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Toxicology Studies on Lewisite and Sulfur Mustard Agents: Mutagenicity of Sulfur Mustard in the *Salmonella* Histidine Reversion Assay

Final Report

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The mutagenic potential of bis 2-chloroethyl sulfide (HD) a bifunctional sulfur mustard was evaluated in the standard plate incorporation version and the preincubation modification of the Salmonella/microsomal assay with tester strains TA97, TA98, TA100 and TA102, with and without S9 activation. HD-induced point mutations in strain TA102 and frameshift mutations in TA97 but showed little or no mutagenicity against strains TA98 and TA100. Extensive HD-induced cell killing was observed with the excision repair deficient strains (TA100, TA98 and TA97) but not with strain TA102, which is wild-activation by Aroclor induced rat liver microsomes (S9).

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FOREWORD

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EXECUTIVE SUMMARY

Chemical warfare agents present an obvious risk to individuals suffering acute exposure, but they may also present long-term environmental or occupational health hazards for workers in operations involving these chemical agents. Occupational health standards have not been established for sulfur mustard [bis-(2-chloroethyl)-sulfide] a strong alkylating agent with known mutagenic and suspected carcinogenic properties. Sulfur mustard is used in a number of research laboratories, stored in depot sites throughout the country and occasionally transported to distant sites. The destruction of current stockpiles of sulfur mustard by the U.S. Army in the near future could create additional environmental and occupational risk. To establish a database for setting environmental and occupational standards, we have conducted studies to evaluate the toxicity, mutagenicity, and reproductive effects of sulfur mustard using *in vitro* and *in vivo* study systems. The purpose of this study was to evaluate the mutagenic potential of sulfur mustard in the standard plate incorporation version and the preincubation version of the *Salmonella*/microsomal assay with tester strains TA97, TA98, TA100 and TA102, with or without S9 activation.

Solutions of sulfur mustard were prepared by diluting the neat agent to the appropriate concentrations in dimethyl sulfoxide (DMSO). Sulfur mustard was tested at 1, 10, 50, 100, and 500 ug/plate in the standard plate incorporation version and the preincubation version of the Ames assay. Sulfur mustard, bacterial tester strain and S9 enzyme in buffer was added to soft agar which was immediately poured onto a minimal agar plate without histidine. Positive and negative controls were included with each assay and two levels of S9 activation were evaluated. Revertant colonies were counted after incubation at 37°C for 48 hours. A preincubation step was added for strains (TA98 and TA100), which initially gave no mutagenic response, whereby all components of the bioassay system were incubated for 1 hour at 37°C prior to plating.

Sulfur mustard induced point mutations in strain TA102 and frameshift mutations in TA97 but showed little or no mutagenicity against strains TA98 and TA100. Based on the average number of revertant colonies/plate, sulfur mustard was about 4 times more potent for the frameshift mutant (TA97) than for the substitution mutant (TA102). The mutagenic response induced by sulfur mustard was dose-dependent over a range of 1 to 50 μg per plate. Extensive sulfur mustard induced cell killing was observed with the excision repair deficient strains (TA100, TA98 and TA97) but not with strain TA102, which is wild-type for excision repair. The mutagenicity of sulfur mustard was independent of metabolic activation by Aroclor induced rat liver microsomes (S9).

TABLE OF CONTENTS

TABLES

TABLES (Continued)

FIGURES

INTRODUCTION

Chemical warfare agents present an obvious risk to individuals suffering acute exposures but may also present certain long-term environmental or occupational health hazards for workers in operations involving these chemical agents. These materials are used in a number of research laboratories, stored in depot sites throughout the country and occasionally transported to distant sites. In addition, stockpiles of agents are scheduled for destruction by the U.S. Army in the near future, creating an additional potential for environmental and occupational exposure. Although considerable information is known concerning the acute effects of these materials including their carcinogenicity, teratogenicity and mutagenicity, especially in mammalian systems. It is therefore necessary that potentially toxic and mutagenic chemicals be identified and that a data base be established for the development of hazard evaluations and occupational health standards for these chemicals.

The two general categories of vesicants are typified by Lewisite [dichloro(2-chlorovinyl)arsine] and sulfur mustard (HD) [bis(2-chloroethyl) sulfide] (Cassarett and Doull, 1986). Contact with these chemicals produces severe skin burns. Recently, a renewed interest in these chemicals was generated by the release of a United Nations report that contained substantial evidence that Iraq was manufacturing and using these agents as chemical warfare agents (Marshall, 1984).

The mustard compounds (both sulfur and nitrogen) are biochemically related to a group of cytotoxic alkylating agents, including the ethylenimines, sulfonic esters, epoxides and n-alkyl-n-nitroso compounds (Wheeler, 1962). These chemicals react rapidly with certain functional groups of proteins (OH, NH2, and SH) to alter their metabolic activity. In aqueous solutions, both sulfur and nitrogen mustard hydrolyze to form cyclic sulfonium or immunium forms, respectively, which, in turn will react with nucleophilic sites. The sulfur mustard reaction proceeds more rapidly to the reaction with nucleophiles than does nitrogen mustard and is independent of the concentration of nucleophiles present (Fox and Scott, 1980). The cytotoxic, mutagenic, and carcinogenic properties of mustard compounds have been studied extensively (Fox and Scott, 1980), but most of these data relate to nitrogen mustard because sulfur mustard is a more toxic and chemically reactive vesicant.

Relevant chemical and physical properties of sulfur mustard are summarized in Table 1. In aqueous solutions, sulfur mustard rapidly hydrolyses to form a cyclic sulfonium salt, b-chloroethyl-ethylenesulfonium chloride. This salt reacts with water to form b-chloroethyl b-hydroxyethyl sulfide and hydrochloric acid. Subsequent hydrolysis of the sulfide, presumably through the intermediation of a second sulfonium salt, forms thiodiglycol (Anslow et al., 1948). These workers have investigated the toxicity of these derivatives of sulfur mustard and a number of other intermediates isolated from hydrolysates of sulfur mustard. They found that two of the derivatives, b-chloroethyl b-hydroxyethl sulfide and thiodiglycol, were relatively nontoxic.

