

US EPA ARCHIVE DOCUMENT

Primary Review by: Stephen C. Dapson, Ph.D. *Stephen C. Dapson 8/11/88*  
Pharmacologist, Review Section V, Toxicology Branch/HED (TS-769C)  
Secondary Review by: Kerry Dearfield, Ph.D. *Kerry Dearfield 8.16.88*  
SMSS, Toxicology Branch/HED (TS-769C)  
Date: August 11, 1988

DATA EVALUATION RECORD
------------------------

CHEMICAL: Methoxychlor

Tox. Chem. No.: 550

Tox Branch Project No: 8-0131

STUDY TYPE: Salmonella/mammalian activation gene mutation assay

MRID NUMBER(S): 28625, 132952 and 133008,

SYNONYMS/CAS No.: methyl-DDT, Marlate, 72-43-5

SPONSOR: United States Environmental Protection Agency  
Health Effects Research Laboratory  
Research Triangle Park, NC 27711

TESTING FACILITY: SRI International  
Menlo Park, California 94025

TITLE OF REPORT: In Vitro Microbiological Mutagenicity and  
Unscheduled DNA Synthesis Studies of Eighteen  
Pesticides

AUTHOR(S): Vincent F. Simmons, Ph.D.

STUDY NUMBER(S): EPA-600/1-79-041  
Contract No. 68-01-2458

REPORT ISSUED: October 1979

CONCLUSION(S) - Executive Summary:

No increase in the number of revertant colonies was noted in Salmonella strains TA98, TA100, TA1535, TA1537 and TA1538 up to 5000 ug/plate with and without metabolic activation. This assay was performed twice with only one plate used per concentration per experiment. Also, there was a lack of positive controls in several instances for particular strains and activation conditions. Due to use of only one plate per experiment (which limits the confidence of negative results) and the lack of positive controls in several instances, this study is unacceptable.

Classification: Unacceptable

Bibliographic Citation

Simmon, V.F. (1979) °In vitro° Microbiological Mutagenicity and Unscheduled DNA Synthesis Studies of Eighteen Pesticides: Report No. EPA-600/1-79-041. (Unpublished study including submitter summary, recieved Apr 3, 1980 under 279-2712; prepared by SRI International, submitted by FMC Corp., Philadelphia, PA.; CDL:099350-A)

Simmon, V. (1979) In vitro Microbiological Mutagenicity and Unscheduled DNA Synthesis Studies of Eighteen Pesticides: Contract No. 68-01-2458. (Unpublished study received Dec 5, 1983 under 239-2471; prepared by SRI International, submitted by Chevron Chemical Co., Richmond, CA; CDL:251894-F)

Simmon V. (1979) In vitro microbiological mutagenicity and unscheduled DNA synthesis studies of eighteen pesticides. By SRI International. Research Triangle Park, N.C.: U.S. Environmental Protection Agency, Office of Research and Development, Health Effects Laboratory, Genetic Toxicology Div. (EPA 600/1-79-041; Contract No. 68-01-2458; also in unpublished submission received Dec 2, 1983 under 279-2038; submitted by FMC Corp., Philadelphia, PA; CDL:251984-C)

A. MATERIALS A copy of the "materials and methods" section from the investigators report is appended.

1. Test Material: Name: Methoxychlor

Description: technical

Batch #6543-108

Purity: Technical

Contaminants: none reported

Solvent used: DMSO

Other comments: Manufactured by E.I. du Pont de Nemours & Co.

2. Control Materials:

Negative: DMSO

Solvent/final concentration: DMSO, 1%

Positive: Non-activation:

Beta-Propiolactone: 50 ug/plate TA1535 and TA100

Activation:

2-Aminoanthracene (2-anthramine) 20 ug/plate  
for strains, see tables

3. Activation: S9 derived from Aroclor 1254 induced rat liver  
S9 mix composition:  
The metabolic activation mixture consists of, for 10 ml:
  - 1.00 ml of freshly thawed S-9 fraction
  - 0.20 ml of MgCl<sub>2</sub> (0.4 M) and KCl (1.65M)
  - 0.05 ml of glucose-6-phosphate (1 M)
  - 0.40 ml of NADP (0.1 M)
  - 5.00 ml of sodium phosphate (0.2 M, pH 7.4)
  - 3.35 ml of H<sub>2</sub>O
  
