TERATOLOGY STUDIES ON LEWISITE AND SULFUR MUSTARD AGENTS: EFFECTS OF SULFUR MUSTARD IN RATS AND RABBITS

FINAL REPORT

P. L. Hackett, R. L. Rommereim, F. G. Burton, R. L. Buschbom, and L. B. Sasser

September 1987

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U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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Sulfur mustard (HD) was administered to rats and rabbits by intragastric intubation. Rats were dosed daily from 6 through 15 days of gestation (dg) with 0. 0.5, 1.0 or 2.0 mg of HD/kg; rabbits were dosed with 0, 0.4, 0.6 or 0.8 mg/kg on 6 through 19 dg. Maternal animals were weighed periodically and, at necropsy, were examined for gross lesions of major organs and reproductive performance; live fetuses were weighed and examined for external, internal and skeletal defects. In rats, reductions in body weights were observed in maternal animals and their female fetuses at the lowest administered dose (0.5 mg/kg), but the incidence of fetal malformations was not increased. In rabbits the highest administered dose (0.8 mg/kg) induced maternal mortality and depressed body weight measures but did not affect fetal development. These results suggest that orally administered HD is not teratogenic in rats and rabbits since fetal effects were observed only at dose levels that induced frank maternal toxicity. Estimations of dose ranges for "no observable effects levels" in rats and rabbits, respectively, were: <0.5 and <0.4 mg/kg in maternal animals and <0.5 and >0.8 mg/kg in their fetuses.

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QUALITY ASSURANCE STATEMENT

Listed below are phases and/or procedures included in the study described in this report which were reviewed specifically for this study by the Quality Assurance Unit during the period, 7/2/84 - 4/10/87, 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.)

PHASES/PROCEDURES REVIEWED	REVIEW DATES	DATE FINDINGS SUBMITTED IN WRITING TO STUDY DIRECTOR/MANAGEMENT
Health Screen	7/02/84	3/06/85
Animal Identification	7/17/84	3/06/85
Body Weights	7/17/84	3/06/85
Dosing	7/26/84	3/06/85
Necropsy	8/12/84	3/06/85
Dose Preparation	9/28/84	3/29/85
Vaginal Lavage	9/28/84	-
Animal Identification	9/28/84	3/29/85
Body Weights	9/28/84	3/29/85
Dosing	10/02/84	3/29/85
Necropsy	10/16/84	10/16/84
Artificial Insemination	11/20/84	8/26/85
Dosing	12/01/84	8/26/85
Necropsy	12/21/84	8/26/85
Artificial Insemination	1/14/85	8/26/85
Dose Preparation	1/23/85	8/26/85
Dosing	1/29/85	8/26/85
Necropsy	2/14/85	8/26/85
Data	3/05/85	3/06/85
Data	3/26-27/85	3/29/85
Data	4/18,5/10,20/85	8/26/85
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Final Report	3/31-4/2/87	*

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* Results of the review have been discussed with the study director and will be reported in writing to management.

Eileen M Crow — 6/15/87

Quality Assurance Specialist — Date

R A Gelman — 6/15/87

Quality Assurance Specialist — Date

EXECUTIVE SUMMARY

Sulfur mustard, which is categorized as an alkylating agent, has been implicated as a potential teratogen because its mode of action in vivo is very similar to that of nitrogen mustard, a known teratogen. Under contract with USABRDL, we therefore conducted studies to evaluate maternal toxicity, intrauterine mortality and developmental toxicity in rats and rabbits following administration of the agent by intragastric intubation. Preliminary dose-range studies were performed with pregnant animals to establish appropriate dose levels for teratology studies.

Solutions of sulfur mustard were prepared for administration by diluting the neat agent with sesame oil. Rats were dosed from 6 through 15 days of gestation (dg) with 0, 0.2, 0.4, 0.8, 1.6, 2.0 or 2.5 mg/kg in range-finding studies and with 0, 0.5, 1.0 or 2.0 mg/kg in the teratology study. Rabbits received 0, 0.5, 1.0, 2.0 or 2.5 mg/kg and 0, 0.4, 0.6 or 0.8 mg/kg for 14 days (6 through 19 dg) in the dose-range and teratology study, respectively. The volume of solution administered was 1 ml/300 g of body weight in rats and 1 ml/4 kg in rabbits.

Body weights were measured on 0, 6 through 16, and 20 dg in rats, and on 0, 6 through 20, and 30 dg in rabbits. At necropsy (20 dg for rats and 30 dg for rabbits), blood samples for hematocrit measurements were obtained from maternal animals, and the animals were examined for gross lesions of major organ systems, particularly of the gastrointestinal tract. Numbers of corpora lutea, implantation sites, resorptions, and live and dead fetuses were determined. Live fetuses were weighed and examined for gross external defects in the dose-range studies. In the teratology studies, additional examinations of the fetal viscera and skeletons were performed to detect morphologic anomalies.

In rats, no maternal deaths were attributable to sulfur mustard at dose levels below 2.5 mg/kg; gastric lesions were observed at a dose level of 2.0 mg/kg, and inflamed mesenteric lymph nodes were commonly observed at necropsy in animals exposed to dose levels in excess of 0.2 mg/kg. Other indicators of maternal toxicity (depressed body weights, extragestational body weights and extragestational gains) were evident at a dose level of 0.5 mg/kg in the teratology study. Hematocrit values decreased following administration of 0.8 or 1.0 mg/kg in the dose-range and teratology studies, respectively.

Reproductive and fetal effects that appear to be a consequence of maternal toxicity were: depressed weights of gravid uteri and placentas in rats of the high dose group (2.0 mg/kg) and significant depressions in fetal weights at lower dose levels (0.5 mg/kg for female fetuses and 1.0 mg/kg for males). Other evidence suggesting retarded fetal development was an increased incidence of reduced vertebral ossification in fetuses of the 0.5-mg/kg group, which was observed when the litter was defined as the unit for testing. Analyses of data based on fetal units indicated that incidences of supernumerary ribs, misaligned sternebrae and reduced ossification of the sternebrae were significantly increased in the high dose group (2.0 mg/kg) and that hydroureter was more apparent in fetuses of the 0.5- and 1.0-mg/kg groups than in fetuses of high-dose or control litters.

Dose levels of 0.8 mg/kg or higher were lethal to maternal rabbits. At necropsy, damage to the gastric mucosa and enlarged Peyer's patches were observed in animals that received the lowest dose (0.4 mg/kg) of sulfur mustard. Body weights, extragestational weight gains and hematocrit levels were depressed in animals of

the highest dose group (0.8 mg/kg) of the teratology study. At this dose level, no significant effects of sulfur mustard treatment on intrauterine mortality, placental and fetal body weights or the incidence of fetal anomalies were observed; however, a significant depression in fetal body weights of litters exposed to 2.0 mg/kg of sulfur mustard was found in the dose-range study.

Under the conditions of this experimental regimen, there was no evidence for a teratogenic response to sulfur mustard since all of the observed effects on fetal development occurred at dose levels that induced frank maternal toxicity. Definitive values for "no observable effect levels" (NOEL) were not obtained in these studies; however, results from the teratology studies permit estimation of specific limits of dose ranges of NOEL in rats and rabbits, respectively, as follows: <0.5 and <0.4 mg/kg in maternal animals and <0.5 and >0.8 mg/kg in their fetuses.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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INTRODUCTION

For decades, the possibility of adverse biological effects resulting from exposure to contaminants in the workplace, or in the environment, has been a matter for concern. In recent years, the potential for exposure of women of childbearing age (whose fetuses are at risk) has increased as a consequence of changing legal and socioeconomic factors. Because increasing numbers of reproductively competent women are now employed, and because employers must protect male and female workers alike, means for accomplishing such protection must be found. For some agents, there is also concern about exposure of large segments of the population to the agents following accidental or deliberate release into the environment. It is therefore necessary to identify potentially toxic and teratogenic chemicals and to establish a data base for the development of hazard evaluations and occupational health standards for them.