TABLE 1. Relevant Chemical and Physical Properties for Sulfur Mustard, Bis(2-Chloroethyl)Sulfide[a]

CAS #:	505-60-2
RTECS #:	WQ0900000
Structural formula:	Cl-CH2-CH2-S-CH2-CH2-Cl
Molecular weight:	159.1 g
Density at 25°C:	1.3 g/ml
State:	Colorless, oily liquid
Vapor pressure at 20°C:	0.072 mm
Decomposition temperature:	149-177°C
Solubility in water at 25°C:	0.68 g/L
Hydrolysis	
Rate (T1/2 at 25°C, pH7):	8.5 min
Products:	Thiodiglycol, chloride
Army Abbreviation	HD

[a]Rosenblatt et al. 1975, Windholz, 1983

The carcinogenicity of nitrogen mustard is well documented, but relatively little data are available for HD. Studies in mice have shown evidence of skin papillomas following subcutaneous HD treatment and lung tumors after intravenous injection or inhalation of HD (Fox and Scott, 1980). Studies conducted by the U.S. Army found little evidence of lesions in rabbits, guinea pigs and dogs after being exposed to HD vapor for up to 52 weeks. Treatment-related skin tumors were observed in rats exposed to 0.1 mg/m^3 HD vapor for as few as 12 weeks (McNamara et al. 1975). In an initiation-promotion study using a mouse-skin model, HD was not found to be an active initiator of tumor development (Berenblum and Shubik, 1949). However, Japanese factory workers, who were involved in the production of chemical agents and who were potentially exposed to unknown quantities of various chemical agents including HD during World War II, show evidence of an increased incidence of respiratory and gastrointestinal tract cancers (Wada et al., 1968; Norman, 1975; Nishimoto et al., 1970; Manning et al., 1981; Yamakido et al., 1985).

The teratogenic potential of HD was studied in rats exposed to two concentrations of inhaled HD (0.001 and 0.1 mg/m^3) during each of the 3 weeks of gestation or throughout the entire gestation period (McNamara et al., 1975). No evidence of dose-related fetal mortality or gross abnormalities was noted. Teratology studies, following the segment II teratology protocol, were recently conducted in rats and rabbits by Hackett et al. (1987). Rats were exposed to 0.5-2.0 mg/kg HD by gastric intubation from 6 to 15 day of gestation (dg) and were killed on dg 20. No evidence of a teratogenic response to HD was observed since fetal effects occurred only at doses exhibiting signs of maternal toxicity. Likewise, fetal development of rabbits exposed to 0.4-0.8 mg/kg HD between 6 and 19 dg was not affected even

though maternal mortality was induced at the highest dose. These results suggest that HD is not teratogenic in rats and rabbits since fetal effects were observed only at dose levels that induced frank maternal toxicity.

Mustard agents (mostly nitrogen mustard) have been found to produce mutagenic affects in a wide variety of animal species and test systems. Reviews on the genetic toxicology of nitrogen mustard and HD have summarized the known effects of these agents in biological systems (Auerbach, 1949; Auerbach, 1976; Fox and Scott, 1980). Dominant lethal, sex-linked recessive and autosomal lethal, and visible mutations as well as major rearrangements and chromosomal aberrations have been reported in the fruit fly.

Relatively little is known concerning the mutagenicity of HD in mammalian species or test systems. Chronic inhalation exposure of male rats to sulfur mustard (0.1 mg/m^3) was reported to produce significant dominant lethal effects, but exposure of pregnant females to the same concentrations for a shorter time interval failed to induce fetal malformations (Rozmiarek et al., 1973). McNamara et al. (1975) subsequently concluded from these same data that there were no differences between the control and experimental groups and no evidence of mutagenesis. It is difficult to resolve the apparent conflict between the conclusions of these two reports, but the fetal mortality values presented in the McNamara report suggest at least a trend for a significant dominant lethal effect. Complete control data are missing from the report and statistical evaluation of the results is not presented, but percentage fetal death at week 12 were 4.12, 4.24, and 21.05 for controls, 0.001 and 0.1 mg/m^3 exposure groups, respectively.

The bifunctional alkylating agent, HD, yields 7-alkylguanine as its principal alkylation product (Fox and Scott, 1980). Approximately 25% of these alkylations result in the formation of the DNA cross link, diguanine-7-ethylmethylamine. DNA cross-links are implicated in the production of chromosomal aberrations and chromosomal rearrangements (Bodell et al., 1985; Tokuda and Bodell, 1987). HD is a known clastogen which produced all of the types of chromatid aberrations commonly seen with ionizing radiation (Fox and Scott, 1980). Conversely, very few, if any, chromosome type aberrations have been observed after HD treatment. Some investigators feel that this observation suggests that only one strand of the DNA helix is affected by the cross-link (Fox and Scott, 1980). Information regarding the dose response relationships of HO induced aberrations is ambiguous and a detailed analysis would require the use of synchronous cell populations and cell progression analysis. HD has been reported to induce a linear increase in the mutation of L5178Y cells as determined by reversion from asparagine dependence (Capizzi et al., 1973).

The purpose of this study was to evaluate the mutagenic potential of sulfur mustard in the standard plate incorporation version and the preincubation version of the *Salmonella*/microsomal assay with tester strains TA97, TA98, TA100 and TA102, with and without S9 Activation.

MATERIALS AND METHODS

The mutagenic potential of sulfur mustard, an alkylating agent, was evaluated in the standard plate incorporation version and the preincubation modification of the *Salmonella*/microsomal assay with tester strains TA97, TA98, TA10D and TA102, with and without S9 activation.

Test System

In-house cultures were obtained from Dr. Bruce Ames' laboratory at the University of California, Berkeley, CA 94720. *S. typhimurium* is routinely used to evaluate the mutagenic potential of test chemicals. Strains TA97, TA98, TA100 and TA102 were selected based on the revised methods for the *Salmonella* mutagenicity test (Maron and Ames, 1983). The two new strains, TA97 and TA102, have been genetically designed to increase their sensitivity to mutagens which previous strains either weakly detected or did not detect at all.

Sulfur Mustard

The HD used in these studies was 2,2', dichlorodiethyl sulfide, also known as bis(2-choroethyl)sulfide or distilled mustard (HD).

The HD was supplied by the U.S. Army Medical Research Institute for Chemical Defense (USAMRICD), Chemical Surety/Safety Office, Aberdeen Proving Ground, Edgewood Arsenal, MD from lot No. HD-U-4244-CTF-N-1, previously designated lot No. ICD-HD-1. The material was prepared August 31, 1981 and analyzed for purity September 4, 1984 by Captain William Beaudry and Linda Szfdraniec (Research Directorate Chemical Research) by nuclear magnetic resonance. Purity, calculated on a weight basis, was 97.3%. There were two impurities with concentrations of 1.2% (assumed to be dithiane) and 1.5% (identity unknown). Material from this lot has been proposed as the standard analytical reference for USAMRDC and USAMRDC has agreed to retain aliquots of this material to comply with the requirements of Good Laboratory Practices (GLP).