4. Test organisms: Salmonella typhimurium strains  
TA98, TA100, TA1535, TA1537, TA1538  
Properly maintained? yes  
Checked for appropriate genetic markers (rfa mutation,  
R factor)? yes  
Obtained from Dr. Bruce Ames
  
5. Test compound concentrations used:  
Non-activated conditions: 1, 10, 50, 100, 500, 1000, 2500 and  
5000 ug/plate  
Activated conditions: 1, 10, 50, 100, 500, 1000, 2500 and 5000  
ug/plate

## B. TEST PERFORMANCE

1. Type of Salmonella assay: standard plate test .

### a. Protocol:

Stock cultures were grown overnight (see attached "materials and methods" for detail). The investigators mixed the culture, agar, the test compound and the metabolic activator (as necessary) together in a tube and added this to minimal agar plates. The plates were then incubated for 2 days at 37°C. The number of his<sup>+</sup>-revertant colonies were then counted.

2. Preliminary cytotoxicity assay:

No preliminary cytotoxicity studies were conducted, two primary studies were conducted (see section 3 following).

3. Mutagenicity assay:

Attached Tables 35 and 36 (appended from the investigator's report) presents the results of the 2 assays conducted. The test article was assayed in two separate experiments with one plate per concentration used. No differences in numbers of revertant colonies were noted at any of the dose levels tested with and without metabolic activation, whereas the positive controls that were used, did cause an increase in numbers of revertant colonies.

4. Reviewer's discussion/conclusions:

The first test assayed to 1000 ug/plate with no apparent toxicity noted. Therefore, the second test (Experiment 2, Table 36) went to the limit dose of 5000 ug/plate with no apparent effects with and without metabolic activation. As only one plate per concentration was used, this would limit the confidence in assessing the apparent negative result. Also, there was a lack of appropriate positive controls in several instances for particular strains and activation conditions (e.g. no positive controls for strains TA1537, TA1538 and TA98 without activation). Therefore, this study is not adequate for regulatory purposes and should be repeated to satisfy regulatory requirements.

5. Was test performed under GLPs (is a quality assurance statement present)? no

6. CBI appendix attached? This is a published study.

1

RAVE SYSTEMS & RESEARCH INC  
 PEREZ-3-13-84-COMC 3

Table 35  
 IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM  
 OF METHOXYCHLOR  
 EXPERIMENT 1

Compound	Metabolic Activation	Micrograms of Compound Added per Plate	Histidine Revertants per Plate					
			TA1535	TA1537	TA1538	TA98	TA100	
Negative control	-		25	10	18	18	30	115
	+		14	6	26	30	30	108
Positive controls	-	50	922					
β-Propionolactone	-	20			19	43		
2-Anthraine	+	20		98	175	216	340	
Methoxychlor	-	1	24	13	15	31	98	
	-	10	29	5	26	30	106	
	-	50	27	8	19	34	118	
	-	100	41	8	17	38	113	
	-	500	29	7	17	29	129	
	-	1000	27	6	23	34	137	
	+	1	13	8	15	29	137	
	+	10	20	9	22	34	96	
	+	50	12	5	22	19	88	
	+	100	15	8	16	17	98	
	+	500	16	8	27	32	99	
	+	1000	15	8	24	24	115	

BEST DOCUMENT AVAILABLE

266

RAV-1 SYSTEMS & RESEARCH INC  
 PEREZ-3-13-86-COMCHA B

Table 36  
IN VITRO ASSAYS WITH SALMONELLA TYPHIMURUM  
OF METHOXYCHLOR  
 EXPERIMENT 2

Compound	Metabolic Activation	Micrograms of Compound Added per Plate	Histidine Reversions per Plate				
			TA1535	TA1537	TA1538	TA100	
Negative control	-		38	13	20	23	149
	+		16	22	35	35	137
Positive controls 2-Anthracine	-	20	53	19	24	29	190
	+	20	585	227	2087	293	2616
	-	50	1560				1120
β-Propiolactone	-	50	42	16	12	16	183
	-	100	68	16	23	16	145
	-	500	56	12	16	27	167
	-	1000	58	10	14	20	168
	-	2500	66	17	14	17	172
	-	5000	50	17	31	24	170
Methoxychlor	+	50	22	10	22	45	164
	+	100	22	11	45	31	150
	+	500	16	9	22	36	151
	+	1000	18	12	33	30	150
	+	2500	27	26	35	28	155
	+	5000	24	19	33	46	160