Sulfur mustard [bis(2-chloroethyl)sulfide], the agent of interest in this study, is a member of a class of chemicals designated as vesicant war gases. Recently, a renewed interest in this chemical was generated by the release of a United Nations report that contained substantial evidence that Iraq was manufacturing sulfur mustard and using it as a chemical warfare agent (Marshall, 1984). In man, sulfur mustard is a putative carcinogen; the incidence of upper respiratory tumors is correlated with the level of exposure to sulfur mustard during World War I (Fox and Scott, 1980).

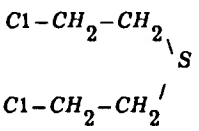
Relevant chemical and physical properties of sulfur mustard are summarized in Table 1. In aqueous solutions, sulfur mustard rapidly hydrolyzes to form a cyclic sulfonium salt, β-chloroethyl-ethylenesulfonium chloride. This salt reacts with water to form β-chloroethyl β-hydroxyethyl sulfide and hydrochloric 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, β-chloroethyl β-hydroxyethyl sulfide and thiodiglycol, were relatively nontoxic.

A review of more recent studies (Fox and Scott, 1980) indicates that the products of sulfur mustard hydrolysis are a small amount of sulfonium ion and, primarily, the carbonium ion that will react rapidly with a nucleophilic site. Thus, sulfur mustard, as well as nitrogen mustard, are biochemically related to a group of cytotoxic alkylating agents, including the ethylenimines, sulfonic esters, epoxides and n-alkyl-n-nitroso compounds. These alkylating agents react readily with certain functional groups of proteins (OH^-, NH_2^- and SH^-) to alter their metabolic activity.

The literature contains very little information concerned with the oral toxicity of sulfur mustard, particularly with regard to the amount of residual injury that might be encountered following short-term, multiple exposures. Published values for the LD_{50} of sulfur mustard administered in a single dose by intragastric (IG) intubation have been cited for rats but not in rabbits; however, a comparison of LD_{50} values following intravenous (IV), subcutaneous (SC) and percutaneous (PC) exposures indicated that the toxic effects of sulfur mustard were observed at a lower dose in the rat than in the rabbit (Table 2). These data also demonstrated that the diluent and the concentration of sulfur mustard in the dosing solution influenced the LD_{50} value (Anslow et al., 1948). The purity of the sulfur mustard used in these studies is not

known, but some of the preparations may approximate the purity of the agent Levenstein mustard, ~70% sulfur mustard, which was used to determine the oral LD_{50} value for rats.

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

CAS #	505-60-2
RTECS #	WQ0900000
Structural formula	
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
Melting point	13 to 14°C
Decomposition temperature	149 to 177°C
Solubility in water at 25°C	0.68 g/L
Hydrolysis	
Rate ($T_{\frac{1}{2}}$ at 25°C, pH 7)	8.5 minutes
Products	Thiodiglycol, chloride

[a]Data from Rosenblatt et al. (1975) and Windholz (1983).

TABLE 2. LD_{50} Values (mg/kg) for Sulfur Mustard

Route of Administration	Vehicle	ml/kg	Rat	Rabbit	Reference
Intravenous	Neat	---	3.3	4.5	Anslow et al., 1948
	Propylene glycol	0.5	0.7	2.7	
	Thiodiglycol	1.0	---	1.1	
Subcutaneous	---	---	1.5	---	Lewis and Tatken, 1980
	Neat	---	5.2	---	Anslow et al., 1948
	Propylene glycol	5.0	3.2	---	
		2.5	9.0	---	
	Sesame oil	5.0	5.0	---	Rosenblatt et al., 1975
	Tributyrin	---	2.5	20-30	
Percutaneous	---	---	~18	~100	Anslow et al., 1948
	---	---	50.9	100	
Intragastric	---	---	17	---	Medical Research Laboratory of the Chemical Warfare Service, 1943

Haskin (1948) reported that extensive edema occurred at the site of administration of nitrogen mustard (intraperitoneal [IP] and SC) and that diarrhea, dyspnea and anorexia were common observations. Death occurred in rats within 3 to 4 days

at dose levels of 1.8 to 3.1 mg/kg and from 5 to 19 days following doses of 1 to 1.2 mg/kg. The cause of death has been attributed to the cytotoxic effects of the mustards on rapidly renewing normal tissues, including lymphoid tissues, stomach, small and large intestine and bone marrow (Fox and Scott, 1980).

Studies of the effects of the mustards on DNA synthesis have led to the conclusion that the cytotoxicity of sulfur and nitrogen mustard is related to their ability to cross-link DNA, thus inhibiting its replication (Fox and Scott, 1980). Mutagenicity of the mustards has been demonstrated in micro-organisms, plants, *Drosophila* and in cultures of mammalian cells. Significant incidences of dominant lethal mutations have been observed following chronic inhalation exposure of male rats to 0.1 mg/m^3 of sulfur mustard; however, exposure of pregnant females to the same atmospheric concentration during the shorter interval of gestation failed to induce fetal malformations (Rozmiarek et al., 1973).

Teratology studies performed in rodents demonstrated that nitrogen mustard was embryocidal and teratogenic (Danforth and Center, 1954; Haskin, 1948). IP administration of nitrogen mustard to rats at 12 days of gestation (dg) at a dose level of 1.4 mg/kg body weight caused 100% intrauterine mortality; 0.7 mg/kg induced syndactyly, encephalocele and cleft palate; and 0.35 mg/kg caused no fetal effects (Murphy et al., 1958). Recent studies with in vitro cultures of 11-dg rat conceptuses have demonstrated that the addition of nitrogen mustard to the culture medium markedly affected embryonic development and organogenesis (Sanyal et al., 1981).

Other than the studies of Rozmiarek et al. (1973) mentioned above, no comparable information concerning the teratogenic potential of sulfur mustard appears to be available in the literature; therefore, the studies reported here were directed toward this objective. The purpose of our study was to determine developmental effects in pregnant rats and rabbits exposed to sulfur mustard during the gestational period that encompasses major organogenesis. To accomplish these exposures, sulfur mustard was administered by IG intubation to pregnant rats and rabbits for periods of 10 or 14 consecutive days, respectively. Preliminary range-finding studies were performed to obtain appropriate dose levels of sulfur mustard for the teratology studies. Target dose levels ranged from a dose that would induce evidence of maternal toxicity or affect fetal development to a dose that would cause no observable fetal effects attributable to sulfur mustard.

MATERIALS AND METHODS

SULFUR MUSTARD

Procurement and Characterization

The sulfur mustard obtained for these studies has the chemical name 2,2'-dichlorodiethyl sulfide and is also known as distilled mustard (HD).

A shipment of 30 ml of HD (USAMRICD Lot No. ICD-HD-1) was provided by the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) and was delivered on 04-25-84 by a team from the U.S. Army Technical Escort Unit. Inspection of the ampule indicated that it was intact. The HD was subsequently divided into two 15-ml aliquots that were pipetted into 30-ml Wheaton bottles, sealed, and stored in secondary unbreakable containers in a refrigerated storage container at ~6°C. The HD was observed to be a colorless liquid at room temperature. Under the storage conditions, it occasionally, but not regularly, solidified. Although no analytical information was received with the shipment, subsequent information supplied by David Stitcher of the U.S. Army Medical Research and Development Command (letter dated 06-19-84) indicated a purity of 97.9 to 98.7 weight percent HD, as determined by nuclear magnetic resonance (NMR). To comply with Good Laboratory Practices (GLP) requirements (21 CFR 58.105), Pacific Northwest Laboratory (PNL) has requested that USAMRICD retain a 50-g aliquot of this lot of HD; they have verbally agreed.