A shipment of 25 ml of HD (in two ampules) was delivered on March 7, 1985 by a team from the U.S. Army Technical Escort Unit. The ampules were inspected and found to be intact. Subsequently the HD was transferred from the ampules into 30-ml Wheaton bottles, sealed and stored in secondary unbreakable containers in a refrigerated storage container at approximately 6°C.

Sulfur mustard is relatively insoluble (680 mg/L) and also is rapidly hydrolyzed in water, therefore dimethyl sulfoxide (DMSO) was employed as the diluent for this study. The HD solutions were prepared in advance and stored in a refrigerator at approximately 6°C overnight. The general procedure was to determine the amount of neat HD needed, based on the volumes to be prepared and the final concentrations desired. This volume was then removed from the bottle of neat HD and thoroughly mixed into a known volume of DMSO.

Aliquots of this intermediate concentration were then diluted further to give the final concentration needed for exposing the cells.

Control Chemicals and S9 Enzyme

All control articles were dissolved in DMSO and tested at the following concentrations listed in Table 2.

Table 2. Concentration of Control Chemicals

Control Article	CAS #	Concentration/ Plate (μg)
2-Aminofluorene (2-AF)	153-78-6	10
Benzo[a]pyrene (BaP)	613-13-8	1.0
N-methyl-N-nitro-N-nitrosoguanidine (MNNG)	70-25-7	1.0
Sodium azide	26628-22-8	1.5
ICR-191	146-59-8	1.0
Mitomycin C	50-07-7	0.5

The S9 enzyme was prepared from 8- to 10-week-old Sprague-Dawley male rats induced with Aroclor 1254 (500 mg/kg) according to the procedure outlined by Ames et al. (1975). All S9 preparations were supplied by Litton Bionetics, 2020 Bridge View Lane, Charleston, SC 29405 and stored at -80°C for no longer than 3 to 4 months. Each batch of S9 enzyme was checked for activity with control mutagens prior to use in the study. These results were compared to the ones supplied by the supplier and to our own histological data base. Only S9 preparations that gave similar mutagenic responses were used for the study.

Experimental Design

Sulfur mustard was tested at 1, 10, 50, 100 and 500 μg/plate in the standard plate incorporation version and the preincubation version of the Ames assay. Preliminary testing to determine appropriate nontoxic doses for testing was conducted with strain TA98 at two dose ranges. These sets of doses were as follows: 0.1, 1.0, 2.5, 5.0 and 10 μg/plate and 0.01, 0.1, 0.25, 0.5 and 1.0 μg/plate.

Sulfur mustard was tested against four Ames tester strains (TA97, TA98, TA100 and TA102) in the plate incorporation version of the Ames assay, with and without metabolic activation, which consisted of Aroclor 1254-induced rat liver microsomal homogenate (S9 enzyme). Two levels of S9 activation (20 and 50 μl/plate) were used for all testing performed. Although sterility controls for each batch of S9 were not included for each experiment, no

evidence of contamination occurred, as indicated in the background controls. Initially, the agent was assayed from 0.1 to 10 μg/plate of sulfur mustard with TA98 to find an acceptable nontoxic dose range. Results of the preliminary screening were used in setting the doses for the mutagenic evaluation of sulfur mustard.

Repeated testing was conducted 1 week later, using the plate-incorporation procedure. Since toxicity occurred only at the 500 μg/plate in the initial test, the same set of dose concentrations was used in the repeat test. Additional testing, using the preincubation modification of the Ames assay, was conducted with strains TA98 and TA100. All exposures were conducted in the Chemical Surety Material (CSM) Facility in a vented hood.

Both mutational background and mutagenicity specificity are criteria required to validate each assay conducted. Mutagenic specificity of the *S. typhimurium* test strains were determined in each experiment by the response of each strain to the positive control chemicals. Positive control chemicals included in this study were sodium azide at 1.5 μg/plate, ICR-191 at 1.0 μg/plate, 2-AF at 10 μg/plate, BaP at 1.0 μg/plate and MNNG at 1.0 μg/plate. Each of the above mutagens was tested for all strains. The quantitative reversion values were determined by incorporating the mutagens into the top agar and counting revertant colonies. Table 3 lists the mutagenic response of each control chemical as reported by Maron and Ames (1983).

Table 3. Mutagenic Response of Control Chemicals

Mutagen	Amount (μg/plate)	S9 (μl)	Tester Strains (Revertants/Plate) TA97	TA98	TA100	TA102
BaP	1.0	20	337	143	937	255
2-AF	10.0	20	1742	6194	3026	261
Sodium azide	1.5	0	76	3	3000	188
Mitomycin C	0.5	0	Inh	Inh	Inh	2772
ICR-191	1.0	0	1640	63	185	0
Background	0	0	90-180	30-50	120-200	240-320

Inh = Inhibitory

Negative solvent controls (DMSO) were included in each experiment to establish the solvent control background. As reported by Maron and Ames (1983), the acceptable ranges for the background mutation, without metabolic activation are shown in Table 3. All strains were checked with each assay for the presence of the following genetic markers: ampicillin resistance, crystal-violet inhibition and histidine independence. Strain TA102 was also checked for tetracycline resistance.

Sample Tube Preparation for Standard Plate Incorporation

Top agar was melted, and 4.5 ml amounts were put in each tube. The tubes were allowed to cool to 50°C. The top-agar tubes were placed in the dry bath outside the fume hood and transferred to the hood as needed. The calculated amount of test article was added to the appropriate tubes. Stock solutions of the test article were prepared at 10.0, 2, 1, 0.2 and 0.02 mg/ml. Fifty-μl aliquots of these stock solutions were tested. Each dose level was assayed in triplicate, with and without the metabolic activating system.

