. 2

	Toxicity Data				Mutagenicity Data			
Conc. (ug/ml) susp.	No. of Cells Remaining in Flask[a]	No. of Plated cells	No. of colonies	Percent Survivors %[b]	Plating efficiency (out of 200)	No. of plated cells ($\times 10^6$)	No. of Mutants	Mutant Freq. ($\times 10^{-6}$)
Technical Grade Maneb + S9								
30	5.3×10^5	200	0/0	0.9	135/122	1.2	1	1.3
	5.1×10^5	200	3/2	2.3	113/118	1.2	0	<1.4
10	5.3×10^5	200	47/50	44.1	120/129	1.2	0	<1.3
	5.6×10^5	200	72/90	73.6	123/103	1.2	28	41.[illegible]
3	3.6×10^6	200	83/62	65.9	101/91	1.2	6	10.4
	2.6×10^6	200	104/98	91.8	99/81	1.2	8	14.8
1	3.0×10^6	200	130/147	125.9	99/133	1.2	17	24.4
	5.4×10^6	200	81/84	75.0	77/76	1.2	4	8.7
0.3	7.2×10^6	200	81/71	69.1	119/105	1.2	3	4.5
	5.1×10^6	200	83/69	69.1	111/110	1.2	7	10.6
1	6.8×10^6	200	75/77	69.1	112/132	1.2	9	12.3
	3.2×10^6	200	178/145	146.8	83/114	1.2	14	23.7
Media + S9 100%	7.0×10^6	200	98/111	100	118/128	1.2	11	14.9
	6.0×10^6	200	99/74		160/116	1.2	5	6.0
	5.7×10^6	200	110/124		140/[c]	1.2	18	21.4
	5.1×10^6	200	135/129		121/122	1.2	14	19.2
DMBA + S9								
15 ug/ml	2.7×10^6	200	67/62	58.6	113/110	1.2	349	522
	2.8×10^6	200	66/66	60.0	113/131	1.2	345	471

DMBA - 9,10-Dimethyl-1,2-benzanthracene

[a] Counts taken at time of toxicity plating.
[b] Percent survivors was calculated using the average number of colonies exposed to 100% Media + S9 as 100% survivors.
[c] Plate not seeded due to technical error.

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TABLE 5

The Effect of Technical Grade Maneb on the Induction of HGPRT Mutants in CHO Cells; Activated Assay (Mouse S9)
Experiment No. 1

	Toxicity Data				Mutagenicity Data			
Conc. (ug/ml) susp.	No. of Cells Remaining in Flask[a]	No. of Plated cells	No. of colonies	Percent[b] Survivors %	Plating efficiency (out of 200)	No. of plated cells (x 10^6)	No. of Mutants	Mutant Freq. (x 10^{-6})
Technical Grade Maneb + S9								
50	$5.7X10^5$	200	0/0	1.3		Lost to Toxicity		
	$6.5X10^5$	200	0/0	1.3		Lost to Toxicity		
30	$7.0X10^5$	200	0/0	1.3		Lost to Toxicity		
	$8.6X10^5$	200	0/0	1.3		Lost to Toxicity		
10	$7.7X10^5$	200	0/0	1.3		Lost to Toxicity		
	$8.0X10^5$	200	0/0	1.3		Lost to Toxicity		
3	$3.8X10^5$	200	16/17	21.5	73/71	1.2	1	2.3
	$3.8X10^5$	200	24/25	31.9	110/129	1.2	0	<1.4
1	$1.9X10^6$	200	83/92	114.0	77/68	1.2	1	2.3
	$1.8X10^6$	200	73/85	102.9	121/130	1.2	1	1.3
[illegible]	$2.6X10^6$	200	213/217	280.1	115/124	1.2	6	8.4
	$3.1X10^6$	200	86/100	121.2	117/116	1.2	2	2.9
0.1	$2.3X10^6$	200	77/91	109.4	87/87	1.2	4	7.7
	$2.3X10^6$	200	112/122	152.4	94/99	1.2	5	8.6
Media + S9 100%	$2.9X10^6$	200	51/86	100	98/116	1.2	9	14.0
	$2.5X10^6$	200	79/92		107/121	1.2	7	10.2
	$2.9X10^6$	200	80/76		91/112	1.2	1	1.6
	$2.3X10^6$	200	78/72		95/119	1.2	2	3.1
DMBA + S9								
5 ug/ml	$1.9X10^6$	200	17/26	28.0	97/93	1.2	191	335
	$1.9X10^6$	200	27/22	31.9	72/66	1.2	140	338

DMBA - 9,10-Dimethyl-1,2-benzanthracene

[a] Counts taken at time of toxicity plating.
[b] Percent survivors was calculated using the average number of colonies exposed to 100% Media + S9 as 100% survivors.