A purity analysis (Appendix A) by gas chromatography (GC) was performed on the neat (undiluted) agent on 07-25-84. The analytical procedure used a 30-m capillary glass column, narrow (0.25-mm) bore, DB-5 bonded phase (J & W Scientific, Inc.) The oven temperature was held at 80°C for 5 minutes, then increased at 5°C/minute until it reached 300°C. Purity, determined by the area-percent method, was 95.9 to 96.1% by this procedure, in reasonable agreement with the NMR determination by USAMRICD. There were two impurities with concentrations (by area percent) in the range of 0.87 to 1.17% and six impurities with concentrations (by area percent) of 0.10 to 0.35%.

Characterization of the Diluent

The selection of a satisfactory vehicle to use in preparing sulfur mustard solutions for administration to the animals was based on the chemical and physical properties of the compound, i.e., its relatively low solubility (0.68 g/L) and rapid hydrolysis ($T_{\frac{1}{2}}$ = 8.5 minutes) in water. Corn oil is commonly the vehicle of choice for the administration of water-insoluble compounds, but several reports have indicated that the use of corn oil may not be appropriate for reproduction studies. Corn oil was reported to induce uterine hypertrophy (Duncan, 1955) and to alter immune function in developing rats (Springer, 1982) and mice (Shepman and Schmidt, 1984). A recent comparison (Kimmel et al., 1985) of reproductive measures in rats dosed with distilled water or corn oil indicated that fetal body weights were lower and that the incidence of malformations was higher in litters of dams dosed with corn oil from 6 through 15 dg. In considering an alternative to corn oil, we found that sesame oil was classified as "generally recognized as safe" by the Food and Drug Administration's (FDA; Furia, 1972) and that the major fatty acid components were quite similar in both oils (Altman and Dittmer, 1972); however, sesame oil contains a lower concentration of sterols (0.49%) than corn oil (0.79%). Sesame oil is readily

available, contains no preservatives and appears to be quite stable when stored under the proper conditions; however, no historical data for reproductive or developmental effects are available in the literature.

During the course of the rat and rabbit studies of HD, a total of 9 quarts of sesame oil were used. The first group of three bottles was purchased 07-02-84, and the second group of six bottles was purchased 10-01-84. The bottles of sesame oil were produced by the Hain Pure Food Company (Los Angeles, CA) and purchased from the Fred Meyer Health Food Department (Richland, WA). An effort was made to obtain bottles from a single lot; however, the presumptive lot number on the bottles, B11421/B (a), has not changed with time and is apparently not a lot number. Occasionally, a small amount of sesame oil was left in a bottle at the end of a gavage study. This was discarded when an extended period of time preceded the next gavage study to ensure that the gavage solutions were prepared from sesame oil with the lowest peroxide content.

Individual bottles of sesame oil used for the studies were analyzed to determine their peroxide content, which serves as a measure of oxidation or rancidity of the oil. The method used quantifies peroxides (and similar substances) that oxidize aqueous iodide under the conditions of the test. The iodine produced is determined potentiometrically by titration with sodium thiosulfate.

Each batch of sesame oil was analyzed prior to its use for the dilution of HD, and again at the end of each study. This procedure gives an accurate estimate of the amount of peroxide in the sesame oil at any time during the study. As in previous studies at PNL, we established 10 meq/kg as the unacceptable level for the peroxide content of an oil.

The results of the analyses for peroxide in sesame oil are given in Table 3. In all cases, the amount of peroxide in the sesame oil was well below the acceptable limits of 10 meq/kg.

TABLE 3. Analysis of Sesame Oil for Peroxide

Assay Date	Container Identification[a]	Peroxide Content (meq/kg ± SD)	PNL Notebook Reference
First Lot			
07-17-84	A	0.22 ± 0.05	50113-3/5
08-15-84	A	0.41 ± 0.02	50113-13/15
09-18-84	B	0.97 ± 0.04	50113-16/18
Second Lot			
10-04-84	A	0.17 ± 0.06	50113-20/22
11-09-84	A	1.07 ± 0.05	50113-25/27
01-11-85	C	0.30 ± 0.04	50113-31/33
03-11-85	E	0.44 ± 0.04	50113-39/43

[a]Denotes source of assay material

Preparation of Solutions for Administration

Specific procedures for preparation of each batch of HD in sesame oil are given in PNL Record Books 50111 and 50844. The general procedure followed was to determine in advance 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 sesame oil. Aliquots of this intermediate concentration were then diluted further to give the final concentrations needed for the dosing solutions. Once the final solutions were prepared, aliquots of the solution were placed in Wheaton bottles with teflon-lined septa and aluminum caps. Each Wheaton bottle contained a sufficient volume of HD/sesame oil for 1 day's use. The bottles were labeled with the name of the agent (HD) and the concentration. The bottles were then placed in a second unbreakable container that was labeled with the following information: solution identification, lot number, concentration, date, PNL Laboratory Notebook number, and initials of preparer. The bottles were then stored in a refrigerated storage container at ~6°C until used.

Analyses for Concentration and Stability of Dilute Solutions

The dose levels to be administered and the corresponding theoretical and actual concentrations, as analyzed, of dilute sulfur mustard solutions for each of the studies are listed in Table 4. The concentration of HD in these solutions was analyzed by a GC procedure that estimates the amounts of HD in the solutions and determines the extent of HD degradation during the storage period of the solution.

TABLE 4. Sulfur Mustard Dose Levels and Solution Concentration for Animal Studies

Study	Dose Level (mg/kg)	HD Concentration (mg/ml)	
		Theoretical	Analyzed[a]
Rat dose-range	0.2	0.06	0.057 ± 0.007
	0.4	0.12	0.116 ± 0.007
	0.8	0.24	0.241 ± 0.018
	1.6	0.48	0.488 ± 0.016
	2.0	0.60	0.586 ± 0.006
	2.5	0.75	0.744 ± 0.004
Rat teratology	0.5	0.15	0.150 ± 0.007
	1.0	0.30	0.297 ± 0.009
	2.0	0.60	0.599 ± 0.026
Rabbit dose-range	0.5	2.0	2.04 ± 0.11
	1.0	4.0	4.00 ± 0.15
	2.0	8.0	7.84 ± 0.31
	2.5	10.0	10.10 ± 1.01
Rabbit teratology	0.4	1.6	1.60 ± 0.05
	0.6	2.4	2.40 ± 0.09
	0.8	3.2	3.20 ± 0.12

[a]Mean ± SD of pooled results of analyses of samples from the day of preparation and the last day of use.

Analytical Procedures

Samples were prepared for analysis in the following way: 1-ml aliquots of the internal standard (ISTD), 144.45 mg/ml of 2,4-dichlorotoluene in isooctane for rat studies (or 1.4445 mg/ml of 2,4-dichlorotoluene in isooctane for rabbit studies), were placed in test tubes. A 1-ml aliquot of the HD/sesame oil gavaging solution was then taken from the gavaging bottle and thoroughly mixed with the ISTD solution. This solution was then divided into two automatic liquid sample (ALS) vials and analyzed by GC. In some cases, two 1-ml aliquots were taken from a single gavaging solution to estimate variability between samples.