Positive-control mutagens (2-AF at 10 μg/plate, BaP at 1.0 μg/plate, MNNG at 1.0 mg/plate, mitomycin C at 0.5 μg/plate, and sodium azide at 1.5 μg/plate were included in each experiment to confirm the mutagenic specificity of the tester strains. Stock concentrations of the control articles were prepared at 10,000 μg/ml and appropriate dilutions were made from these stock solutions. A 50-μl aliquot of the test chemicals and negative solvent controls was used with each experiment. For indirect activation (i.e., mutagen is activated by S9 enzyme to active metabolites), a volume of 0.5 ml S9 buffer was added to each tube of top agar with the appropriate volume of Aroclor 1254-induced S9 enzyme. For direct activation (i.e., mutagen does not require S9 enzyme for activation), only S9 buffer was added to the top agar. The same lot of S9 enzyme was used throughout any given experiment.

A volume of 0.1 ml of *S. typhimurium* (Ames) tester strain culture was added to each tube. The final concentration was approximately 2.5 x 10^8 cells/ml of top agar. A volume of 0.5 ml of S9 buffer and either 20 or 50 μl/plate of Aroclor-induced S9 enzyme were added to each tube for indirect activation. For direct activation (without metabolic activation), only 0.5 ml buffer solution was added. The top agar was gently mixed on a vortex mixer, then poured onto minimal agar plates. When the agar was solidified, the plates were transferred in sealed plastic jars to the incubators and incubated at 37°C for 48 hours.

The revertant colonies were counted on each plate, using a Biotran III electronic plate counter. Plate counts were transferred directly to an Apple II Plus computer for storage, statistical analysis and subsequent retrieval. The background bacterial lawn was also examined under magnification to check the cytotoxicity of the chemical; a sparse bacterial lawn with pinpoint-size visible colonies indicated a toxic dose. Revertant colonies (at least 50 colonies) were transferred from plates that exhibited a mutagenic response to a minimal agar plate without histidine to check for histidine independence.

Sample Tube Preparation for Preincubation Modification

This assay was conducted as described above, except the components of the system without top agar were incubated for 1 hour at 37°C before plating. At the time of plating, viability determinations were also conducted by the serial-plate-dilution method, using nutrient agar.

Statistical Analysis

Simple linear regression analysis of dose-response data were performed with an Apple II Plus computer, using a program written for processing data in this laboratory. These results have been verified by using a standard program for linear regression analyses written for the Hewlett-Packard® calculator.

RESULTS

In Tables 4 and 5, dose-response values of the first and second testing are presented. Linear regression analysis was performed on these average values for four consecutive concentrations of sulfur mustard, then for five consecutive concentrations, then for all six concentrations. This type of analysis was selected because, at higher concentrations of test chemical, cytotoxicity sometimes occurs, and linear regression analysis may not reflect the mutagenic potential for a given compound.

Dose-response data for each *Salmonella* tester strain indicated that sulfur mustard, at the concentrations used, is toxic for most strains tested (Tables 4 and 5). At the highest dose, 500 μg/plate, all strains except TA102 exhibited a reduced mutagenic response, indicating some degree of cytotoxicity. However, preliminary range finding tests (as indicated in Table 6) with TA98 to determine a set of doses to be used indicated no toxicity or any mutagenic response up to 10 μg/plate. Therefore, this set of doses (1-, 10-, 50-, 100- and 500-μg/plate) was chosen to insure that a mutagenic response would be induced and to detect some level of cytotoxicity.

Strains TA97 and TA102 both exhibited a dose-response relationship for at least three consecutive doses, with and without S9 activation, in both Test #1 and Test #2. Figures 1, 2, and 3 illustrate the average number of revertants/μg for all strains tested in the standard plate incorporation method. With S9 activation, strains TA97 and TA102 gave an average mutagenic response of eight revertants/μg. Without S9 activation, strain TA97 appeared to give a higher average number of revertants/μg than TA102. Figures 4 and 5 illustrate the dose-response relationships for strains TA97 and TA102, respectively. Strain TA102 exhibited a greater dose-response relationship than strain TA97 at these test doses. Cytotoxicity with TA97 was evident at 500 μg/plate of sulfur mustard, as indicated by a 10-fold reduction in response. Strain TA98 showed only a slight mutagenic response (less than one revertant/μg) at a concentration of 50 to 100 μg/plate; strain TA100 showed no mutagenic response to sulfur mustard.

As indicated in the Protocol, a preincubation modification of the Ames test was conducted only with the stains that did not give a mutagenic response. The preincubation data for strains TA98 and TA100 are presented in Tables 7 and 8. Only strain TA98 showed borderline mutagenic activity (two times the experimental background) in this assay. Cytotoxicity, as indicated by viability determination, occurred at the higher concentrations (50, 100 and 500 μg/plate) of sulfur mustard. Results for positive and negative controls are presented in Tables 4 to 8. Although the responses in general are lower than the ones reported by Maron and Ames (1983), each tester strain gave a mutagenic response pattern that indicated strain specificity. Responses of *Salmonella* tester strains with MNNG, another known alkylating agent, also agree with this laboratory historical data base and provided confirmation of mutagenic specificity of the tester strains.

TABLE 4. Mutagenic Response of Sulfur Mustard in the *Salmonella* Histidine Reversion Assay With and Without S9 Activation in Test No. 1

Control or Test Agent	Concentration (μg/Plate)	S9 (μl)	Revertants/Plate ± SD (N = 3) TA97	TA98	TA100	TA102
BaP	1.0	20	147 ± 7	125 ± 9	365 ± 21	353 ± 28
2-AF	10	20	542 ± 76	1608 ± 101	1734 ± 76	464 ± 24
Sodium azide	1.5	0	68 ± 11	21 ± 4	793 ± 11	235 ± 23
Mitomycin C	0.5	0	5 ± 2	25 ± 0	17 ± 4	789 ± 82
MNNG	1.0	0	79 ± 8	NT	1569 ± 38	1636 ± 132
ICR-191	1.0	0	638 ± 155	69 ± 6	310 ± 13	240 ± 16
Sulfur mustard	0	20	75 ± 10	29 ± 3	233 ± 10	242 ± 9
	1		160 ± 37	29 ± 8	226 ± 2	299 ± 16
	10		401 ± 10	36 ± 8	283 ± 33	388 ± 36
	50		487 ± 55	44 ± 9	269 ± 23	690 ± 63
	100		412 ± 66	39 ± 9	143 ± 66	938 ± 101
	500		49 ± 16	15 ± 4	98 ± 85	1276 ± 88
Sulfur mustard	0	50	86 ± 22	24 ± 2	200 ± 10	236 ± 4
	1		170 ± 6	28 ± 5	240 ± 11	272 ± 34
	10		310 ± 45	30 ± 2	276 ± 21	422 ± 9
	50		433 ± 100	43 ± 11	302 ± 34	784 ± 38
	100		436 ± 36	35 ± 5	229 ± 7	994 ± 34
	500		73 ± 20	15 ± 2	68 ± 8	1494 ± 71
Sulfur mustard	0	0	49 ± 6	27 ± 4	299 ± 30	257 ± 19
	1		152 ± 15	22 ± 12	297 ± 13	224 ± 23
	10		233 ± 30	29 ± 6	322 ± 4	294 ± 27
	50		496 ± 82	49 ± 5	313 ± 20	503 ± 35
	100		270 ± 67	36 ± 7	232 ± 19	828 ± 15
	500		20 ± 7	18 ± 3	63 ± 25	1312 ± 97