BEST DOCUMENT AVAILABLE

6

RECEIVED SYSTEMS & RESEARCH INC  
PETRI - -3-13-84-CGACK B

METHODS

Microbiological Assays

The in vitro microbiological assay systems used to examine the 18 pesticides for mutagenicity were Salmonella typhimurium (TA1535, TA1537, TA1538, TA98, and TA100), Escherichia coli WP2, repair-deficient and -proficient strains of Bacillus subtilis (H17 and M45) and of E. coli (W3110 and p3478); and the yeast Saccharomyces cerevisiae D3. In each procedure except the relative toxicity assays, an Aroclor 1254-stimulated, rat liver homogenate metabolic activation system was included to provide metabolic steps that the microorganisms either are incapable of conducting or do not carry out under the assay conditions.

The assay procedure with S. typhimurium has proven to be 80 to 90% accurate in detecting carcinogens as mutagens, and it has about the same accuracy in identifying chemicals that are not carcinogenic.<sup>2,3</sup> The assay procedure with S. cerevisiae is about 55% accurate in detecting carcinogens as agents that increase mitotic recombination.<sup>4</sup> E. coli WP2 and the relative toxicity assays are three additional methods of detecting mutagens; however, the reliability of these test methods has not been adequately validated yet. The combination of these five assay procedures significantly enhances the probability of detecting potentially hazardous chemicals.

Salmonella typhimurium Strains TA1535, TA1537, TA1538, TA98, and TA100

The Salmonella typhimurium strains used at SRI are all histidine auxotrophs by virtue of mutations in the histidine operon. When these histidine-dependent cells are grown on a minimal media petri plate containing a trace of histidine, only those cells that revert to histidine independence (his<sup>+</sup>) are able to form colonies. The small amount of histidine allows all the plated bacteria to undergo a few divisions; in many cases, this growth is essential for mutagenesis to occur. The his<sup>+</sup> revertants are easily scored as colonies against the slight



RAYE SYSTEMS & RESEARCH INC  
PERC - -3-13-84-CGICHA B

17

background growth. The spontaneous mutation frequency of each strain is relatively constant, but when a mutagen is added to the agar, the mutation frequency is increased 2- to 100-fold, usually in a dose-related manner.

We obtained our S. typhimurium strains from Dr. Bruce Ames of the University of California at Berkeley.<sup>3,5-10</sup> In addition to having mutations in the histidine operon, all the indicator strains have a mutation (rfa) that leads to a defective lipopolysaccharide coat; they also have a deletion that covers genes involved in the synthesis of the vitamin biotin (bio) and in the repair of ultraviolet (uv)-induced DNA damage (uvrB).<sup>5</sup> The rfa mutation makes the strains more permeable to many large aromatic molecules, thereby increasing the mutagenic effect of these molecules. The uvrB mutation causes decreased repair of some types of chemically or physically damaged DNA and thereby enhances the strains' sensitivity to some mutagenic agents. Strain TA1535 is reverted to his<sup>+</sup> by many mutagens that cause base-pair substitutions. TA100 is derived from TA1535 by the introduction of the resistance transfer factor, plasmid pKM101. This plasmid is believed to cause an increase in error-prone DNA repair that leads to many more mutations for a given dose of most mutagens.<sup>7</sup> In addition, plasmid pKM101 confers resistance to the antibiotic ampicillin, which is a convenient marker to detect the presence of the plasmid in the cells. We have shown that TA100 can detect mutagens such as benzyl chloride and 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF2) that are not detected by TA1535. The presence of this plasmid also makes strain TA100 sensitive to some frameshift mutagens (e.g., ICR-191, benzo(a)pyrene, aflatoxin B<sub>1</sub>, and 7,12-dimethylbenz(a)anthracene). Strains TA1537 and TA1538 are reverted by many frameshift mutagens. Strain TA98 is derived from TA1538 by the addition of plasmid pKM101, which makes it more sensitive to some mutagenic agents.