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TABLE 6

The Effect of Technical Grade Maneb on the Induction of HGPRT Mutants in CHO Cells; Activated Assay (Mouse S9) Experiment No. 2

Conc. (ug/ml) susp.	Toxicity Data: No. of Cells Remaining in Flask[a]	No. of Plated cells	No. of colonies	Percent Survivors[b] %	Mutagenicity Data: Plating efficiency (out of 200)	No. of plated cells ($\times 10^6$)	No. of Mutants	Mutant Freq. ($\times 10^{-6}$)
Technical Grade Maneb + S9								
	1.4×10^6	200	79/81	76.4	68/85	1.2	9	19.6
10	1.7×10^6	200	78/85	77.8	82/116	1.2	1	1.7
	4.3×10^6	200	118/150	127.9	55/66	1.2	6	16.5
5	4.7×10^6	200	109/104	101.7	90/83	1.2	1	1.9
	6.2×10^6	200	145/122	127.4	65/67	1.2	4	16.1
3	6.6×10^6	200	102/101	96.9	98/81	1.2	6	11.2
	6.4×10^6	200	108/97	97.9	79/75	1.2	7	15.2
1	7.3×10^6	200	110/102	101.2	58/55	1.2	1	2.9
	5.3×10^6	200	119/125	116.5	92/93	1.2	5	9.0
0.3	7.7×10^6	200	118/106	106.9	82/86	1.2	12	23.8
	7.8×10^6	200	92/83	83.5	80/104	1.2	5	9.1
0.1	8.3×10^6	200	97/94	91.2	105/116	1.2	6	9.0
ia + S9	8.0×10^6	200	73/88		64/67	1.2	3	7.6
100%	5.9×10^6	200	100/103		81/94	1.2	0	<1.9
				100				
	4.7×10^6	200	117/134		63/92	1.2	2	4.3
	8.0×10^6	200	118/105		71/79	1.2	9	20.0
DMBA +S9								
15 ug/ml	4.1×10^6	200	60/59	56.8	88/89	1.2	159	239
	4.9×10^6	200	58/48	50.6	96/104	1.2	147	245

DMBA - 9,10-Dimethyl-1,2-benzanthracene

[a] Counts taken at time of toxicity plating.
[b] Percent survivors was calculated using the average number of colonies exposed to 100% Media +S9 as 100% survivors.

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APPENDIX

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2.0 MATERIALS

2.1 Test Substance

Name:	Technical grade Maneb
ABC No.:	85-11A
Lot No.:	MT 01
Physical State:	powder
Color:	yellow
Purity:	81.1% Technical 11.9% Inerts
Composition:	see purity
Stability of Test Article	determined by sponsor
Stability of Formulations:	determined by sponsor
Solubility:	slightly soluble in water (culture medium)
Storage Conditions:	4°C
Safety Precautions:	avoid topical and respiratory contact

The test article was assayed in suspension using culture medium as a vehicle. Stock suspensions with concentrations of 1 mg/ml were used for both the activated and nonactivated assays. All further dilutions were made in culture medium. The initial stock suspension and all dilutions were prepared immediately prior to use. All stock suspensions were thoroughly agitated immediately prior to use; however, due to the insolubility of the test sample in water, particles settled out immediately. Concentrations were based on the weight of the test sample as received; no corrections were made using the purity of the active ingredient.

The Sponsor has assumed responsibility for performing analysis to determine the characterization and stability of the test article and concentration analysis of the test article mixture, as appropriate. Concentration and homogeneity analyses of the suspension were not performed prior to the test.

2.2 Negative Control

Untreated cells (nonactivated assay) and cells exposed only to the metabolic activation system (activated assay) provided the negative control data.

2.3 Positive Control Substances

Activated Assay

Name:	9,10-Dimethyl-1,2-Benzanthracene (DMBA)
Lot No.:	44F-0813
Supplier:	Sigma
Physical State:	powder
Color:	yellow tint
Purity:	at least 98%
Solubility:	soluble in DMSO
Composition:	on file with manufacturer
Stability:	at least 1 yr. (solid)
Stability of Formulation:	1 month at 4°C (solution)
Storage Conditions:	room temperature
Safety Precautions:	avoid topical and respiratory contact

Non-Activated Assay

Name:	Ethylmethanesulfonate(EMS)
Lot No.:	83F-0279
Supplier:	Sigma Chemical Co.
Physical State:	liquid
Color:	yellow tint
Purity:	reagent grade
Composition:	on file with manufacturer
Stability:	at least one year
Stability of formulations:	5 hours at room temperature
Specific Gravity:	1.167
Solubility:	soluble in H_2O (culture medium)
Storage Conditions:	4°C
Safety Precautions:	avoid topical and respiratory contact

Stock solutions of the positive control agents were prepared and aliquots added to the exposure medium. A 1.5 mg/ml stock solution of DMBA in DMSO was used to provide a final concentration of 15 ug/ml. EMS was dissolved directly in the exposure medium at a stock concentration of 11.7 mg/ml (final concentration 234 ug/ml). Stock solutions of DMBA were stored at 4°C. Stock solutions of EMS were prepared fresh prior to use.