NT = Not tested

TABLE 5. Mutagenic Response of Sulfur Mustard in the *Salmonella* Histidine Reversion Assay With and Without S9 Activation in Test No. 2

Control or Test Agent	Concentration (μg/Plate)	S9 (μl)	Revertants/Plate ± SD (N = 3) TA97	TA98	TA100	TA102
BaP	1.0	20	243 ± 69	128 ± 21	430 ± 93	209 ± 58
2-AF	10	20	932 ± 120	2059 ± 435	1822 ± 126	465 ± 69
Sodium azide	1.5	0	91 ± 6	19 ± 3	719 ± 18	236 ± 47
Mitomycin C	0.5	0	6 ± 2	10 ± 0	8 ± 1	789 ± 82
MNNG	1.0	0	189 ± 21	23 ± 4	902 ± 45	1483 ± 147
ICR-191	1.0	0	1580 ± 253	83 ± 23	268 ± 14	222 ± 39
Sulfur mustard	0	20	91 ± 18	26 ± 5	198 ± 21	241 ± 19
	1		177 ± 9	28 ± 14	293 ± 15	272 ± 17
	10		421 ± 11	37 ± 8	323 ± 6	347 ± 8
	50		666 ± 23	52 ± 7	317 ± 20	662 ± 7
	100		409 ± 35	51 ± 6	221 ± 25	847 ± 92
	500		66 ± 18	21 ± 7	65 ± 3	1269 ± 65
Sulfur mustard	0	50	110 ± 23	19 ± 1	196 ± 16	244 ± 6
	1		180 ± 37	36 ± 7	263 ± 21	151 ± 26
	10		449 ± 33	43 ± 5	301 ± 39	241 ± 8
	50		688 ± 38	60 ± 3	323 ± 37	452 ± 44
	100		520 ± 105	48 ± 11	258 ± 65	752 ± 136
	500		66 ± 27	19 ± 5	60 ± 21	1288 ± 111
Sulfur mustard	0	0	77 ± 3	24 ± 5	237 ± 25	243 ± 23
	1		158 ± 26	27 ± 5	308 ± 52	145 ± 5
	10		428 ± 35	37 ± 8	354 ± 9	171 ± 7
	50		710 ± 81	57 ± 3	367 ± 18	366 ± 8
	100		588 ± 106	52 ± 8	301 ± 22	605 ± 34
	500		67 ± 10	12 ± 2	109 ± 25	897 ± 149

TABLE 6. Preliminary Toxicity Results of Sulfur Mustard

Control or Test Agent	Concentration (μg/Plate)	S9 (μl)	TA98 Revertants/ Plate ± SD (N = 3)
BaP	1.0	20	148 ± 8
2-AF	10.0	20	1701 ± 95
Sodium azide	1.5	0	21 ± 8
ICR-191	1.0	0	52 ± 4
Mitomycin C	0.5	0	13 ± 2
Sulfur mustard	0	20	22 ± 2
	0.1		26 ± 4
	1.0		32 ± 6
	2.5		38 ± 8
	5.0		55 ± 20
	10.0		55 ± 13
Sulfur mustard	0	50	25 ± 8
	0.1		28 ± 3
	1.0		30 ± 2
	2.5		35 ± 8
	5.0		42 ± 5
	10.0		41 ± 4
Sulfur mustard	0	0	23 ± 4
	0.1		24 ± 9
	1.0		32 ± 6
	2.5		29 ± 4
	5.0		34 ± 5
	10.0		36 ± 3

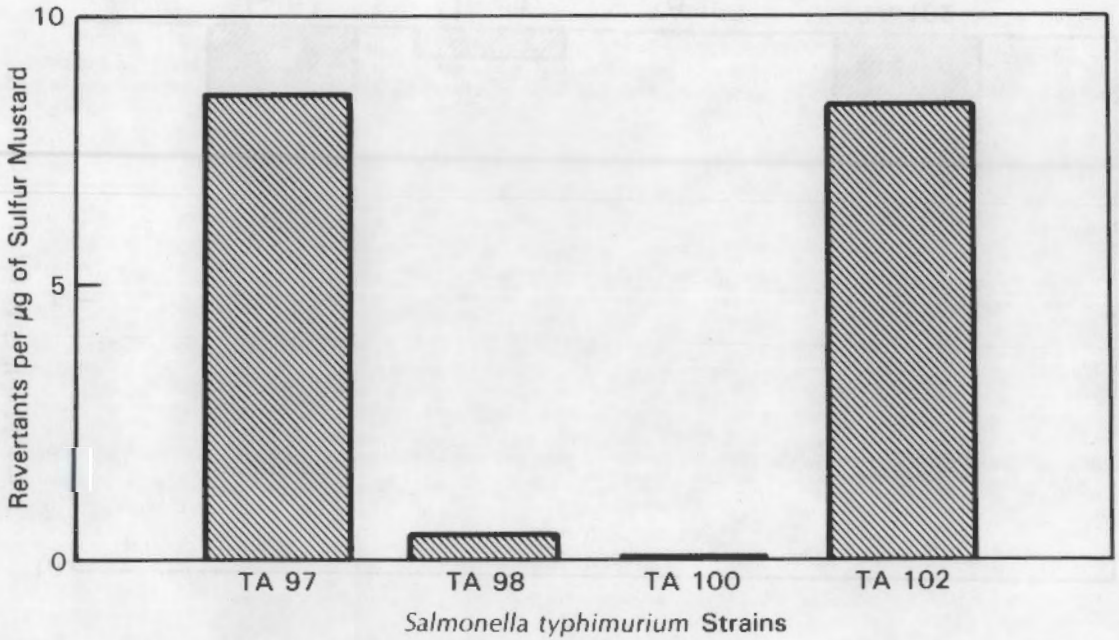

FIGURE 1. Average Number of Revertants Per Microgram of Sulfur Mustard at Low Metabolic Activation (20 µl/Plate) in the Standard Plate Incorporation Assay