To quantitate the results of the GC, the data from the chromatograms were used to determine the ratio of the area of the HD to that of the ISTD. These data were fit to a linear regression curve using a Hewlett-Packard (HP) 25 calculator to determine the slope, the intercept and the coefficient of determination (r^2). Since the rat data were obtained using an ISTD solution that was precisely one-tenth the concentration of that used in the rabbit study, the slope values for the rabbit data included an additional factor of 10.

Data obtained from the linear regression curves for all gavage-solution preparations for both rats and rabbits are given in Table 5. In all cases, values for r^2 were acceptable, both within and among individual studies. Because no external standard was available for comparisons and an internal standard was used in the analyses, some variability in values for intercepts and slopes were attributed to changes in the sensitivity of the gas chromatograph.

TABLE 5. Summary of Data from Curve-Fitting for Sulfur Mustard Studies

Study	Intercept	Slope	r^2
Rat dose-level (combined data from three preparations)	-0.02	3.75	0.994
Rat teratology			
First preparation	-0.04	3.89	0.992
Second preparation	-0.01	3.64	0.994
Rabbit dose-level			
First preparation	-0.013	3.56	0.992
Second preparation	-0.056	3.79	0.982
Rabbit teratology			
First preparation	0.006	3.18	0.984
Second preparation	-0.018	3.67	0.979

For these studies, the HD solutions to be administered to the animals were made up in advance and stored in a refrigerator at ~6°C until used. For each of the studies reported, the required amount of solution was prepared just prior to use, in two lots; one volume sufficient for each half of the dosing interval. As a result, some of the gavaging solution was held in refrigerated storage for as long as 10 days. Since

GLP regulations require that the stability of the test material be determined in the carrier, the stability of the HD in sesame oil over periods of 10 days or longer was determined.

The initial study, entitled "Distilled Mustard Stability Study" and reported on 09-28-84 (see Appendix A), compared the samples prepared on 07-20-84, 07-31-84 and 08-20-84. Thus, some samples were a month older than other samples. Aliquots of each of the dosing solutions were saved in the refrigerated storage container until 08-28-84, at which time they were mixed with an aliquot of the 2,4-dichlorotoluene ISTD. They were then analyzed in duplicate on the gas chromatograph. Although there was some variability between samples (attributable to handling during preparation), there was no evidence of significant degradation in any of the samples over this time interval. The r^2 for all the samples was 0.994. The slope was 3.75, which is not significantly different from values determined later in the study for samples that were analyzed shortly after preparation.

A second study, titled "Question: Is Sulfur Mustard (HD) Stable in Sesame Oil over a 10-Day Period?" involved analysis of the gavage solutions each day following the dosing; this was reported on 11-03-84 (see Appendix A). An aliquot of the gavaging solution was mixed with an aliquot of the 2,4-dichlorotoluene ISTD and analyzed. Data were obtained for the period from Day 3 to Day 8 or Day 9 (two separate preparations were analyzed) after the solution was prepared (on Day 0). The results of the analyses were submitted to a statistician to determine, by regression analysis, whether the data indicated a decay with time (i.e., whether the slope was significantly different from zero). After statistical analysis, the statistician stated that the "results suggest that no change in HD values occurred over the course of the study."

In addition to these specific studies, HD/sesame oil dosing solutions for all rabbit studies were analyzed by GC, using an ISTD, on the date of preparation, part-way through the dosing interval, and on the final day of dosing. In no case was there evidence of degradation of the HD in the sesame oil.

We therefore conclude that the HD is stable in the refrigerated sesame oil for at least a 10-day period and may be stable for periods in excess of 1 month.

ANIMAL MAINTENANCE

Procedures for Rats

Young, adult CD female (7 to 8 weeks old, 170 to 175 g) and male (7 to 8 weeks old, 200 to 225 g) rats of Sprague-Dawley derivation were obtained from Charles River Breeding Laboratories, Inc., Portage, MI Facility. Groups of 65 females and 55 males (for the dose-range study) and 206 females and 111 males (for the teratology study) were purchased to provide sufficient pregnant animals for the studies, to provide additional animals for evaluation of health status, and to compensate for any losses in shipment.

Upon arrival at PNL, the rats were placed in an isolated facility and remained within this facility until sacrifice. Specified environmental conditions for animal rooms were temperatures of 72 ± 3°F, relative humidities (RH) of 50 ± 15% and a lighting cycle of 12 hours on/12 hours off. During quarantine, the animals were group-housed in stainless-steel, wire-mesh cages placed in automatic-flush racks provided with an automatic watering system. Females in which sperm were

detected were housed in individual cages in similar cage racks. Purina Certified Rodent Chow (#5002) and water were provided ad libitum.

Purchase requisitions specified that the rats were to be free of *Mycoplasma*, Sendai virus and Corynebacterium. Upon receipt, six rats (one female and five males) from the shipment for the dose-range study were removed for health evaluations. Their sera were tested for antibodies to Sendai virus, pneumonia virus of mice (PVM), rat corona virus/sialodacryoadenitis virus (RCV/SDA), H-1 virus and Kilham rat virus (KRV) by Microbiological Associates (Bethesda, MD). Ten rats (five females and five males) from the shipment for the teratology study were tested for Sendai virus, PVM, RCV/SDA and *M. pulmonis* by serological methods in our laboratory. Additional evaluations included cultures of nasopharyngeal and lung washes; examination of animals for internal and external parasites; and histopathologic examination of lungs, trachea, Harderian gland, heart, liver, kidney, ileum and colon. No significant pathogens or pathologic lesions were detected in rats of either shipment.

Following an isolation period of 3 weeks, the females were individually identified by means of a numbered eartag and weighed. The rats were allowed to mate overnight by caging one male with one female. Copulation was established by detecting the presence of sperm in the vagina, as determined by microscopic examination of a slide prepared from a lavage suspension of normal saline delivered into, then recovered from, the vagina with a pipette. Mating was continued nightly until sufficient sperm-positive rats were obtained for the study. The morning of observation of sperm was designated as 0 dg. At that time, females that had mated the previous night were weighed and assigned to treatment groups by means of formal randomization, blocking on weight and using a statistical software package. Each treatment group of rats was identified by an appropriate toe clip, in addition to the individual identification by numbered eartags. Cage cards for individual rats indicated the animal number, treatment group and gestational group.

Procedures for Rabbits

Sexually mature, New Zealand White rabbit does (5 to 6 months of age; body weight about 3 kg) were obtained from R & R Rabbitry (Stanwood, WA). In addition to the 112 does used in the studies, 10 additional does were obtained to be used for training bucks to the artificial vagina (AV), or for replacements to compensate for shipping losses or in the event that the rabbits' health status might interfere with the study results. Six mature, naive bucks (6 to 7 months old; body weight 3 to 4 kg) of the same stock were purchased for breeding in the dose-range study; an additional six bucks were purchased for the teratology study. All rabbits were identified by the supplier with a uniquely numbered, stainless-steel eartag.

Upon arrival at PNL, the rabbits were placed in an isolated facility, where they remained until sacrifice. Specified environmental conditions for animal rooms were temperatures of 70 ± 4°F, RH of 50 ± 15% and a lighting cycle of 16 hours on/8 hours off. The animals were housed individually in stainless-steel, wire-mesh cages in automatic-flush racks. They were provided with Purina Certified Rabbit Chow (#5322) and water ad libitum. Food consumption was not measured, but estimates of the amount eaten by each animal were obtained by observing the feeders, which were completely filled with food each day. The rabbits were considered to be anorectic if their food, which had been leveled at the top and bottom of the feeder, had not been disturbed.