2.4 CHO Cell Culture

Cells used in this assay were obtained from Dr. Sheila Galloway, Litton Bionetics, Kensington, MD. Routine cell culture methods, preparation of media, metabolic activation and quality control measures are detailed in American Biogenics Corporation's standard operating procedures.

Master vials were stored in liquid nitrogen or in a freezer at -80°C; stock cultures were replaced from the frozen vials. All the frozen cultures were prescreened for mycoplasma contamination and the spontaneous background mutant frequency was acceptably low. Working and experimental cultures were maintained in cell culture incubators in F12 medium (without hypoxanthine) containing 5% dialyzed fetal calf serum. The medium in the experimental and expression flasks was supplemented with HEPES buffer (20 mM).

2.5 Microsomal Activation System

Two sources of S9 microsome fraction prepared from the liver of Aroclor 1254 induced Sprague-Dawley rats and mice (B6C3F1) were used in the activated assays. The S9 fraction was combined with a solution of cofactors and culture medium to form the metabolic activation system. A mixture of 10% 10X Isocitrate Cofactors and 90% serum-free F12 medium was prepared and used as the exposure medium. Following the addition of the test sample, an aliquot of the S9 fraction was added to each flask. The final concentrations in each flask were 4.5 mg/ml Isocitric acid (trisodium salt), 2.4 mg/ml NADP and 20 ul/ml S9 fraction. The cofactor/medium mixture was prepared just prior to use and the S9 fraction was maintained on ice until use.

The following S9 fractions were used in the study:

Rat Liver S9

Source: Microbiological Associates, Bethesda, MD
Lot/Batch No.: R-218
Storage Conditions: -80°C

Mouse Liver S9

Source: Microbiological Associates, Bethesda, MD
Lot/Batch No.: M-107
Storage Conditions: -80°C

2.6 Identification of Test System

All the experimental vessels were labeled with the last five digits of the project number and a code number. The key to code numbers is given in the raw data sheets.

3.0 EXPERIMENTAL DESIGN

3.1 Toxicity Determination

Initial range-finding experiments delineated the concentrations used in subsequent mutagenesis assays. Standard activated (rat liver S9) and nonactivated test procedures were used. Parallel toxicity and mutagenicity studies were conducted to confirm the toxicity of each concentration used in assays with and without activation. The following method of toxicity assessment was used for the range-finding studies and also to confirm toxicity in mutagenesis studies:

Half a million ($5x10^5$) CHO cells were seeded in plastic T25 flasks. Approximately 24 hours later, the cells were exposed to a series of Technical grade Maneb concentrations. Exposure times for the activated and nonactivated assays were 4 and 16 hours, respectively.

The day of sample removal in the nonactivated assay and the day following the exposure for the activated assay 200 cells (total count) were seeded in duplicate tissue culture dishes. After at least one week of incubation the cells were fixed, stained and scored.

3.2 Mutation Induction

CHO cells were seeded at a density of approximately 1.5×10^6 in T75 flasks and grown for 1 day before being exposed to the test article and controls. The exposure times were 4 and 16 hours for activated and nonactivated assays, respectively. All test article concentrations and the positive control were tested in duplicate flasks. Two sets of duplicate flasks were tested for the negative control. All flasks were maintained independently throughout the expression period. The day of removal of the test article in the nonactivated assay and the day following the removal of the test article in the activated assay, the cells were replated for toxicity. All of the remaining cells or an aliquot of the remaining cell suspension (if the cell suspension was very dense) were replated into flasks for expression time. The expression flasks were replated as necessary to maintain their maximum growth rate; the cells were maintained for at least 7 days. The cells were then plated at 2×10^5 per dish (six dishes per flask) in medium containing 2 ug/ml 6-thioguanine (6TG). Concomitantly with the selection step, 200 cells from each flask were plated in two tissue culture dishes in medium without 6TG to determine the cloning efficiency of the cells. After at least 1 week the colonies were fixed with methanol, stained with Giemsa and scored. A group of cells containing a minimum of 50 cells was counted as a mutant colony. The activated part of the assay was performed by exposing cells in serum-free medium to the test article and controls in the presence of a metabolic activation system.

$$\text{Mutant Frequency} = \frac{\text{No. of Mutants}}{\text{No. of cells plated in selective medium} \times \text{PE}}$$

$$\text{PE (plating efficiency)} = \frac{\text{Avg. No. Colonies}}{\text{No. of plated cells}}$$

$$\text{Relative Percent Survivors} = 100 \times \frac{\text{Avg. No. Colonies of Sample}}{\text{Avg. No. Colonies of Negative Control}}$$