All indicator strains are kept at 4° C on minimal agar plates, supplemented with an excess of biotin and histidine. The plates with the plasmid-carrying strains contain, in addition, ampicillin (25 µg/ml) to ensure stable maintenance of plasmid pKM101. New stock culture plates are made every four to six weeks from single colony reisolates

6  
BEST DOCUMENT AVAILABLE

140

that have been checked for their genotypic characteristics (his, rfa, uvrB, bio) and for the presence of the plasmid. For each experiment, an inoculum from the stock culture plates is grown overnight at 37° C in nutrient broth (Oxoid, CM67). After stationary overnight growth, the cultures are shaken for 3 to 4 hours to ensure optimal growth.

To a sterile 13 x 100 mm test tube placed in a 43° C heating block, we add in the following order:

- (1) 2.00 ml of 0.6% agar\*
- (2) 0.05 ml of indicator organisms
- (3) 0.50 ml of metabolic activation mixture (optional)†
- (4) 0.05 ml of a solution of the pesticide dissolved in DMSO.

For negative controls, we use steps (1), (2), and (3) (optional) and 0.05 ml of the solvent used for the test chemical. For positive controls, we test each culture by specific mutagens known to revert each strain, using steps (1), (2), (3) (optional), and (4).

This mixture is stirred gently and then poured onto minimal agar plates.† After the top agar has set, the plates are incubated at 37° C for 2 days. The number of his<sup>+</sup> revertant colonies is counted and recorded.

A positive response in the Salmonella/microsome assay is indicated by a reproducible, dose-related increase in the number of revertants in one or more of the tester strains.

Escherichia coli WP2

~~The E. coli WP2 (uvrA) strain used at SRI was obtained originally from Dr. D. McCalla. It is a cryptophan auxotroph (trp) by virtue of a~~

\*0.6% agar contains 0.05 mM histidine, 0.05 mM biotin, and 0.6% NaCl.

† See page 9.

‡ Minimal agar plates consist of, per liter, 15 g of agar, 20 g of glucose, 0.2 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 2 g of citric acid monohydrate, 10 g of K<sub>2</sub>HPO<sub>4</sub>, and 3.5 g of NaH<sub>2</sub>PO<sub>4</sub>·4H<sub>2</sub>O.

110  
V-3715 3-13-88-CCMERA B INC

strains. Therefore, if a chemical interacts with DNA, strain p3478 should be more sensitive than strain W3110 to any toxic effect due to this interaction.

The *S. subtilis* strains H17 and M45 were obtained from Dr. T. Kada.<sup>17</sup> Strain M45 (rec<sup>-</sup>) is derived from H17 but is deficient in the genetic recombination mechanism necessary to repair DNA damage. Cells deficient in this repair mechanism are killed more easily by chemical mutagens than are wild-type cells (rec<sup>+</sup>). If the chemical is toxic to rec<sup>-</sup> cells but at the same concentration is not toxic to rec<sup>+</sup> cells, the chemical is assumed to interact with DNA.

For each experiment, an inoculum from frozen stock cultures is grown overnight at 37° C with shaking in nutrient broth consisting of 1% tryptone and 0.5% yeast extract. A 0.1-ml aliquot of this bacterial culture (approximately 3 x 10<sup>8</sup> cells) is added to 2 ml of nutrient broth containing 0.6% agar. The suspension is mixed and poured onto the surface of a plate containing the same ingredients as the broth plus 2% agar (25 ml). When the top agar has solidified, a sterile filter disc impregnated with the test substance is placed in the center of the plate. The plates are incubated at 37° C for 16 hours; then the width (diameter) of the zone of inhibition of growth is measured. Several concentrations of the substance are usually tested. We routinely use DMSO as diluent and as solvent for crystalline chemicals.