To obviate the possibility of pseudopregnancy and to permit frequent observations of their health status, the does were maintained for 21 days (dose-range study) and 24 days (teratology study) before use. For the first 10 days of this period, oxytetracycline was added to the drinking water for prophylaxis. The rabbits were examined by the PNL Clinical Veterinarian and were found to be acceptable for these studies. A bilateral ocular discharge was noted in one doe (#7228) of the group used in the teratology study.

Following quarantine and acclimation, the does were weighed and assigned to treatment groups by means of a formal computerized-randomization program. Artificial insemination (AI) was performed in the afternoon of the day designated as 0 dg.

Bucks to be used as semen donors were trained to service an AV during a 2- to 3-week period (Gregoire et al., 1958). On the day of AI, semen samples from at least three bucks were collected into an AV equipped with a reservoir warmed to 42 to 45°C just prior to use (Adams, 1961; Hafez, 1970; Tesh and Tesh, 1971; Hagen, 1974). The individual samples were evaluated for motility, sperm concentration and the presence of urine, bacteria, erythrocytes and leukocytes. Semen samples of satisfactory quality were pooled and diluted with buffered citrate/egg-yolk extender to a concentration of 21 to 30 million sperm/ml (Table 6).

TABLE 6. Characteristics of Extended Rabbit Seminal Fluids used for Artificial Insemination[a]

Study	Day of Insemination	Number of Does Inseminated	Number of Donor Bucks	Sperm Characteristics		
				Concentration (10^6/ml)	Motility	
					Grade[b]	Percent
Dose range	1	20	3	21	3	73
	2	19	3	21	3	83
Teratology	1	24	5	30	3	84
	2	24	4	30	3	89
	3	25	4	30	3	78

[a]Pooled semen samples diluted with buffered citrate/egg-yolk extender
[b]Graded from 0 (no motility) to 4 (excellent motility)

The does were inseminated with approximately 0.5 ml of the extended semen within 2 hours of semen collection. To induce ovulation, 100 USP units of chorionic gonadotropin (APL; Ayerst, 500 USP units/ml in saline) were administered to each doe by IV injection immediately after insemination.

ADMINISTRATION OF SULFUR MUSTARD

Solutions of appropriate concentrations of sulfur mustard in sesame oil were administered to the animals by IG intubation, in the morning, on consecutive days. Rats were dosed for 10 days (6 through 15 dg); rabbits were dosed for 14 days (6 through 19 dg). Daily doses for individual animals were calculated from their body

weight, which was determined just prior to dosing. The dosage volume/body weight was 1 ml/300 g for rats and 1 ml/4 kg of body weight for rabbits.

The dosing solution was measured with a syringe and delivered to the rats with an 18-ga, 3-in. feeding needle terminating in a 2.25-mm ball. For rabbits, a #8 French 22-in. feeding tube was used to ensure delivery of the dose solution into the stomach. To minimize the possibility of injury to the animals during dosing, the rats were restrained by the technician who delivered the dose. Rabbits were restrained by enclosure in a canvas bag, which was hand-held in a plastic box by an assistant to the technician who delivered the dose.

TOXICOLOGIC AND DEVELOPMENTAL EVALUATIONS

All animals were observed for clinical signs of toxicity in the morning prior to, and following, the administration of the sulfur mustard, and observed again in the afternoon. The condition of the animals was observed twice daily on experimental days on which no chemical was administered. Body weights of rats were determined prior to mating and on 0, 6 through 16, and 20 dg; body weights of rabbits were measured prior to AI and on 0, 6 through 20, and 30 dg.

Necropsies were performed on animals found dead and those that were euthanized because they were judged too moribund to complete the scheduled experimental regimen. All gross observations for abnormalities were recorded and grossly abnormal tissues were preserved in 10% neutral buffered formalin (NBF). Ovaries were removed, and the corpora lutea were counted; the uterus was opened and examined for the number and position of viable fetoplacental units and of resorption sites.

At scheduled sacrifice (20 dg for rats and 30 dg for rabbits), the animals were killed in a randomly determined order by introduction of carbon dioxide into a euthanasia chamber. The animals were identified only by their unique identification number to assure that the treatment group was not known to the prosectors. Foot markings of the rats were observed only by the prosectors performing the necropsy of the adult animals, so that the exposure group was not known to those evaluating reproductive status and fetal measures. Body weights and uterine weights were measured and recorded. All animals were examined for evidence of infections or lesions. Grossly abnormal tissues were preserved in 10% NBF.

The uterus, with ovaries attached, was removed from each animal and weighed. The ovaries were excised, identified as to right and left, and the number of corpora lutea estimated by counting. Uteri of all apparently nonpregnant females were stained with ammonium sulfide and examined for implantation sites (Kopf et al., 1964). In pregnant animals, the excised uterus was opened. Beginning at the right ovary, numbers were assigned consecutively to each implantation site down the right horn to the cervix. Consecutive numbers for implantation sites in the left horn proceeded from ovary to cervix. The membranes and amniotic fluid were observed for abnormalities, and living and dead fetuses and resorptions were counted. For the rat, mortality in utero was classified and recorded as "early" (placenta and conceptus indistinguishable, or metrial gland), "late" (placenta distinct, embryo or fetus partially to fully formed), or "dead" (no movement detected at necropsy). For rabbits, mortality in utero was classified as "early" (placenta and conceptus indistinguishable, or metrial gland), "mid" (placenta distinct, embryo partially to fully formed), "late" (fully formed with evidence of resorption), or "dead" (no move-

ment detected at necropsy) to provide more information concerning the time of death.

For dose-range and teratology studies, live and recently dead fetuses were removed in serial order, freed of adherent material and weighed. Each fetus was examined for gross external abnormalities. The sex of each rat fetus was determined by external inspection of the anogenital distance; the sex of rabbit fetuses was determined by internal examination because of the variability in anogenital distance encountered in this species (Njielsen and Torday, 1983). For teratology studies, the crown-rump length of each fetus was measured. Concurrently, the placentas were removed, weighed and examined; abnormal placentas were preserved in 10% NBF. Fetuses of both species were randomly divided into two equal groups for more detailed teratologic examination. Complete skeletons were examined in one group; in the second group, the heads were removed and placed in Bouin's fixative for subsequent examination of serial razor-blade-cut sections by the methods of Wilson and Warkany (1965) and van Julsingha and Bennett (1977) for rats and rabbits, respectively.

All fetuses in the teratology studies were examined for internal abnormalities by dissection, using Staples' (1974) technique, which is a modification of that of Barrow and Taylor (1969), and is similar to those described by Stertz (1977) and Stuckhardt and Poppe (1984). The sex of each fetus was determined by visceral examination of the gonads. All fetuses were eviscerated; rat fetuses were skinned and immediately fixed in alcohol; rabbit fetuses were skinned and air-dried prior to fixation in alcohol. Following staining with Alizarin red S, maceration with KOH and clearing in glycerol (Staples and Schnell, 1964), each skeleton was examined for abnormalities in size, shape, relative position and degree of ossification. Results from fetal morphologic examinations were grouped into three categories (major malformations, minor anomalies or morphologic variations) according to degree of severity, locus of fetal structural change and incidence of these changes (Palmer, 1977, 1978; Perraud, 1976).

Tabulated data for body weights of adult animals are from pregnant animals only. Although formal randomization of body weights was used to select animals for the experimental groups, the fact that data from pregnant animals only were analyzed tended to produce apparent deviations in initial body-weight values for some groups of animals. Extragestational body weight (body weight at necropsy minus weight of gravid uterus) and extragestational gain (extragestational weight minus body weight on 0 dg) were calculated for each maternal animal.