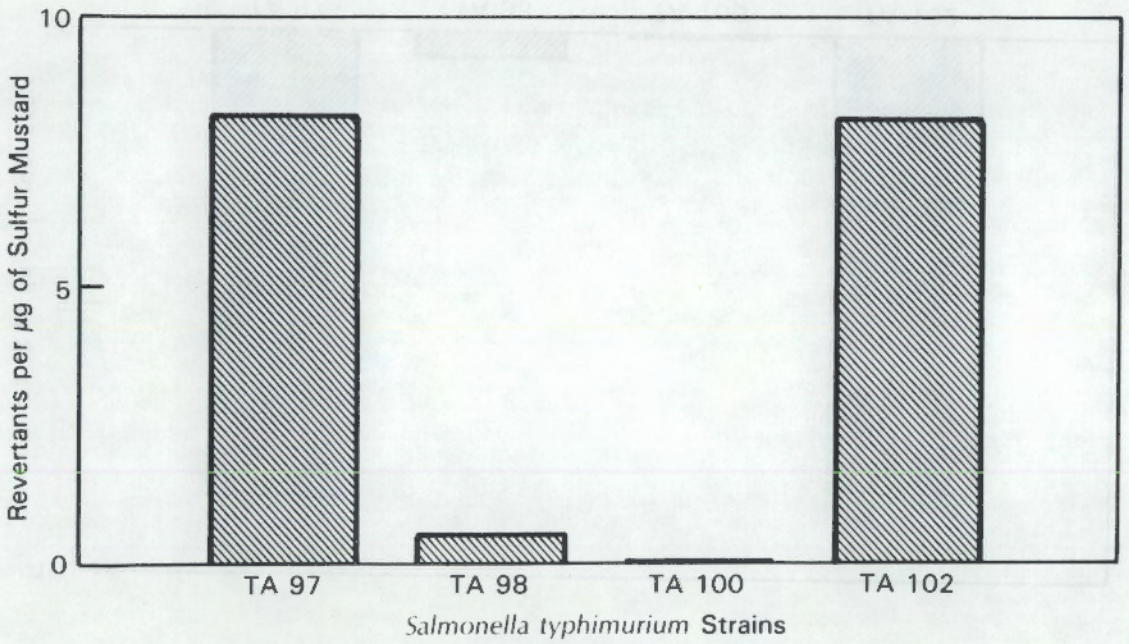

FIGURE 2. Average Number of Revertants Per Microgram of Sulfur Mustard at High Metabolic Activation (50 µl/Plate) in the Standard Plate Incorporation Assay

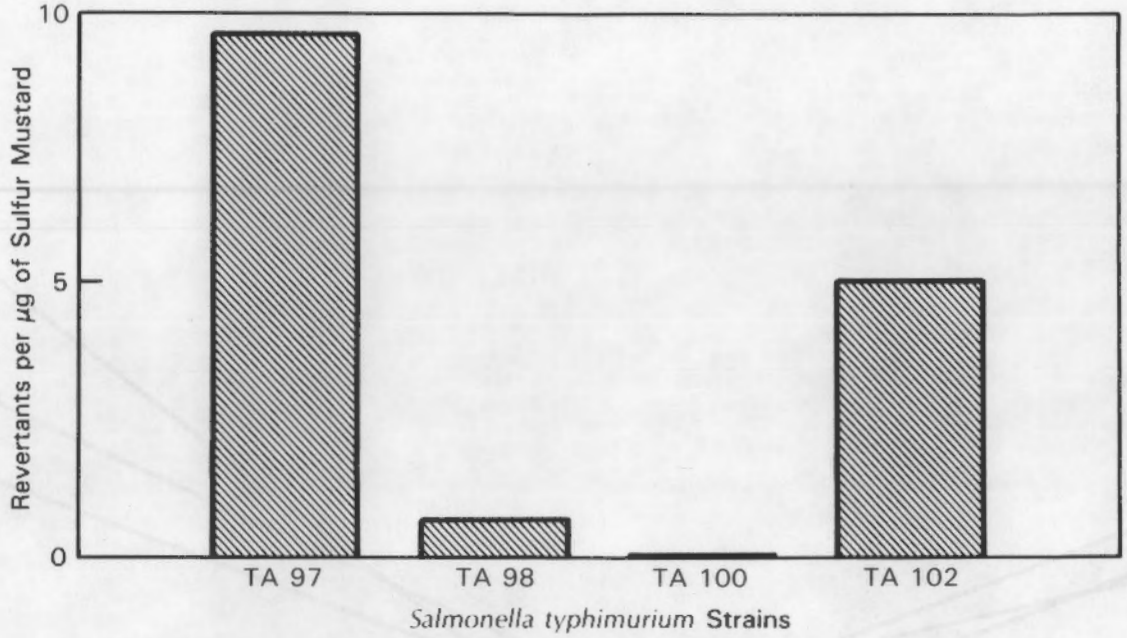

FIGURE 3. Average Number of Revertants Per Microgram of Sulfur Mustard Without Metabolic Activation in the Standard Plate Incorporation Assay