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DATA EVALUATION REPORT

A. Study Type: Mutagenicity - Ames Test

B. Compound: MANEB Technical, 88.1% a.i.

C. Study Report Citation:

Title: Salmonella/Microsome Mutagenesis Assay on Technical Grade Maneb

Author: Marybeth Thomas, B.A. (Study Director)

Laboratory: American Biogenics Corporation

Project Number: 850047-40

Date: 11/26/85

D. Reviewed By: Alan C. Katz, M.S., D.A.B.T.
Toxicologist
Toxicology Branch
Hazard Evaluation Division (TS-769C)

(Signature)

7/20/87
(Date)

E. Secondary Review: Marcia vanGemert, Ph.D.
Head, Section III
Toxicology Branch (TS-769C)

(Signature)

07/23/87
(Date)

F. Classification: Acceptable

G. Conclusion:

Under the conditions of these assays, Maneb was found to be negative for induction of mutagenicity with and without metabolic activation.

H. Materials and Methods:

An Ames test was conducted to investigate the potential of the test substance to induce point mutations in bacteria with and without rat or mouse liver microsomal (S9) activation. The test substance was assayed at levels up to and including 100 ug per plate. Salmonella typhimurium strains TA 98, TA 100, TA 1535, TA 1537, and TA 1538 were used. These strains were selected for detection of base pair substitutions and frame-shift mutations. Separate S9 mixes were prepared from the livers of adult Sprague-Dawley rats and $B6C3F_1$ mice which had been injected i.p. with Arochlor 1254 for enzyme induction. Sodium azide, 2-nitrofluorene, 5-nitro-o-anisidine, and/or 2-anthramine were used as positive controls. Details of the materials and methods, as excerpted from the study report, are presented in the Appendix.

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I. Results:

In a preliminary test, Maneb showed significant cytotoxicity, reduced bacterial lawn, and absence of revertant colonies at a concentration of 100 ug/plate with and without rat or mouse liver S9 activation. This concentration was selected as the highest level for the mutagenicity assays.

Results of the mutagenicity assays are presented in Table 1. Results of the assays with positive controls appeared to validate the sensitivity of these experiments, although this reviewer notes that the solvent (DMSO) used in preparation of all of the positive controls is different from the negative (deionized water) control. At all concentration levels of Maneb, the numbers of revertants per plate were found comparable to those of the negative controls, except where reduced numbers were attributable to cytotoxicity as indicated in the table.

TABLE 1. MUTATION QUOTIENTS* (Mean Values of Duplicate Tests)

Maneb Conc. (ug/plate)	TA 98 -S9	TA 98 +S9 Rat	TA 98 +S9 Mouse	TA 100 -S9	TA 100 +S9 Rat	TA 100 +S9 Mouse	TA 1535 -S9	TA 1535 +S9 Rat	TA 1535 +S9 Mouse	TA 1537 -S9	TA 1537 +S9 Rat	TA 1537 +S9 Mouse	TA 1538 -S9	TA 1538 +S9 Rat	TA 1538 +S9 Mouse
3	0.8	1.7	0.9	1.0	1.1	0.8	0.7	0.6	0.8	0.8	2.2	0.9	1.0	1.3	1.0
10	0.7	0.8	0.7	1.0	1.1	0.7	0.9	0.6	0.7	1.4	1.2	0.8	0.8	1.3	0.6
15	0.5	0.9	0.8	1.3	1.1	1.0	0.5	0.7	0.6	1.2	1.3	1.0	0.9	0.7	0.8
30	1.4	0.7	0.7	1.1	1.3	0.9	0.5†	0.5†	0.2†	1.0	1.5	1.0	0.6	1.1	0.6
50	0.7	1.4	1.3	1.3	1.1	0.9	0.3†	0.4†	0.1†	1.4	0.8	0†	0.8	1.0	0.6†
100	0.6	0.6	0.2†	0.7†	1.2	0.3†	<0.1†	0†	0†	0.6†	0.2†	0.3†	0.3†	0.4†	0.2†
Positive control	44.0	46.0	21.7	10.8	17.9	9.3	32.3	5.5	11.1	53.2	35.2	6.5	72.3	57.1	27.5

* Mutation Quotient: $\frac{\text{Mean \# of mutants/plate in test group or positive controls}}{\text{Mean \# of mutants/plate in negative (solvent) controls}}$

† Cytotoxicity reported

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APPENDIX

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2.0 MATERIALS

2.1 Test Substance

Name:	Technical Grade Maneb
ABC No.:	85-11A
Lot/Batch No.:	MT 01
Physical State:	powder
Color:	yellow
Purity:	88.1% a.i. Technical, 11.9% Inerts
Composition:	see purity
Solubility:	slightly soluble in water
Storage Conditions:	4°C
Safety Precautions:	Avoid topical and respiratory contact

A 1 mg/ml suspension in deionized water was used for activated and nonactivated assays. All further dilutions were made fresh in deionized water. All stock suspensions were vortexed immediately prior to use, however, due to the insolubility of the test sample in water, particles settled out immediately.