STATISTICAL METHODS

The computer software program (DRANDBLK) for randomizing animals into experimental groups is based on a single blocking factor, animal weight at 0 dg. Animal weights for a given study are ordered from lightest to heaviest; blocks of animal weights are then randomly assigned to the treatment groups and the control group. Block sizes are governed by the number of test groups.

Analysis of variance, which was used to analyze continuous variable data, employed the General Linear Model (GLM) procedure in the Statistical Analysis System (SAS, 1985). If the F statistic was significant, Duncan's (1955) multiple range test was used to delineate intergroup differences. A comparisonwise error rate was set at 0.05 for Duncan's test. By using this comparisonwise error rate in the Duncan's multiple range test, more significant differences may be detected than would result

from the use of a multiple comparison procedure that sets the experimentwise error at 0.05. Data were also compared by orthogonal contrasts to determine if there was a trend with respect to increasing dose.

A randomization test was used to analyze body-weight measurements. This test is a nonparametric statistical test that is based on the absolute area between growth curves. The test allows for correlation of body-weight measurements over time (Zerbe, 1979).

Fetal body weight and crown-rump lengths for live fetuses were analyzed by nested analysis of variance. The analysis takes into account the effects of treatment, litter and sex on the body-weight and crown-rump length measurements.

Pairwise comparison of binary response variables between groups was done by chi-square tests or Fisher's Exact Test (Siegel, 1965), using the P4F program in the BMDP statistical software (Dixon et al., 1983). Chi-square tests were used for individual comparisons of fetal data, and Fisher's Exact Test was used for litter data, where a litter with one or more fetuses responding was considered a positive litter response.

DOSE-RANGE AND TERATOLOGY STUDIES IN RATS

Fifty-six adult female rats were mated for 7 consecutive nights to provide 43 sperm-positive animals for the dose-range study. These animals were weighed and assigned to treatments groups that received the following dose levels of sulfur mustard: 0 (vehicle control), 0.2, 0.4, 0.8 and 1.6 mg/kg (Table 7). None of the rats had died by the conclusion of this study; therefore, two additional groups of animals were dosed with 2.0 or 2.5 mg/kg. All of these rats were from the same shipment of animals; rats in the latter two groups were 1 month older than those in the original dose groups at the time of dosing.

TABLE 7. Experimental Design for Sulfur Mustard Studies with Rats

Dose Level (mg/kg)	Number of Sperm-Positive Females	
	Dose-Range Study	Teratology Study
0	9	25
0.2	8	--
0.4	9	--
0.5	-	25
0.8	9	--
1.0	-	27
1.6	8	--
2.0	3	25
2.5	3	--

For the teratology study, 200 adult female rats were mated for 4 consecutive nights to obtain the 102 sperm-positive females used in this study. The rats were weighed and assigned to treatment groups by formal randomization. Data from the dose-range study was used to establish the following dose levels: 0 (vehicle control), 0.5, 1.0 and 2.0 mg sulfur mustard/kg (Table 7).

Sulfur mustard solutions were administered by IG intubation in the morning on consecutive days from 6 through 15 dg. The animals were weighed prior to mating and on 0, 6 through 16, and 20 dg and observed for behavior and signs of toxicity at least twice daily. At scheduled sacrifice (20 dg), maternal animals were weighed and examined for gross abnormalities and reproductive status. Procedures for these observations and for the fetal evaluations for each study have been detailed in previous sections.

DOSE-RANGE AND TERATOLOGY STUDIES IN RABBITS

For the dose-range study, 39 mature New Zealand White rabbit does were artificially inseminated on 2 consecutive days (20 does on the first day and 19 on the second day). On each day, the does were randomly distributed into five treatment groups on the basis of their weight on 0 dg. Dose levels of sulfur mustard for this study were 0 (vehicle control), 0.5, 1.0, 2.0 and 2.5 mg/kg (Table 8).

TABLE 8. Experimental Design for Sulfur Mustard Studies with Rabbits

Dose Level (mg/kg)	Number of Inseminated Does	
	Dose-Range Study	Teratology Study
0	8	19
0.4	-	18
0.5	8	--
0.6	-	18
0.8	-	18
1.0	8	--
2.0	8	--
2.5	7	--

In the teratology study, 73 females were artificially inseminated on 3 consecutive days (24, 24 and 25 does on Days 1 to 3, respectively). The does were randomly assigned to four dose groups: 0 (vehicle control), 0.4, 0.6 and 0.8 mg/kg (Table 8). The selection of these dose levels was based on information obtained in the dose-range study.

The sulfur mustard solutions were administered to the rabbits by IG intubation in the morning on consecutive days from 6 through 19 dg. The animals were weighed prior to AI and on 0, 6 through 20, and 30 dg and observed for clinical signs of

toxicity at least twice daily. At scheduled sacrifice (30 dg), maternal animals were weighed and examined for gross abnormalities and reproductive status. Details of these procedures and for fetal evaluations for each study appear in previous sections.

All facets of these studies were conducted in compliance with FDA GLP regulation 21 CFR 58. All tissue and fetal specimens will be shipped to a storage site designated by the U.S. Army Biomedical Research and Development Laboratory (USABRDL). Documentation, records, raw data and Final Reports will be placed in Room 1433 of the Life Sciences Laboratory (LSL)-II building at PNL according to 21 CFR 58.195 for at least 2 years following completion of the study or until USABRDL requests transfer of the data. USABRDL will be notified of the completion of the storage period so that they may specify the terms of the disposition of these records.

RESULTS

DOSE-RANGE STUDY IN RATS

The numbers of experimental animals, the numbers of rats surviving the experimental regimen and their reproductive status are shown in Table 9. One of three rats in the 2.5-mg/kg dose group died on 12 dg from treatment-related effects, which included sloughing of the gastric mucosa, pericardial and gastrointestinal inflammation and inflammation of lymph nodes and renal papillae (Table 10). Lesions of the gastric mucosa were observed in one additional rat of this group and in one of the 2.0-mg/kg dose group. The most common finding at necropsy in all groups exposed to sulfur mustard at dose levels greater than 0.2 mg/kg was inflammation of the mesenteric lymph nodes. Lung lesions were encountered in two rats of the 0.4-mg/kg dose group and two of the 0.8-mg/kg group; these were single, small, red foci that may have been induced by the carbon dioxide euthanasia. Observations at necropsy for individual rats are listed in Table B1 of Appendix B.

TABLE 9. Status of Rats for the Dose-Range Study of Sulfur Mustard

Status	Dose Level (mg/kg)						
	0	0.2	0.4	0.8	1.6	2.0[a]	2.5[a]
Number of rats dosed	9	8	9	9	8	3	3
Not pregnant	2	3	2	2	1	1	1
Pregnant	7	5	7	7	7	2	2
Number of survivors	9	8	9	9	8	3	2
Not pregnant	2	3	2	2	1	1	1
Pregnant	7	5	7	7	7	2	1

[a]Groups were dosed in a separate study

TABLE 10. Summary of Observations at Necropsy of Maternal Rats in the Dose-Range Study of Sulfur Mustard[a]

Dose (mg/kg)	Number of Animals	Number with Abnormalities	Observations
Vehicle	9	1	Ovarian cyst
0.2	8	0	---
0.4	9	4	Inflamed mesenteric lymph nodes
		1	Esophageal lesion
0.8	9	9	Inflamed mesenteric lymph nodes
		1	Enlarged lumbar lymph nodes
		1	Slight renal cavitation
1.6	8	6	Inflamed mesenteric lymph nodes
		1	Enlarged lumbar lymph nodes
2.0	3	1	Bilateral renal cavitation; esophageal blister (mesenteric); petechial hemorrhage and sloughing of gastric mucosa
		2	Inflamed mesenteric lymph nodes
		1	Inflamed renal papillae
2.5	3	1	(Died, 12 dg) Pericardial and gastrointestinal inflammation; inflamed thymus and Peyer's patches; gastric mucosa sloughing; inflamed renal papillae
		3	Inflamed mesenteric lymph nodes
		1	Petechial hemorrhage and thickened gastric mucosa

[a]Observations at necropsy for individual rats are shown in Table B1, Appendix B.

