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FINAL

DATA EVALUATION REPORT

OIL OF CITRONELLA

Study Type: Mutagenicity: Salmonella typhimurium/Mammalian Microsome Mutagenicity Assay

Prepared for:

Health Effects Division
Office of Pesticide Programs
Environmental Protection Agency
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Work Assignment Number: 1-76
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GUIDELINE SERIES 84: MUTAGENICITY
SALMONELLA

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DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity: Salmonella typhimurium/mammalian microsome mutagenicity assay

EPA IDENTIFICATION Numbers:

Tox Chem. Number: 21901

MRID Number: 421513-10

TEST MATERIAL: TREO SPF 15 lotion

SYNONYMS: Oil of citronella

SPONSOR: Primavera Laboratories, Inc., New York, NY

STUDY NUMBER: 063629-2

TESTING FACILITY: United States Testing Company, Inc., Hoboken, NJ

TITLE OF REPORT: Ames Salmonella Microsome Mutagenesis Assay on TREO SPF 15 Lotion

AUTHOR: Wang, X.M.

REPORT ISSUED: December 2, 1991

CONCLUSIONS--EXECUTIVE SUMMARY: No conclusions can be reached from the Salmonella typhimurium/mammalian microsome plate incorporation assay conducted with TREO SPF 15 lotion. The study was seriously compromised for the following reasons:

1--Unacceptably high spontaneous reversion counts for strains TA98 and TA100.

2--Marked reductions in the sensitivity of strains TA98 and TA100 to direct-acting mutagens.

3--The use of an excessive concentration of the S9-activated positive control (10 µg/plate 2-anthramine).

(See Section C, Reported Results, for a detailed discussion.)

Based on the above considerations, it was concluded that the study was unacceptable, and, therefore, did not satisfy Guideline requirements (§84-2) for genetic effects Category I, Gene Mutations.

STUDY CLASSIFICATION: Unacceptable. The study should be repeated using established procedures for the Salmonella typhimurium/mammalian microsome plate incorporation assay.[1] The purity of the test compound should also be included with the report of the repeat study.

A. MATERIALS:

1. Test Material: TREO SPF 15 lotion

Description: Off-white emulsion
Lot number: Not reported
Purity: Not reported
Receipt date: Not reported
Stability: Not reported
Contaminants: None listed
Solvent used: Dimethyl sulfoxide (DMSO)
Other provided information: The storage conditions and frequency of dose solution preparation were not reported.

2. Control Materials:

Negative: Not done

Solvent/final concentration: DMSO/≤0.1 mL

Positive:

Nonactivation:

N-Methyl-N-nitro-N-nitrosoguanidine (MNNG)	10	µg/plate TA1535, TA100
2-Nitrofluorene (2-NF)	50	µg/plate TA1538, TA98
9-Aminoacridine (9-AA)	100	µg/plate TA1537

Activation:

2-Anthramine (2-AA)	10	µg/plate all strains

3. Activation: S9 derived from adult male

x Aroclor 1254	x induced	x rat	x liver
_____ phenobarbital	_____ noninduced	_____ mouse	_____ lung
_____ none		_____ hamster	_____ other
_____ other		_____ other	

[1]Maron, D.M. and Ames, B.N. (1983). Revised Methods for the Salmonella Mutagenicity Test. Mutat. Res. 113:173-215.

Neither the strain of rats nor the source of the S9 were reported.

S9 mix composition:

Component:	Concentration/mL
Sodium phosphate buffer (pH 7.4)	100 µmoles
Glucose 6-phosphate	5 µmoles
NADP	4 µmoles
KCl	33 µmoles
$MgCl_2$	8 µmoles
S9	100 µL

4. Test Organism Used: S. typhimurium strains
_____ TA97 __x__ TA98 __x__ TA100 _____ TA102 _____ TA104
__x__ TA1535 __x__ TA1537 __x__ TA1538
list any others:

Test organisms were properly maintained: Overnight cultures were prepared from master plates held at 4°C for no more than 1 month. There is some ambiguity about the source of cultures for the master plates. The study author stated that "master plates are prepared from frozen permanents kept at 0°C," but also stated that cultures are stored "in liquid nitrogen at approximately -190°C."

Checked for appropriate genetic markers (rfa mutation, R factor): Yes.

5. Test Compound Concentrations Used:

(a) Preliminary cytotoxicity assay: Up to 10,000 µg/plate; the concentrations tested were not specified.

(b) Mutation assay: Five doses (50, 150, 500, 1500, and 5,000 µg/plate) were evaluated in triplicate in the presence and absence of S9 activation; all tester strains were used.

B. TEST PERFORMANCE:

1. Type of Salmonella Assay:
__x__ Standard plate test
_____ Pre-incubation (_____) minutes
_____ "Prival" modification
_____ Spot test
_____ Other (describe)

2. Methods:

(a) Preliminary cytotoxicity/mutation assays: The procedure used for the preliminary cytotoxicity assay was not specified.

For the mutation assay, 0.1 mL of a 16±4-hour culture of the appropriate tester strain, and up to 100 µL of the appropriate test

material dose, solvent, or positive controls were added to tubes containing 2-mL volumes of molten top agar. For the S9-activated tests, 0.5 mL of the S9-cofactor mix was added. Tester strains, and test and control solutions were added as described. The contents of the tubes were mixed, poured over Vogel-Bonner minimal medium E plates, and incubated at 37°C for 48±4 hours. Triplicate plates per strain, per dose, per condition were used for the test compound, solvent, and positive controls.