The sponsor has assumed responsibility for performing analysis to determine the characterization and stability of the test sample, and concentration and stability analysis of the test article mixture, as appropriate. Homogeneity analysis of the suspension was nor performed prior to the test.

2.2 Negative Control Substance

Deionized water, the resistivity of which is greater than 10 megaohms.

2.3 Positive Control Substances

	Nonactivated	Nonactivated	Nonactivated
Identification:	5-nitro-o-anisidine	sodium azide	2-nitrofluorene
Supplier:	Lancaster	Sigma	Aldrich
Lot/Batch #:	4842	113F-0252	1511CJ
Physical State:	solid	solid	solid
Color:	orange	white	white
Purity:	97%	99%	98%
Composition:	On file at manufacturer		
Stability:			
(Solid)	at least 1 yr	at least 1 yr	at least 1 yr
(Solution)	at least 24 hrs.	1 yr at -20°C	1 yr at -20°C
Storage Conditions:			
(Solid)	RT	RT	RT
(Solution)	-20°C	-20°C	-20°C
Solubility:	DMSO	DMSO, water	DMSO
Safety precautions:	Avoid topical and respiratory contact		
Stock Concentration:	20 mg/ml	25 ug/ml	100 ug/ml

Stock solutions of sodium azide and 2-nitrofluorene were prepared in DMSO and stored at -20°C for up to one year. The stock solution of 5-nitro-o-anisidine was prepared fresh in DMSO prior to use. Aliquots of the stock solutions are added to the plates.

	Activated
Identification:	2-anthramine
Supplier:	Sigma
Lot/Batch #:	33F-0816
Physical State:	solid
Color:	gold
Purity:	99%
Composition:	on file at manufacturer
Stability:	
(Solid)	at least 1 yr
(Solution)	at least 6 hrs at room temperature
Storage Conditions:	
(Solid)	4°C (Dessicate)
Solubility:	DMSO
Safety precautions:	Avoid topical and respiratory contact
Stock Concentration:	10 ug/ml

The stock solution of the activated positive control agent was prepared in DMSO prior to use. Aliquots of the stock solution are added to the plates.

2.4 Bacterial Culture

The Salmonella typhimurium strains TA98, TA100, TA1538, TA1537, and TA1535 used in this assay were obtained from Dr. Bruce Ames at the University of California, Berkeley, CA.

Master vials are stored at -80°C (10% DMSO added), and working plates are prepared monthly and stored at 4°C. Daily cultures are grown overnight in Oxoid nutrient broth from colonies on nutrient agar plates.

2.5 Microsomal Activation System

Two sources of metabolic activation were used in the mutagenesis assay: Aroclor 1254 induced mice (B6C3F1) and rats (Sprague-Dawley). The S9 mix consisted of 3% rat liver S9 fraction or 1% mouse liver S9 fraction in a cofactor mixture of 8mM $MgCl_2$, 33mM KCl, 5mM glucose-6-phosphate, 4mM NADP and 100mM Na_2HPO_4, pH 7.4. An aliquot of the rat or mouse S9 mix (0.5 ml) was then added to the appropriate plates. The final concentration of rat liver S9 fraction was 15 ul/plate and the final concentration of mouse liver S9 fraction was 5 ul/plate. Both the rat liver and mouse liver S9 mixtures were prepared fresh and maintained on ice during use.

The following rat liver S9 fraction was used in the study:

Source: Microbiological Associates
Lot/Batch No.: R192
Storage Conditions: -80°C

The following mouse liver S9 fraction was used in the study:

Source: Microbiological Associates
Lot/Batch No.: M107
Storage Conditions: -80°C

2.6 Identification of Test System

All the experimental vessels were labelled with the last five digits of the project number, strain number (where appropriate) and a code number. The key to the code numbers are given in the raw data sheets.

3.0 EXPERIMENTAL DESIGN:

3.1 Range-finding Assay

For the range-finding assay the method used for the mutagenesis assay was followed using *Salmonella* strain TA100 under activated (rat liver and mouse liver S9) and nonactivated conditions. Duplicate plates were employed. Toxicity was evaluated on the basis of the number of revertants per plate relative to the negative control and the condition of the bacterial background lawn. Sample sterility was also assessed. The results of the range-finding assay delineated the concentrations used in the mutagenesis assay.

3.2 Mutagenesis Assay

Five *Salmonella* strains were used: TA98, TA100, TA1535, TA1537 and TA1538. Bacterial strains were checked the day of the assay to ensure that all five strains were sensitive to crystal violet (rfa mutation) and that TA98 and TA100 contained the R-factor (ampicillin resistance). Test sample concentrations and the positive and negative controls were tested in all strains in the presence and absence of a metabolic activation system.

