Guideline Series 84: MUTAGENICITY

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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 Microbological 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 (Unpublished study including submitter summary, recieved Apr 3, 1980 under 279-2712; prepared by SRI International, submitted by FMC Corp., Philadelphia, PA.; CDL:099350-A)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 (Unpublished study received Dec 5, 1983 under 239-2471; prepared by SRI International, submitted by Chevron Chemical Co., Richmond, CA; CDL:251894-F)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 (EPA 600/1-79-041; Contract No. 68-01-2458; also in unpublished submission received Dec 2, 1983 under 279-2038; submitted by FMC Corp., Philadelphia, PA; CDL:251984-C)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 $MgCl_2$ (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_2O

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^+ revertant colonies were then counted.

2. Preliminary cytotoxicity assay:

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

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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.

Table 35

IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM

OF METHOXYCHLOR

EXPERIMENT 1

Compound	Metabolic Activation	Microgram of Compound Added per Plate	Histidine Revertants per Plate TA1535	TA1537	TA1538	TA98	TA100
Negative control	-		25	10	18	18	115
	+		14	6	26	30	108
Positive controls							
β-Propiolactone	-	50	922				
2-Anthramine	-	20			19	43	
	+	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

Table 36

IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM

OF METHOXYCHLOR

EXPERIMENT 2

Compound	Metabolic Activation	Micrograms of Compound Added per Plate	Histidine Revertants per Plate TA1535	TA1537	TA1538	TA98	TA100
Negative control	-		38	13	20	23	149
	+		16	22	35	35	137
Positive controls							
2-Anthramine	-	20	53	19	24	29	190
	+	20	585	227	2087	295	2616
β-Propiolactone	-	50	1560				1120
Methoxychlor	-	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
	+	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

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.[3,8] The assay procedure with *S. cerevisiae* is about 55% accurate in detecting carcinogens as agents that increase mitotic recombination.[4] *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*$^+$) 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*$^+$ revertants are easily scored as colonies against the slight

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.[2,8-10] 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). 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^+ 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.[7] 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_1, 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

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$^+$ 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.[11] It is a tryptophan 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 $MgSO_4 \cdot 7H_2O$, 2 g of citric acid monohydrate, 10 g of K_2HPO_4, and 3.5 g of $NaNH_4PO_4 \cdot 4H_2O$.

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 B. subtilis strains H17 and M45 were obtained from Dr. T. Kada.[17] Strain M45 (rec$^-$) 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$^+$). If the chemical is toxic to rec$^-$ cells but at the same concentration is not toxic to rec$^+$ 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 10^8 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.[8-10] Some of these intermediate metabolites are very potent mutagens in the S. typhimurium

test. Ames has described the liver metabolic activation system that we use.[10] 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 $MgCl_2$ (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_2O.

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.[10] 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

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