The positive control for this assay is 1-phenyl-3,3-dimethyltriazine. The negative control is chloramphenicol, which should cause equal zones of inhibition in both strains because it is toxic to bacteria but does not kill by interacting with DNA.

Aroclor 1254-Stimulated Metabolic Activation

Some carcinogenic chemical (e.g., of the aromatic amino type or polycyclic hydrocarbon type) are inactive unless they are metabolized to active forms. In animals and man, an enzyme system in the liver or other organs (e.g., lung or kidney) is capable of metabolizing a large number of these chemicals to carcinogens.<sup>18-20</sup> Some of these intermediate metabolites are very potent mutagens in the *S. typhimurium*

4 STEAKS FEE... INC  
PERIC - - 3-13-86 - COMCIB 3

test. Ames has described the liver metabolic activation system that we use.<sup>10</sup> In brief, adult male Sprague-Dawley rats (250 to 300 g) are given a single 500-mg/kg intraperitoneal injection of a polychlorinated biphenyl, Aroclor 1254. This treatment enhances the synthesis of enzymes involved in the metabolic conversion of chemicals. Four days after the injection, the animals' food is removed, but drinking water is provided ad libitum. On the fifth day, the rats are killed and the liver homogenate is prepared as follows.

The livers are removed aseptically and placed in a preweighed sterile glass beaker. The organ weight is determined, and all subsequent operations are conducted in an ice bath. The livers are washed in an equal volume of cold, sterile 0.15 M KCl (1 ml/g of wet organ), minced with sterile surgical scissors in three volumes of 0.15 M KCl, and homogenized with a Potter-Elvehjem apparatus. The homogenate is centrifuged for 10 minutes at 9000 x g, and the supernatant, referred to as the S-9 fraction, is quickly frozen in dry ice and stored at -80° C.

The metabolic activation mixture consists of, for 10 ml:

- 1.00 ml of freshly thawed S-9 fraction
- 0.20 ml of MgCl<sub>2</sub> (0.4 M) and KCl (1.65 M)
- 0.05 ml of glucose-6-phosphate (1 M)
- 0.40 ml of NADP (0.1 M)
- 5.00 ml of sodium phosphate (0.2 M, pH 7.4)
- 3.35 ml of H<sub>2</sub>O.

Unscheduled DNA Synthesis Assays

Many mutagenic and carcinogenic agents have been shown to induce unscheduled DNA synthesis (UDS) in an in vitro tissue culture system of mammalian cells.<sup>11</sup> UDS is a form of mammalian repair synthesis that involves at least two processes: first, the agent interacts with DNA, resulting in damage to the DNA; then follows incorporation of nucleotide(s) to repair the DNA. UDS, which occurs in a wide variety of mammalian cell types, is considered to be a fairly universal

## REFERENCES

1. V. F. Simon, A. D. Mitchell, and T. A. Jorgenson. Evaluation of select pesticides as chemical mutagens: In vitro and in vivo studies. Environmental Health Effects Research Series EPA-600/1-77-028, May 1977.
2. J. McCann, E. Choi, E. Yamasaki, and B. N. Ames. Detection of carcinogens as mutagens in the Salmonella microsome test: Assay of 300 chemicals. Proc. Nat. Acad. Sci. USA 72, 5135-5139 (1975).
3. I. F. M. Purchase, E. Longstaff, J. Ashby, J. A. Styles, D. Anderson, P. A. Lafevre, and F. R. Westwood. Evaluation of six short term tests for detecting organic chemical carcinogens and recommendations for their use. Nature 254, 624-627 (1976).
4. V. F. Simon. In vitro assays for recombinogenic activity of chemical carcinogens and related compounds with Saccharomyces cerevisiae D3. J. Nat. Cancer Inst. 62 (in press) (1979).
5. B. N. Ames, E. G. Curney, J. A. Miller, and M. Bartsch. Carcinogens as frameshift mutagens: Metabolites and derivatives of 2-acetylaminofluorene and other aromatic amine carcinogens. Proc. Nat. Acad. Sci. USA 69, 3126-3132 (1972).
6. B. N. Ames, F. D. Lee, and W. E. Durston. An improved bacterial test system for the detection and classification of mutagens and carcinogens. Proc. Nat. Acad. Sci. USA 70, 782-786 (1973).
7. J. McCann, L. E. Spingarn, J. Koberi, and B. N. Ames. The detection of carcinogens as mutagens: Bacterial tester strains with R factor plasmids. Proc. Nat. Acad. Sci. USA 72, 979-983 (1975).
8. B. N. Ames, W. E. Durston, E. Yamasaki, and F. D. Lee. Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. Proc. Nat. Acad. Sci. USA 70, 2241-2285 (1973).
9. L. D. Kier, E. Yamasaki, and B. N. Ames. Detection of mutagenic activity in cigarette smoke condensates. Proc. Nat. Acad. Sci. USA 71, 4159-4163 (1974).
10. B. N. Ames, J. McCann, and E. Yamasaki. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Res. 31, 347-364 (1975).
11. D. R. McCalla and D. Voutsinos. On mutagenicity of nitrofurans. Mutation Res. 26, 3-16 (1974).
12. B. A. Bridges. Simple bacterial systems for detecting mutagenic agents. Lab. Pract. 21, 413-419 (1972).