The following components were added sequentially to 2 ml aliquots of molten top agar containing 50 uM biotin and 50 uM histidine: 0.1 ml of test sample or control concentration and 0.1 ml overnight bacterial culture. In the activated assay, 0.5 ml S9 mix was also added to the tube. The contents of the tubes were mixed and plated on minimal medium bottom agar petri dishes. After 2-3 days of incubation at 37°C, plates were scored for visible colonies.

DATA EVALUATION REPORT

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A. Study Type: Mutagenicity

B. Compound: MANEB Technical, 88.1% a.i.

C. Study Report Citation:

Title: In Vitro Sister Chromatid Exchange Assay in Cultured Chinese Hamster Ovary (CHO) Cells Treated With Technical Grade Maneb

Author: Marybeth Thomas, B.A. (Study Director)

Laboratory: American Biogenics Corporation

Project Number: 850047-30

Date: 2/26/86

D. Reviewed By: Alan C. Katz, M.S., D.A.B.T.
Toxicologist
Toxicology Branch
Hazard Evaluation Division (TS-769C)

(Signature)
7/20/87
(Date)

E. Secondary Review: Marcia vanGemert, Ph.D.
Head, Section III
Toxicology Branch (TS-769C)

(Signature)
7/23/87
(Date)

M. vanGemert
7/23/87

F. Classification: Acceptable

G. Conclusion:

Under the conditions of this assay, Maneb was found to be negative for induction of Sister Chromatid Exchange (SCE) in CHO cells in the absence of a metabolic activation system and positive with activation.

H. Materials and Methods:

A description of materials and methods, as excerpted from the study report, is presented in the Appendix.

Deionized water was used as the vehicle and negative control. The assays were conducted with and without metabolic activation. The activated systems utilized the S9 microsome fraction from the livers of Sprague Dawley rats and $B6C3F_1$ mice (in separate assays) induced with Aroclor 1254. Fifty cells were scored for each concentration level of Maneb tested. Cyclophosphamide (2.5 ug/ml) and mitomycin C (0.05 ug/ml) served as positive controls in the assays with activated and non-activated systems, respectively.

Range-finding experiments were performed to evaluate the effects of the test material on the CHO cells. These preliminary experiments were conducted with and without rat S9. The range of concentrations used with mouse S9 was based on the results of the rangefinding studies with rat S9.

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I. Results:

Results of the nonactivated assay are summarized in Table 1, as excerpted from the study report. At a concentration of 10 ug/ml, the number of cells in metaphase was insufficient for evaluation. With the 4 concentrations tested in the range of 0.5 to 5 ug/ml, the mean numbers of SCE's per cell were comparable to the negative (solvent) control value. Mitomycin C was demonstrated to be an effective positive control.

In an initial rat S9 activated assay (Table 2A), slightly increased frequencies of SCE's were reported for concentrations of Maneb from 10 to 30 ug/ml. These increases ranged from 10 to 25 percent above solvent control values. At 50 ug/ml, results were reportedly not analyzed due to an extremely low mitotic index. No difference from the solvent control was found at 3 ug/ml. In a repeated assay with rat S9 (Table 2B), SCE's were increased by 18 and 26 percent at Maneb concentrations of 20 and 30 ug/ml, respectively (the 2 highest concentration levels tested). Cyclophosphamide was shown to be effective as a positive control in each of these assays.

In the mouse S9 activated assay (Table 3), mean SCE's were increased by 10 to 33 percent when tested at 4 concentration levels from 1 to 10 ug/ml; however, these increases were not found to be concentration-dependent. Cyclosphosphamide exhibited strong activity (i.e., a 667 percent increase in SCE's).

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TABLE 1

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The Effects of Technical Grade Maneb on the Imduction of Sister Chromatid Exchanges in CHO Cells

Nonactivated Assay

	Mitotic Index	Cell Cycle Stages(%) M1	M2	Total# Cells Scored	No. of SCE's	SCE/ Cell	% Increase Over Solvent Control
							Solvent Control= 7.7
Solvent Control: 100% Medium	0.020	14	86	50	386	7.7	
Positive Control: 0.05 ug/ml Mitomycin C	0.016	0	100	10	563	56.3	631
Technical Grade Maneb (ug/ml suspension)							
10	Insufficient number of cells suitable for analysis						
5	0.054	10	90	50	354	7.1	-
3	0.018	20	80	50	364	7.3	-
1.5	0.036	8	92	50	375	7.5	-
0.5	0.046	2	98	50	367	7.3	-

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TABLE 2A

The Effects of Technical Grade Maneb on the Induction of Sister Chromatid Exchanges in CHO Cells

Activated Assay (Rat S9)

	Mitotic Index	Cell Cycle Stages(%) M1	M2	Total# Cells Scored	No. of SCE's	SCE/ Cell	% Increase Over Solvent Control
Solvent Control: 10C% Medium + S9	0.115	2	98	50	406	8.1	Solvent Control= 8.1
Positive Control: 2.5 ug/ml Cyclophosphamide	0.065	0	100	10	380	38.0	369
Technical Grade Maneb (ug/ml suspension)							
50	Extremely low mitotic index; not analyzed						
30	0.018	14	86	50	505	10.1	25
15	0.054	28	72	50	496	9.9	22
10	0.037	24	76	50	447	8.9	9.9
3	0.074	0	100	50	405	8.1	-