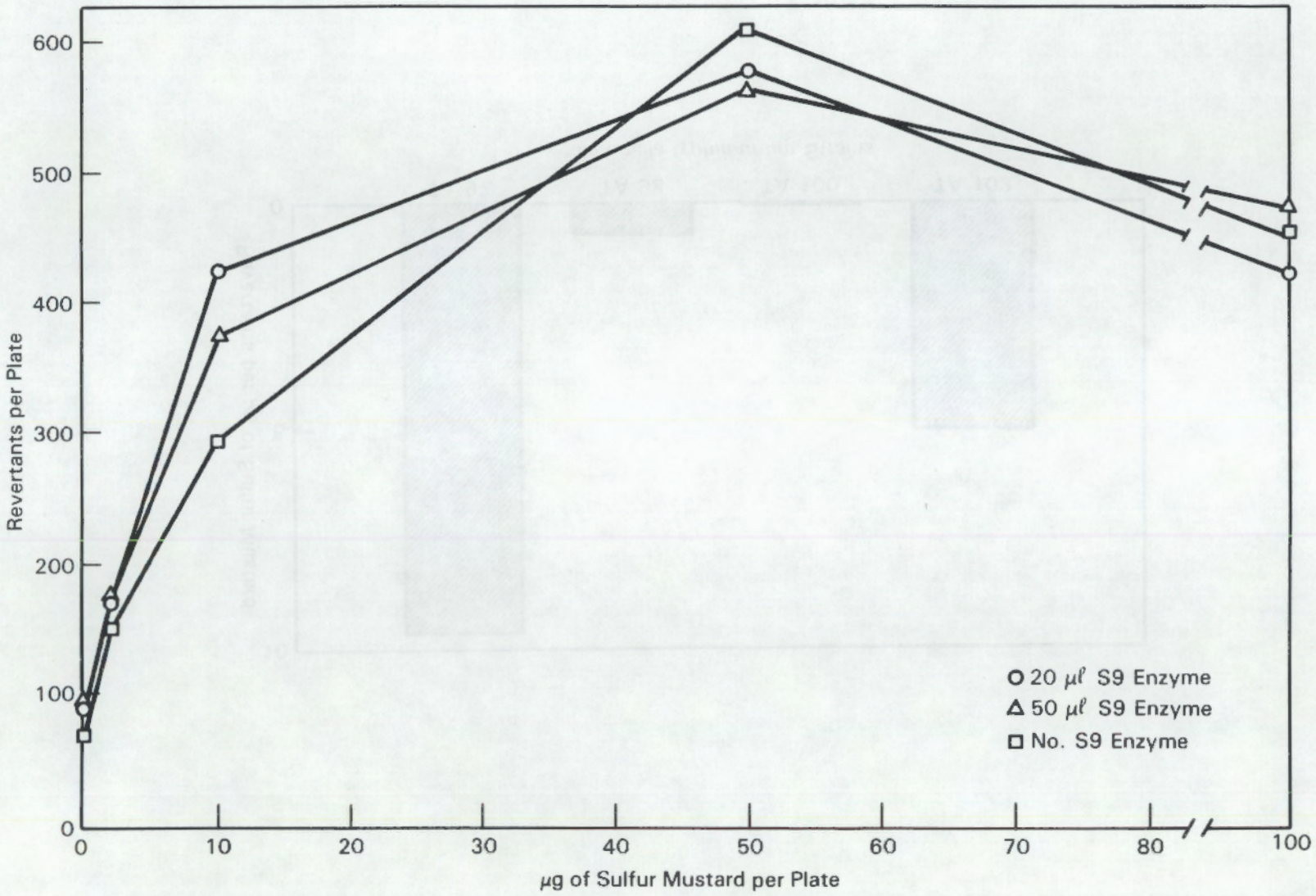

FIGURE 4. Mutagenicity of Sulfur Mustard with TA97

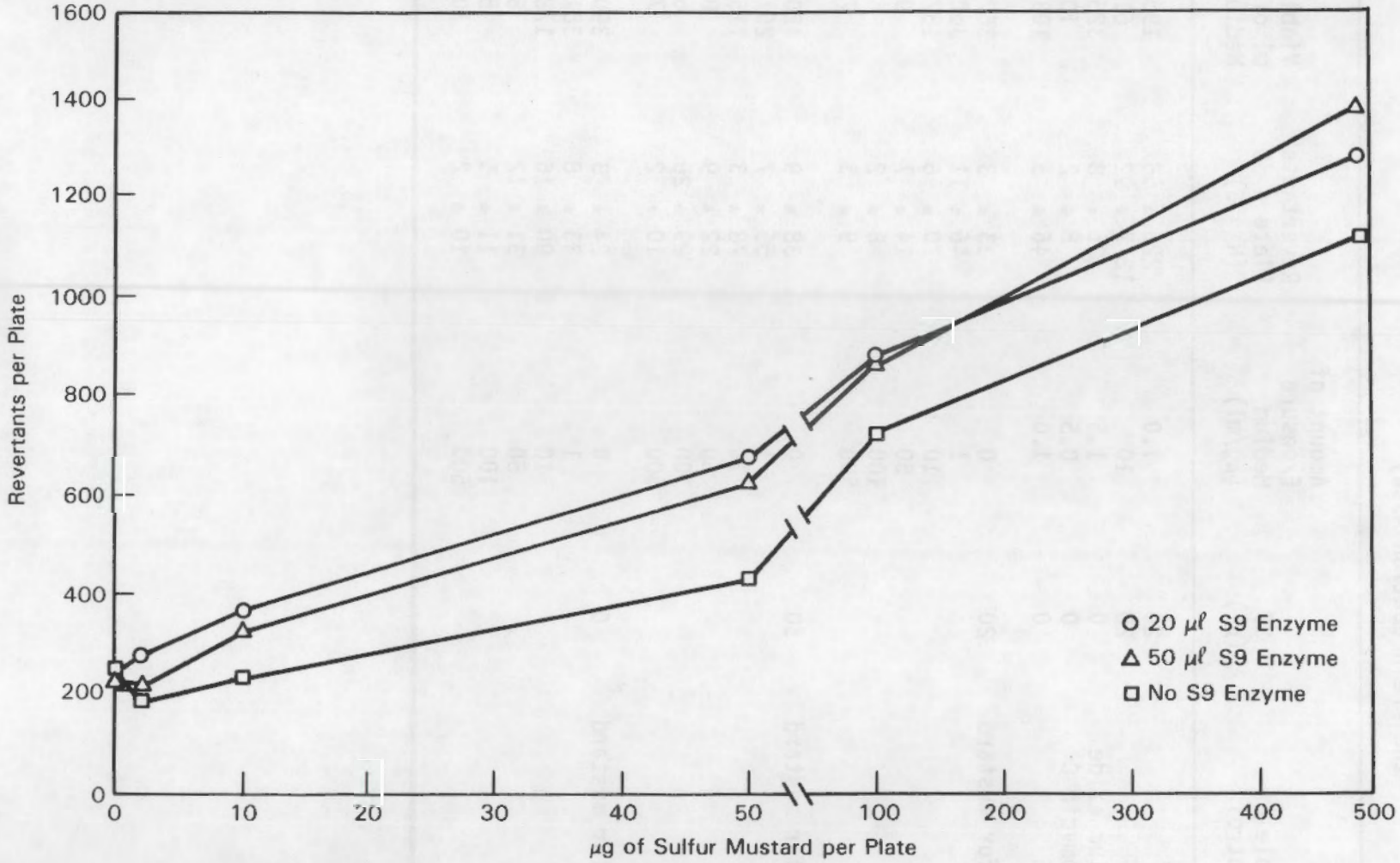

FIGURE 5. Mutagenicity of Sulfur Mustard with TA102

TABLE 7. Mutagenicity of Sulfur Mustard for *Salmonella typhimurium* TA98 in the Preincubation Assay