Body weights of pregnant survivors tended to be lower than control values in the higher dose groups after 11 dg, or when sulfur mustard had been administered for 5 consecutive days (Table 11). Body weights for the rats receiving 1.6 mg/kg were significantly lower than those of control animals from 12 dg to sacrifice (20 dg). No statistical comparisons were made for the 2.0- and 2.5-mg/kg dose groups because these rats were 1 month older than rats of the other five groups at the time dosing was initiated. Changes in body weights during gestation, expressed as percentage of the weight on 0 dg, are shown for all treatment groups in Figure 1.

TABLE 11. Body Weights (g, Mean ± SE) of Pregnant Survivors in the Dose-Range Study of Sulfur Mustard in Rats

	Dose Level (mg/kg)						
dg[a]	0	0.2	0.4	0.8	1.6	2.0[b]	2.5[b]
0	243 ± 11	246 ± 2	243 ± 12	244 ± 5	247 ± 4	280 ± 31	271
6	279 ± 11	283 ± 1	282 ± 10	287 ± 4	287 ± 3	303 ± 24	308
7	281 ± 12	282 ± 3	282 ± 12	281 ± 3	280 ± 4	297 ± 30	304
8	283 ± 11	287 ± 3	284 ± 11	277 ± 4	275 ± 4	282 ± 29	288
9	288 ± 11	287 ± 6	285 ± 9	274 ± 3	269 ± 5	289 ± 26	279
10	293 ± 13	289 ± 7	287 ± 10	280 ± 4	270 ± 5	289 ± 27	274
11	301 ± 13	295 ± 6	292 ± 9	285 ± 5	276 ± 5	290 ± 27	274
12	306 ± 13[c]	301 ± 6[c,d]	296 ± 10[c,d]	285 ± 4[c,d]	278 ± 5[d]	295 ± 29	273
13	309 ± 13[c]	305 ± 6[c]	297 ± 11[c,d]	289 ± 4[c,d]	275 ± 5[d]	299 ± 28	264
14	315 ± 14[c]	306 ± 6[c]	302 ± 10[c,d]	293 ± 5[c,d]	274 ± 5[d]	300 ± 23	262
15	325 ± 14[c]	311 ± 7[c]	302 ± 11[c,d]	298 ± 4[c,d]	280 ± 6[d]	298 ± 27	264
16	337 ± 16[c]	324 ± 7[c]	312 ± 11[c,d]	306 ± 4[c,d]	281 ± 6[d]	295 ± 32	270
20	398 ± 19[c]	379 ± 10[c,d]	366 ± 14[c,d]	377 ± 7[c,d]	354 ± 5[d]	362 ± 36	328

[a]dg = day of gestation

[b]Groups were dosed in a separate study; no statistical comparisons were made.

[c,d]Values that do not share a common superscript letter are significantly different ($P \leq 0.05$) from one another (Duncan's multiple range test).

Weights of the gravid uteri were not different among treatment groups (Table 12). Values for extragestational body weights of animals in the 1.6-mg/kg dose group were significantly lower than those of the control and 0.2-mg/kg dose group, and extragestational gains were significantly depressed in animals that received 0.4, 0.8 and 1.6 mg/kg. Hematocrit values were not consistently affected by the sulfur mustard dose; they were significantly decreased in the 0.8-mg/kg group, and in the 1.6- and 0.2-mg/kg groups tended to be lower than control values.

The mean number of corpora lutea/dam tended to be higher in the 0.2-mg/kg dose group (Table 13). This value may reflect the variability in the small sample size (five) and probably has no relationship to treatment, since exposure to sulfur mustard was not initiated until 6 dg. The number of implantations/dam was similar for

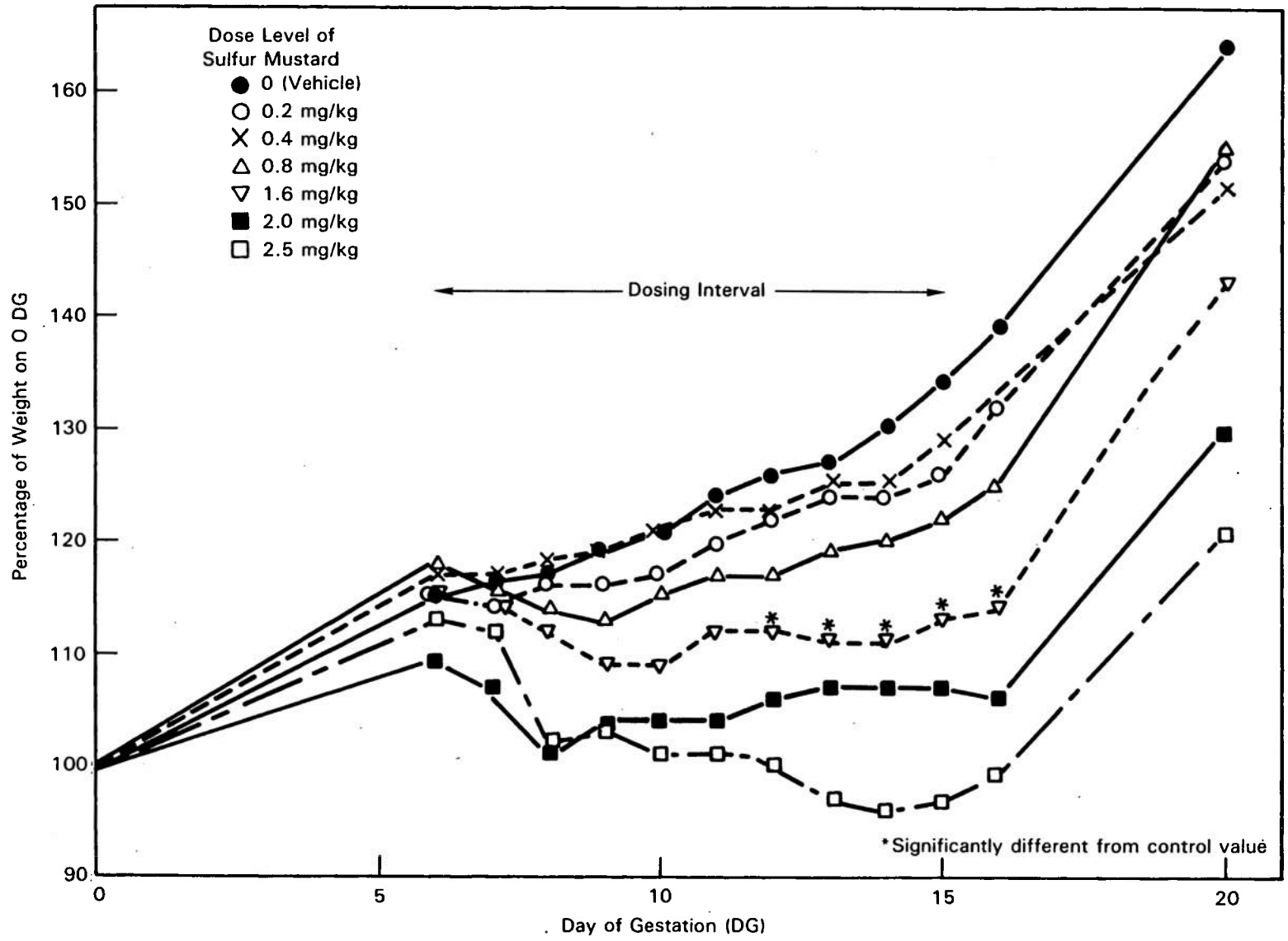


FIGURE 1. Weight Gain of Pregnant Survivors in the Dose-Range Study of Sulfur Mustard in Rats. (Analyses by Duncan's multiple range test.)