13. F. K. Zimmermann and R. Schwaber. A genetic effect of symmetric dimethylhydrazine: Induction of mitotic recombination. *Naturwissenschaften* 54, 251 (1967).
14. D. J. Brusick and V. W. Mayer. New developments in mutagenicity screening techniques with yeast. *Envir. Health Perspectives* 6, 83-96 (1973).
15. E. E. Slater, M. D. Anderson, and W. S. Posenkrans. Rapid detection of mutagens and carcinogens. *Cancer Res.* 31, 970-973 (1971).
16. J. Cross and M. Cross. Genetic analysis of an *E. coli* strain with a mutation affecting DNA polymerase. *Nature* 224, 1166-1168 (1969).
17. T. Kada. Mutagenicity testing of chemicals in microbial systems. In *New Methods in Environmental Chemistry and Toxicology*. International Academic Printing Co., Totaka, Tokyo, 1973.
18. R. M. C. Lee and W. F. Stich. DNA repair synthesis of cultured human cells as a rapid bioassay for chemical carcinogens. *Int. J. Cancer* 16, 284-291 (1975).
19. B. Djordjevic and L. Tolmach. Response of synchronized populations of HeLa cells to ultraviolet irradiation at selected stages of the generation cycle. *Radiat. Res.* 32, 327 (1967).
20. R. E. Rasmussen and R. B. Painter. Radiation-stimulated DNA synthesis in cultured mammalian cells. *J. Cell Biol.* 29, 11 (1966).
21. G. Schmidt and S. J. Thannhauser. A method for the determination of deoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues. *J. Biol. Chem.* 161, 83-89 (1945).
22. G. M. Richards. Modification of the diphenylamine reaction giving increased sensitivity and simplicity in the estimation of DNA. *Anal. Biochem.* 57, 369-374 (1974).
23. N. R. Spiegel. *Theory and Problems of Statistics*. Schaum Publishing Co., New York, 1961, pp. 241-248.
24. W. C. Guenther. *Analysis of Variance*. Prentice-Hall, Inc., Englewood Cliffs, N. J., 1964, pp. 20-21, 31-43.
25. S. Siegel. *Nonparametric Statistics for the Behavioral Sciences*. McGraw-Hill Book Company, New York, 1956, pp. 184-194.
26. S. S. Epstein, E. Arnold, J. Andrea, W. Bass, and V. Bishop. Detection of chemical mutagens by the dominant lethal assay in the mouse. *Toxicol. Appl. Pharmacol.* 23, 288-325 (1972).
27. V. V. Brashchik. Possibility of induction of mutations in *Drosophila melanogaster* with chlorophos. *Mud. Parasit. Parazit. Belez.* 42, 703-706 (1973).
28. IARC. 2,4-D and Esters. In *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man Vol. 15*, IARC, Lyon, France, 1977, pp. 111-139.
29. Y. Shirasu. Significance of mutagenicity testing on pesticides. *Environ. Qual. Sat.* 4, 226-231 (1975).