Sample or Control	S9 (μl)	Amount of Exposure Medium (μg/ml)	Revertants/ Plate ± SD (N = 3)	Viable Cells/ ml of Exposure Medium x 10^6 ± SD
BaP	20	1.0	226 ± 68	195 ± 22
2-AF	20	10	1277 ± 92	61 ± 3
Sodium azide	0	1.5	25 ± 8	325 ± 66
Mitomycin C	0	0.5	8 ± 2	10 ± 8
MMNG	0	1.0	46 ± 5	103 ± 30
Sulfur mustard	20	0	33 ± 3	367 ± 15
		1	46 ± 11	362 ± 60
		10	70 ± 6	137 ± 6
		50	14 ± 7	9 ± 2
		100	8 ± 2	6 ± 2
		500	9 ± 3	7 ± 5
Sulfur mustard	50	0	38 ± 9	420 ± 21
		1	55 ± 7	261 ± 6
		10	78 ± 3	115 ± 6
		50	22 ± 9	8 ± 4
		100	29 ± 26	6 ± 4
		500	10 ± 2	7 ± 4
Sulfur mustard	0	0	24 ± 3	390 ± 42
		1	33 ± 8	381 ± 26
		10	90 ± 16	178 ± 14
		50	31 ± 12	8 ± 4
		100	11 ± 5	5 ± 1
		500	10 ± 2	10 ± 4

TABLE 8. Mutagenicity of Sulfur Mustard for *Salmonella typhimurium* TA100 in the Preincubation Assay

Sample or Control	S9 (μl)	Amount of Exposure Medium (μg/ml)	Revertants/ Plate ± SD (N = 3)	Viable Cells/ ml of Exposure Medium x 10^6 ± SD
BaP	20	1.0	596 ± 41	130 ± 24
2-AF	20	10	809 ± 84	42 ± 8
Sodium azide	0	1.5	445 ± 13	149 ± 9
Mitomycin C	0	0.5	11 ± 3	10 ± 5
MMNG	0	1.0	981 ± 19	61 ± 18
Sulfur mustard	20	0	159 ± 17	236 ± 7
		1	189 ± 8	107 ± 20
		10	266 ± 55	79 ± 2
		50	107 ± 21	16 ± 7
		100	22 ± 6	11 ± 4
		500	11 ± 1	8 ± 3
Sulfur mustard	50	0	167 ± 17	252 ± 8
		1	188 ± 10	112 ± 3
		10	260 ± 23	50 ± 5
		50	101 ± 10	13 ± 3
		100	18 ± 5	9 ± 2
		500	12 ± 3	10 ± 0
Sulfur mustard	0	0	173 ± 17	254 ± 17
		1	162 ± 10	140 ± 17
		10	236 ± 25	60 ± 10
		50	76 ± 8	10 ± 3
		100	90 ± 32	5 ± 2
		500	11 ± 2	8 ± 4

DISCUSSION

A mutagenic response to sulfur mustard was detected with *Salmonella* strains TA97, TA98 and TA102. No mutagenic response was seen with TA100. Both strains TA97 and TA98 detect frameshift mutagens, but TA97 has been genetically designed to contain an added cytosine at the site of the hisD6610 mutation, allowing for greater sensitivity to frameshift mutagens. Strain TA102 detects a variety of oxidative mutagens and crosslinking agents, such as mitomycin C, and requires in intact excision repair system. The data from the assay without activation suggest that sulfur mustard acts directly as in intercalating or crosslinking agent. As evidenced by the minimal or total lack of response with strains TA98 and TA100, sulfur mustard does not exhibit activity at mutational sites of alternating G-C. This lack of response in reversion of these strains may be related to the lethal effects of this agent. It has been reported by Fox and Scott (1980) that crosslinking agents may cause a complete block to DNA synthesis, allowing for little or no possibility of replication across unexcised monofunctionally alkylated guanines.

In our laboratory, a chemical is considered mutagenic if: 1) it induces a response that is greater or equal to two times the experimental background (solvent control) for the day; 2) if the colonies formed were prototrophic (i.e., they were histidine revertants), and 3) if it shows an increasing dose response for two or more concentrations (μg/plate) in the dose-response range.

Using these criteria, sulfur mustard gave a mutagenic response with strains TA97 and TA102, with and without S9 activation. Strain TA98 did not exhibit a dose-response relationship for two doses but did give a response of two times the experimental background in both the standard plate and preincubation versions of the *Salmonella* histidine reversion assay. All colonies tested for prototropy were true revertants.

In summary, sulfur mustard gave a mutagenic response with tester strains TA97 and TA102 under the conditions in our laboratory. No mutagenic response was evident with TA100, and only a slight mutagenic response occurred with TA98.

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Study Dates: Initiated May 5, 1985
Completed July 24, 1985

Data are property of the U.S. Army and will be archived under the Army's direction at approved facilities.

Dorothy L Stewart
D.L. Stewart

6/7/84
Date

Mutagenicity of Sulfur Mustard in the *Salmonella* Reversion Test

Quality Assurance Statement

Listed below are the phases and/or procedures included in the study described in this report which were reviewed by the Quality Assurance Unit during the period, 5/15/85 - 6/1/85, specifically for this study and the dates the reviews were performed and findings reported to management.** (All findings were reported to the study director or his designee at the time of the review.)

Phase/Procedure Reviewed	Review Date	Date Findings Submitted in Writing to Study Director/Management
Plate Incorporation Procedure	5/21/85	5/22/85
Evaluation of Cell Growth	5/23/85	6/11/85
Data	10/4/85	10/8/85
Draft Report	10/5,7&8/85	10/8/85
Final Report	6/1/89	6/7/89

** Our observation of activities in the Chemical Surety Facility were limited to those conducted in the hood in room 196. These activities were observed by closed-circuit television.

R L Gelman
Quality Assurance Specialist

6-7-89
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