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TABLE 2B

The Effects of Technical Grade Maneb on the Induction of Sister Chromatid Exchanges in CHO Cells

Repeat Activated Assay (Rat S9)

	Mitotic Index	Cell Cycle Stages(%) M1	M2	Total# Cells Scored	No. of SCE's	SCE/ Cell	% Increase Over Solvent Control
							Solvent Control= 10.1
Solvent Control: 100% Medium + S9	0.048	0	100	50	506	10.1	
Positive Control: 2.5 ug/ml Cyclophosphamide	0.051	0	100	10	492	49.2	387
Technical Grade Maneb (ug/ml suspension)							
30	0.046	4	96	50	636	12.7	26
20	0.059	0	100	50	596	11.9	18
15	0.054	0	100	50	437	8.7	-
5	0.052	0	100	50	542	10.8	7

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TABLE 3

The Effects of Technical Grade Maneb on the Induction of Sister Chromatid Exchanges in CHO Cells

Activated Assay (Mouse S9)

	Mitotic Index	Cell Cycle Stages(%) M1	M2	Total# Cells Scored	No. of SCE's	SCE/ Cell	% Increase Over Solvent Control
Solvent Control: 100% Medium + S9	0.043	0	100	50	366	7.3	Solvent Control= 7.3
Positive Control: 2.5 ug/ml Cyclophosphamide	0.058	4	96	10	560	56	667
Technical Grade Maneb (ug/ml suspension)							
30	No metaphase cells						
10	0.021	0	100	50	409	8.2	12
5	0.072	2	98	50	487	9.7	33
3	0.074	0	100	50	398	8.0	10
1	0.035	0	100	50	431	8.6	18

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APPENDIX

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2.0 MATERIALS

2.1 Test Substance

Name:	Technical Grade Maneb
ABC No.:	85-11A
Lot No.:	MT 01
Physical State:	powder
Color:	yellow
Purity:	88.1% a.i. Technical, 11.9% Inerts
Compostion:	See purity
Stability of Test Article:	determined by Sponsor
Stability of Formulations:	determined by Sponsor
Solubility:	slightly soluble in water
Storage Conditions:	4°C
Safety Precautions:	avoid topical and respiratory contact

The test sample was assayed in suspension using deionized water as a vehicle. A 1 mg/ml stock suspension was prepared for the nonactivated assay, the activated assay using rat liver S9 and the activated assay using mouse liver S9. All further dilutions were made in deionized water. The initial stock and all dilutions were prepared immediately prior to use. All stock suspensions were thoroughly agitated immediately prior to each use, however, due to the insolubility of the test sample in water, particles settled out immediately. Suspensions were based on the weight of the test sample as received; no corrections were made using the purity of the active ingredient.

The sponsor has assumed responsibility for performing analysis to determine the characterization and stability of test article and concentration analysis of the test article mixtures, as appropriate. Homogeneity analysis of the suspension was not performed prior to the test.

2.2 Negative Control Substance

Name: Sterile deionized water, the resistivity of which is greater than 10 megaohms.

2.3 Positive Control Substances:

Name:	Cyclophosphamide(CP)	Mitomycin C
Supplier:	Sigma Chem. Co.	Sigma Chem. Co.
Lot. No.:	33F-0157	123F-0463
Physical state:	powder	powder
Color:	white	bluish tint
Purity:	reagent grade	reagent grade
Solubility:	soluble in H_2O	soluble in H_2O
Stability:		
(solid)	at least 1 yr at 4°C	at least 1 yr at 4°C
(solution)	at least 4 mo at 4°C	at least 4 mo at 4°C

Composition: on file with manufacturer
Storage Conditions: 4°C 4°C
Safety Precautions: avoid topical and respiratory contact

Stock solutions of the positive controls were prepared and aliquots added to the exposure medium. A 5.0 ug/ml stock solution of Mitomycin C in deionized water was used to provide a final concentration of 0.05 ug/ml. A 250 ug/ml stock solution of Cyclophosphamide in deionized water was used to provide a final concentration of 2.5 ug/ml.

2.4 CHO Cell Culture

Cells used in this assay were obtained from Dr. Sheila Galloway at Litton Bionetics, Kensington, MD.

Master vials are stored in liquid nitrogen or in a freezer at -30°C at passage 9. Stock cultures are used at passage 9-15 for the Sister Chromatid Exchange Assay. All the frozen cultures have been prescreened for mycoplasma contamination. Working cultures are maintained in cell culture incubators in McCoy's 5A medium plus 10% fetal calf serum.