3. Evaluation Criteria: The test material was considered positive for a particular strain and condition if it caused at least a doubling in the number of revertants of strains TA98 or TA100, or a tripling in the number of revertants of strains TA1535, TA1537, or TA1538.

4. Statistical Analysis: The data were not analyzed statistically.

5. Protocol: See Appendix B.

C. REPORTED RESULTS

1. Preliminary Cytotoxicity Assay: Neither the data nor the details of the preliminary cytotoxicity assay were reported. The study author stated that the test material was evaluated in strains TA100 and TA1537 at concentrations up to 10,000 µg/plate. The study author further stated that cytotoxicity was observed between 1000 and 10,000 µg/plate. Accordingly, the dose range selected for the mutation assay was 50-5,000 µg/plate +/-S9.

2. Mutation Assay: Representative results from the mutation assay with TREO SPF 15 lotion are presented in Table 1. As shown, solvent control colony counts for strain TA98 +S9 and strain TA100 +/-S9 were well beyond the expected range reported by Maron and Ames[2] (TA98:30-50 revertants/plate; TA100: 120-200 revertants/plate) and either approached the acceptability limit for TA98 (15-75) or exceeded the range for TA100 (60-220) established by other investigators.[3] The results with the nonactivated positive controls (10 µg/plate MNNG and 50 µg/plate 2-NF) further indicated that the higher-than-expected background counts for TA98 and TA100 coincided with a reduction in sensitivity.

Table 1 also shows that there was a marked difference in the number of revertant colonies of TA1538 and TA98 induced by 50 µg/plate 2-NF. Exposure of these strains to comparable doses of 2-NF generally results in the production of approximately equivalent numbers of revertant colonies. While the response (i.e., fold increase) varies as a function of spontaneous mutant counts, the actual number of mutant colonies induced by 2-NF is consistently close. Similarly, marked differences

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[2]Maron, D.M. and Ames, B.N. (1983).

[3]deSerres, F.J. and Shelby, M.D. 1979. "Recommendations on Data Production and Analysis Using the Salmonella/Microsome Mutagenicity Assay." Environmental Mutagenesis 1:87-92.

between the number of mutant colonies induced in strains TA1535 and TA100 by 10 µg/plate MNNG were observed in this study, even though a similar phenomenon exists for these strains.

Excessive levels of 2-AA (10 µg/plate) were used for the S9-activated positive control. Such high levels can be expected to be cytotoxic, resulting in reduced revertant levels. Finally, the reporting of colony counts >3000/plate is an unacceptable practice. The generally accepted rule, whether counting manually or using an automatic colony counter, is that counts ≥3000 colonies/plate can not be accurately determined.

Based on the above considerations, it was concluded that the study is invalid.

D. REVIEWERS' DISCUSSION/CONCLUSIONS: The numerous technical deficiencies identified in the review of the study precludes an evaluation of the relevance of the findings with the test material. The study should be repeated using established procedures for the S. typhimurium/mammalian microsome mutagen-icity assay.[4] Additionally, information about the purity of the test material should be submitted with the repeat study.

E. QUALITY ASSURANCE MEASURES: Was the test performed under GLP? Yes. (A quality assurance statement was signed but not dated.)

F. CBI APPENDICES: Appendix A, Introduction and Materials and Methods, CBI pp. 7-9; Appendix B, Protocol, CBI pp. 20-23.

CORE CLASSIFICATION: Unacceptable. The study does not satisfy the data Guideline requirement (§84-2) for genetic effects Category I, Gene Mutations.

[4]Maron, D.M. and Ames, B.N. (1983).

TABLE 1: Representative Results of the Salmonella typhimurium/Mammalian Microsome Mutation Assay with TREO SPF 15 Lotion

Substance	Dose/Plate	S9 Activation	Revertants per Plate of Bacterial Tester Strain[a] TA1535	TA1537	TA1538	TA98	TA100
Solvent Control							
Dimethyl sulfoxide	≤100 µL	-	19±6	16±3	29±4	43±2	275±16
	≤100 µL	+	37±4[b]	13±2	31±7	74±8	370±6
Positive Controls							
N-methyl-N-nitro-N-nitrosoguanidine	10 µg	-	4019±113	--	--	--	1027±123
9-Aminoacridine	100 µg	-	--	641±405	--	--	--
2-Nitrofluorene	50 µg	-	--	--	3044±154	1544±169	--
2-Anthramine	10 µg	+	469±201	1151±13	3549±44	3753±192	3270±103
Test Material							
TREO SPF 15 lotion	500 µg[c]	-	25±5	15±6	30±7	47±5	275±12
	1500 µg	-	18±9	7±2	19±7	36±4	309±46
	5000 µg	-	12±3	0±0	10±5	33±3	286±28
	150 µg[c]	+	28±1	24±3	24±6	109±4	370±23
	500 µg	+	26±11	20±6	28±3	96±13	328±16
	1500 µg	+	28±1	20±6	30±3	90±13	343±32
	5000 µg	+	22±5	20±2	32±10	75±3	306±18

[a]Means and standard deviations of the counts from triplicate plates.
[b]Calculated by our reviewers; erroneously reported by the study author as 27±21.
[c]Results for lower doses (50 and 150 µg/plate -S9, and 50 µg/plate +S9) did not suggest a mutagenic effect.

APPENDIX A

INTRODUCTION AND MATERIALS AND METHODS
CBI pp. 7-9

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Page_____ is not included in this copy.

Pages 9 through 16 are not included.

The material not included contains the following type of information:

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