Guideline Series 84: MUTAGENICITY

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Date: August 11, 1988

DATA EVALUATION RECORD

CHEMICAL: Methoxychlor

Tox. Chem. No.: 550

Tox Branch Project No.: 8-0131

STUDY TYPE: Rec-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, N.C. 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:

At dose levels up to 5 mg/disk, there was no increase in the zone of inhibition by Methoxychlor in either the *E. coli* or *B. subtilis* strains, whereas the positive control and chloramphenicol did cause an increase in the zone of inhibition. However, there was no indication as to how many plates per dose were used and metabolic conditions were not used.

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 (Unpublished study including submitter summary, received Apr 3, 1980 under 279-2712; prepared by SRI International, submitted by FMC Corp., Philadelphia, Pa.; CDL:099350-A)

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 (Unpublished study received Dec 5, 1983 under 239-2471; prepared by SRI International, submitted by Chevron Chemical Co., Richmond, CA; CDL:251894-F)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 (EPA 600/1-79-041; Contract No. 68-01-2458; also In unpublished submission received Dec 2, 1983 under 279-2038; submitted by FMC Corp., Philadelphia, PA; CDL:251984-C)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 "material 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: Manufacturer: E.I. du Pont de Nemours & Co.

2. Control Materials:
   Negative: chloramphenicol: 20 ug/disk (toxic to bacteria not via interaction with DNA) - tests sensitivity of system
   Solvent/final concentration: DMSO 10 ul
   Positive: 1-phenyl-3,3-dimethyltriazine: 2ug/disk

3. Activation: None

4. Test organisms: Escherichia coli strains[+] W3110 and p3478
   Bacillus subtilis strains[++] H17 and M45
   Properly maintained? yes
   [+]Obtained from Dr. H. Rosenkranz
   [++]Obtained from Dr. T. Kada

5. Test compound concentrations used:
   0.01, 0.10, 1.0 and 5.0 mg/disk

B. TEST PERFORMANCE

1. Type of Rec-assay: standard plate test

a. Protocol:

Inoculum of frozen culture were grown overnight at 37°C. An aliquot was removed and placed in nutrient broth with agar and then poured on a plate containing a similar surface. A filter disc containing the test compound was placed on the solidified plate and then incubated at 37°C for 16 hours. The width of the inhibitory zone was then measured.

2. Preliminary cytotoxicity assay: None conducted.

3. Mutagenicity assay: The following table (extracted from the investigators report) presents the results of the study:

DIFFERENTIAL TOXICITY OF REPAIR-PROFICIENT AND -DEFICIENT MICROORGANISMS

Compound	mg of Compound in 10 ul of DMSO Applied to Disc	Diameter of Zone of Inhibitions (mm)* B. subtilis H17	B. subtilis M45	E. coli W3110	E. coli p3478
Negative control:					
Chloramphenicol	20 ug	38	40	40	40
Positive control:					
1-Phenyl-3,3-dimethyl-triazene	2	12	20	13	20
Methoxychlor	0.01	6	6	6	6
	0.10	6	6	6	6
	1.0	6	6	6	6
	5.0	6	6	6	6

* The diameter of the disc was 6 mm.

From the data presented, there was no effect of Methoxychlor at dose levels tested on the zone of inhibition in any of the test strains. The negative and positive control did increase the zone of inhibition.

4. Reviewer's discussion/conclusions:

No effect was noted at any of the dose levels of Methoxychlor tested in the E. coli and B. subtilis strains, whereas the positive control and chloramphenicol did cause an increase in the zone of inhibition of the plates. However, there was no positive indication as to how many plates per dose were used and metabolic activation conditions were not used. The study is not adequate for regulatory purposes and ~~must~~ should be repeated.

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

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

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.[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

The suspension mixture is incubated at 30° C for 4 hours on a roller drum. The sample is diluted serially in sterile physiological saline, and 0.2-ml aliquots of the 10^{-4} and 10^{-5} dilutions are spread on tryptone-yeast agar plates; five plates are used for the 10^{-4} dilution and three plates are used for the 10^{-5} dilution. The plates are incubated for 2 days at 30° C, followed by 2 days at 4° C to enhance the development of the red pigment indicative of adenine-deficient homozygosity. Plates of the 10^{-5} dilution are scanned with a dissecting microscope at 10X magnification, and the number of red colonies or red sectors (mitotic recombinants) is recorded. The surviving fraction of organisms is determined from the number of colonies appearing on the plates of the 10^{-5} dilution. The number of mitotic recombinants is calculated per 10^{5} survivors.

A positive response in this assay is indicated by a dose-related increase in the absolute number of mitotic recombinants per milliliter as well as in the relative number of mitotic recombinants per 10^{5} survivors.

Escherichia coli W3110/p3478 and Bacillus subtilis H17/M45

The E. coli strains W3110 and p3478 that are used at SRI were obtained from Dr. H. Rosenkranz, who devised the DNA polymerase repair assay.[9] Strain p3478 is a DNA polymerase-deficient (polA^{-}) derivative of W3110 and is very sensitive to the effects of some physical and chemical agents that react with cellular DNA. The repair assay is based on the finding that when exposed to agents that alter the DNA, bacteria tend to protect themselves by removing the altered DNA segment and then by resynthesizing the correct DNA sequence. Thus, their survival is enhanced. The enzyme DNA polymerase is involved in this resynthesizing process.[10] The extent of chemically induced DNA damage can be measured by comparing the relative toxicity (zone of growth inhibition) of the two

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 10⁸ 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

REFERENCES

1. V. F. Simmon, 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. H. Purchase, E. Longstaff, J. Ashby, J. A. Styles, D. Anderson, P. A. Lefevre, and F. R. Westwood. Evaluation of six short term tests for detecting organic chemical carcinogens and recommendations for their use. Nature 264, 624-627 (1976).

4. V. F. Simmon. 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. Gurney, J. A. Miller, and H. Bartsch. Carcinogens as frameshift mutagens: Metabolites and derivatives of 2-acetylaminofluorene and other aromatic amine carcinogens. Proc. Nat. Acad. Sci. USA 69, 3128-3132 (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. Kobori, 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, 2281-2285 (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. Schwaier. 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 H. S. Rosenkranz. 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., Totsuka, Tokyo, 1973.

18. R. M. C. San and M. 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. Telmach. 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. M. 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 Y. Bishop. Detection of chemical mutagens by the dominant lethal assay in the mouse. Toxicol. Appl. Pharmacol. 23, 288-325 (1972).

27. V. V. Brahechii. Possibility of induction of mutations in Drosophila melanogaster with chlorophos. Med. Parasit. Parazit. Bolez. 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).