2.5 Microsomal Activation System

Two sources of S9 microsome fraction prepared from the liver of Aroclor 1254 induced Sprague-Dawley rats and mice (B6C3F1) were used in the activated assays. The S9 fraction was combined with a solution of cofactors and culture medium to form the metabolic activation system. A mixture of 10% 10X Isocitrate Cofactors and 90% serum-free McCoy's 5A medium was prepared and used as the exposure medium. Following the addition of the test sample, an aliquot of the S9 fraction was added to each flask. The final concentrations in each flask were 4.5 mg/ml Isocitric acid (trisodium salt), 2.4 mg/ml NADP and 20 ul/ml S9 fraction. The cofactor/medium mixture was prepared just prior to use and the S9 fraction was maintained on ice until use.

The following S9 fractions were used in the study:

Rat Liver S9
Source: Microbiological Associates, Bethesda, MD
Lot/Batch No.: R-216
Storage Conditions: -80°C

Mouse Liver S9
Source: Microbiological Associates, Bethesda, MD
Lot/Batch No.: M-107
Storage Conditions: -80°C

2.6 Identification of Test System

All the experimental vessels were labeled with the last five digits of the project number, assay number and a code number. The keys to both the assay and the code numbers are given in the raw data sheets.

3.0 EXPERIMENTAL DESIGN

3.1 Range-finding Experiments

The effect of Maneb on the cell cycle of CHO cells was initially characterized under standard activated (rat liver S9) and nonactivated conditions. Initial range-finding experiments delineated the concentrations used in subsequent sister chromatid exchange assays. The methods used in the range-finding experiments (growing cells, chemical exposure and cell harvest) are outlined below. The cell cycle stage of cells exposed to Maneb was determined by counting 100 metaphase cells.

3.2 SCE Induction Assay

Nonactivated Assay

CHO cells were seeded at a density of 1.5 x 10^6 in plastic T75 flasks. One day later the cells were exposed in McCoy's 5A Medium supplemented with 10% fetal calf serum, 20 mM HEPES buffer and 1% Penicillin-streptomycin to a minimum of 5 test sample concentrations, the positive and negative controls. Two hours later BrdU (final concentration 1×10^{-5}M) was added to the exposure medium and the cells were incubated for an additional 26 hours. The cells were washed with phosphate buffered saline and fresh medium containing BrdU (final concentration 1×10^{-5} M) and vinblastine sulfate (final concentration 0.26 ug/ml) was added. The cells were harvested for the preparation of slides 2-2½ hours later.

At the end of the incubation period, metaphase cells were collected by treatment with trypsin and concentrated by centrifugation with a table top centrifuge for 5 minutes. The cells were suspended in hypotonic solution (0.03 M KCl and 0.01 M sodium citrate) for 12 minutes at 37°C and fixed 3 times in 3:1 methanol: acetic acid. Drops of the concentrated cell suspension were placed on glass slides and air dried. Slides were stained in a solution of 50 ug/ml 33258 Hoechst in phosphate buffered saline. A cover slip was placed over the slide and the cells were irradiated with a UV lamp for 30 minutes. The cover slips were removed, the slides were rinsed with deionized water and incubated in 2X SSC for 15 minutes at 65°C (SSC: 0.015 M sodium citrate, 0.15 M NaCl). The slides were rinsed with H_2O and stained with 5% Giemsa for approximately 5 minutes at room temperature.

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Activated Assay

The methods used in the activated assay followed that of the nonactivated assay with the following changes. The exposure time was 2 hours in serum-free medium in the absence of BrdU. Following the removal of the test sample, the cells were washed with phosphate buffered saline and grown for approximately 28 hours in medium containing serum and BrdU. The cells were harvested at the end of the growth period as outlined above.

Analysis

For a minimum of four test sample concentrations and the negative control, 50 cells per flask were analyzed using a 100X objective; for the positive controls ten cells were analyzed. The mitotic index (minimum of 500 cells) and cell cycle stage (minimum of 50 cells) were recorded for each concentration analyzed.

Mitotic Index (MI) = # metaphase cells/total # cells

SCE/cell = Total # SCE/# cells scored

$$\text{\% increase over negative control} = \frac{\text{SCE/cell(test sample)} - \text{SCE/cell (negative control)}}{\text{SCE/cell (negative control)}}$$

M1 - first division metaphase cells
M2 - second division metaphase cells
(needed to score SCEs)

A test sample was considered to be negative if less than a 20% increase in SCE/cell compared to the negative control was obtained. An increase in SCE/cell over the negative control greater than 20% was required before a test sample was considered positive. Other criteria such as a concentration dependent increase and the number of concentrations displaying a significant increase were considered before a positive conclusion was made.

To verify a positive response data obtained from the three highest concentrations analyzed and the negative controls were analyzed statistically according to the methods described in a paper by Margolin et al., Environmental Mutagenesis, vol. 8, 1986.

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