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: Escherichia coli WP2 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. Simmon, Ph.D.

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

REPORT ISSUED: October 1979

CONCLUSION(S) - Executive Summary:
No effect of Methoxychlor was noted on the numbers of tryptophan revertants in the E. coli WP2 reversion assay up to 5000 ug/plate unactivated and 1000 ug/plate metabolically activated. This assay was performed with only one plate used per concentration per experiment. Also, there was a lack of positive controls in most instances. Due to the use of only one plate per experiment (which limits the confidence of negative results), the loss of the highest concentration (5000 ug/plate) to contamination, with activation (therefore, not adequate top concentration), the lack of positive controls in many instances and the uncertainty of the solvent used, 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 (Unpublished study including submitter summary, received Apr 3, 1980 under 279-2712; prepared by SRI International, submitted by FMC Corp., Philadelphia, Pa.; CDL:099350-A)

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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 (EPA 600/1-79-041; Contract No. 68-01-2458; also In unpublished submission received Dec 2, 1983 under 279-2038; submitted by FMC Corp., Philadelphia, PA; CDL:251984-C)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: not stated
   Other comments: Manufacturer: E.I. du Pont de Nemours & Co.

2. Control Materials:
   Negative: not stated
   Solvent/final concentration: ?
   Positive: Non-activation: none
   Activation:
   2-Aminoanthracene (2-anthramine): 2.5 ug/plate

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 $MgCl_2$ (0.4 M) and KCl (1.65 M)
   0.05 ml of glucose-6-phosphate (1 M)
   0.40 ml of NADF (0.1 M)
   5.00 ml of sodium phosphate (0.2 m pH 7.4)
   3.35 ml of H_2O

4. Test organisms: Escherichia coli WP2 (uvrA)
   Properly maintained? yes
   Checked for appropriate genetic markers? yes
   Obtained from Dr. D. McCalla

5. Test compound concentrations used:
   Non-activated conditions: 1, 10, 50, 100, 500, 1000 and 5000 ug/plate
   Activated conditions: 1, 10, 50, 100, 500, 1000 and 5000 ug/plate

B. TEST PERFORMANCE

1. Type of E. coli assay: standard plate test

   a. Protocol:

An inoculum from the stock culture was grown overnight at 37°C . Supplemented agar with a trace of tryptophan, the test compound, metabolic activator (as necessary) and the indicator culture were placed in a test tube, then added to minimal agar plates and incubated for 2 days at 37°C. The number of revertant colonies were then counted.

2. Preliminary cytotoxicity assay

None conducted, two primary tests were conducted (see section 3 following).

3. Mutagenicity assay:

The following table (extracted from the investigators report) presents the results of 2 assays.

Controls	Metabolic Activation	Micrograms of Compound Added per Plate	Tryptophan Revertants per Plate
Negative control	-		23
	+		26
Positive controls:			
2-Anthramine	+	2.5	370
Experiment 1	-	1	19
	-	10	38
	-	50	20
	-	100	33
	-	500	18
	-	1000	23
	+	1	30
	+	10	29
	+	50	22
	+	100	19
	+	500	34
	+	1000	20
Negative control	-		45
	+		48
Experiment 2	-	10	28
	-	50	32
	-	100	25
	-	500	41
	-	1000	34
	-	5000	28
	+	10	48
	+	50	31
	+	100	48
	+	500	34
	+	1000	53
	+	5000	Contaminated

From the data presented, no effect of Methoxychlor on the total number of tryptophan revertants were noted at dose levels tested. However, only Experiment 1 had a positive control and only under metabolically activated conditions. Experiment 2-had no positive control groups and the highest dose tested under metabolically activated conditions was contaminated. Only one plate per concentration was used in each experiment.

4. Reviewer's discussion/conclusions:

Although no effects on the numbers of tryptophan revertants were noted at dose levels of Methoxychlor tested in this study, there was a lack of positive control groups. As only one plate per concentration was used, this would limit the confidence in assessing the apparent negative result. Also, the contamination at the highest concentration with activation reduced the adequacy of an appropriate top concentration (another short coming of using one plate per concentration). Further, the identity of the solvent is unclear. This study is not adequate for regulatory purposes and should be repeated to satisfy regulatory requirments.

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

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

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METHODS

Microbiological Assays

The *in vitro* microbiological assay systems used to examine the 18 pesticides fo· 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.[2,3] 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

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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

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*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 $NaHNH_4PO_4 \cdot 4H_2O$.

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base-pair substitution mutation in the tryptophan operon. In addition, WP2 is deficient in the repair of some physically or chemically induced DNA damage (uvrA).[12] This uvrA mutation makes the strain more sensitive to certain mutagens.

A procedure similar to the Ames Salmonella assay is used to measure the reversion of WP2 to tryptophan independence. However, the minimal agar is supplemented with 1.25 g of Oxoid nutrient broth (CM67) per liter to provide each plate with the trace of tryptophan required for enhancement of any mutagenic effect of the test chemical.[12] No additional tryptophan is added to the top agar.

Saccharomyces cerevisiae D3

The yeast S. cerevisiae D3 is a diploid microorganism heterozygous for a mutation leading to a defective enzyme in the adenine-metabolizing pathway.[13] When grown on medium containing adenine, cells homozygous for this mutation produce a red pigment. These homozygous mutants can be generated from the heterozygotes by mitotic recombination. The frequency of this recombinational event may be increased by incubating the organisms with various mutagens. The degree of mutagenicity of a compound or of its metabolite is determined from the number of red-pigmented colonies appearing on the plates.[14]

The S. cerevisiae tester strain is stored at -80° C. For each experiment, the tester strain is inoculated in 1% tryptone and 0.5% yeast extract and grown overnight at 30° C with aeration.

The in vitro yeast mitotic recombination assay in suspension is conducted as follows. The overnight culture is centrifuged, and the cells are resuspended at a concentration of 10^8 cells/ml in a 67 mM phosphate buffer (pH 7.4). To a sterile test tube are added:

- 1.30 ml of the resuspended culture
- 0.50 ml of either the metabolic activation mixture or buffer
- 0.20 ml of a solution of pesticide dissolved in DMSO or 0.20 ml of DMSO alone.

Several doses of the pesticide (up to 5%, w/v or v/v) are tested in each experiment, and appropriate controls are included.

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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.

<u>Aroclor 1254-Stimulated Metabolic Activation</u>

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*

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test. Ames has described the liver metabolic activation system that we use.[16] 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.[18] 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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