TABLE 12. Maternal Measures (Mean ± SE) for the Dose-Range Study of Sulfur Mustard in Rats

Observation	Dose Level (mg/kg)						
	0	0.2	0.4	0.8	1.6	2.0[a]	2.5[a]
Number of pregnant survivors	7	5	7	7	7	2	1
Body weight (g)							
0 dg	243 ± 11	246 ± 2	243 ± 12	244 ± 5	247 ± 4	280 ± 31	271
20 dg	398 ± 19[c]	379 ± 10[c,d]	366 ± 14[c,d]	377 ± 7[c,d]	354 ± 5[d]	362 ± 36	328
Extragestational[b]	316 ± 13[c]	312 ± 8[c]	295 ± 7[c,d]	295 ± 7[c,d]	281 ± 4[d]	290 ± 20	260
Extragestational gain[b]	73 ± 5[c]	66 ± 8[c,d]	53 ± 7[d]	52 ± 5[d]	34 ± 2[e]	10 ± 11	-11
Weight of gravid uterus (g)	82 ± 6	67 ± 7	70 ± 9	82 ± 6	72 ± 6	65 ± 19	68
Hematocrit (%)	37 ± 0.4[c]	36 ± 1.6[c,d]	37 ± 1.6[c]	33 ± 1.0[d]	35 ± 1.2[c,d]	36 ± 0.7	38

[a]Groups were dosed in a separate study; no statistical comparisons were made.
[b]Extragestational weight = weight on 20 dg minus weight of gravid uterus; extragestational gain = extragestational weight minus weight on 0 dg.
[c-e]Values that do not share a common superscript letter are significantly different ($P \leq 0.05$) from one another (Duncan's multiple range test).

all treatment groups. Implantation success, as measured by percentage of implantations per corpus luteum, tended to be depressed in the rats of the 0.2-mg/kg dose group. This is a consequence of the larger number of corpora lutea observed in this group and there is no evidence to suggest that it is treatment-related. Reproductive data for individual animals appears in Table C1 of Appendix C.

Intrauterine mortality (percentage of resorptions/implantations) and the number of live fetuses/litter were not affected by treatment (Table 13). The number of litters with resorptions was low in the group that received 0.4 mg/kg; therefore, the percentage of resorptions in these litters was significantly higher than the values for all other dose groups except those of the 1.6-mg/kg dose level.

Exposure of the dam to 0.2 to 1.6 mg of sulfur mustard/kg had no apparent effect on fetal body weights (Table 14). Fetal weights in treatment groups that received 2.0 or 2.5 mg/kg tended to be low, but the values may have been influenced by the small number of litters examined and the fact that the dams were older. Gross observations of fetuses revealed that no morphologic variations occurred in any treatment group.

Results from this preliminary study suggested that the selection of dose levels of 0.5, 1.0 and 2.0 mg of sulfur mustard/kg for the teratology study would produce effects ranging from minimal maternal and developmental toxicity to overt maternal toxicity with some observable effects on fetal development.

TERATOLOGY STUDY IN RATS

Ninety-four percent of the animals survived to scheduled sacrifice (Table 15). None of the deaths were attributed to chemical exposure; all appeared to be caused by dosing trauma (perforated esophagus or lung and a ruptured thoracic blood vessel). Lesions observed at the scheduled sacrifice in the control and 0.5-mg/kg dose groups included three survivors with esophageal lesions (perforated or cystic). Peri-

TABLE 13. Reproductive Measures (Mean ± SE) for the Dose-Range Study of Sulfur Mustard in Rats

Observation	Dose Level (mg/kg)						
	0	0.2	0.4	0.8	1.6	2.0[a]	2.5[a]
Number of:							
Pregnant survivors	7	5	7	7	7	2	1
Corpora lutea/dam	19 ± 12[b,c]	22 ± 2.1[b]	18 ± 1.7[b,c]	17 ± 0.6[c]	16 ± 0.6[c]	14 ± 1.5	14
Implantations/dam	16 ± 0.8	13 ± 1.6	12 ± 1.7	15 ± 1.2	15 ± 1.1	14 ± 1.5	14
Implantations/corpus luteum (%)	87 ± 7[b,c]	62 ± 11[c]	73 ± 12[b,c]	89 ± 6[b]	89 ± 4b	100 ± 0	100
Resorptions/litter (%):							
Early	4.78 ± 2.11	6.58 ± 3.65	6.87 ± 5.32	4.45 ± 1.68	5.43 ± 2.11	0	0
Late	0	2.22 ± 2.22	0	0	3.39 ± 2.32	0	0
Total	4.78 ± 2.11	8.80 ± 3.31	6.87 ± 5.32	4.45 ± 1.68	8.82 ± 3.13	0	0
Litters with resorptions (%)	57	80	29	57	71	0	0
Resorptions in litters with resorptions (%)	8.37 ± 2.34[b]	11.0 ± 3.19[b]	24.0 ± 13.5[c]	7.77 ± 1.11[b]	12.3 ± 2.96[b,c]	0	0
Number/litter:							
Dead fetuses	0	0	0	0	0	0	0
Live fetuses	15.3 ± 0.97	11.6 ± 1.36	11.6 ± 1.37	14.1 ± 1.32	13.3 ± 1.14	14.5 ± 1.50	14

[a]Groups were dosed in a separate study; no statistical comparisons were made.
[b-c]Values that do not share a common superscript letter are significantly different ($P \leq 0.05$) from one another (Duncan's multiple range test).

TABLE 14. Fetal Measures (Mean ± SE) for the Dose-Range Study of Sulfur Mustard in Rats

Observation	Dose Level (mg/kg)						
	0	0.2	0.4	0.8	1.6	2.0[a]	2.5[a]
Number of:							
Litters examined	7	5	7	7	7	2	1
Fetuses examined	107	58	81	99	93	29	14
Body weight (g)							
Female	3.53 ± 0.13	3.80 ± 0.17	3.60 ± 0.13	3.79 ± 0.06	3.57 ± 0.14	3.25 ± 0.12	3.19
Male	3.70 ± 0.12	3.81 ± 0.18	3.90 ± 0.20	4.05 ± 0.07	3.74 ± 0.15	3.29 ± 0.14	3.66
Both sexes	3.62 ± 0.13	3.81 ± 0.17	3.83 ± 0.19	3.92 ± 0.06	3.66 ± 0.13	3.27 ± 0.14	3.39
Sex ratio (% males)	55 ± 6.4	47 ± 8.8	59 ± 10	51 ± 3.1	58 ± 6.0	47 ± 16	43

[a]Groups were dosed in a separate study; no statistical comparisons were made.

cardial effusion, possibly resulting from injury to the thorax during administration of the dose, was evident in three survivors (Table 16; see Table B2 of Appendix B for individual animals). In one rat with pericardial infusion, there was no gross evidence of injury or trauma to the esophagus, trachea or lungs; histopathologic examinations of these tissues might resolve the origin of this lesion. Inflamed mesenteric lymph nodes were a consistent observation at 20 dg in animals dosed with sulfur mustard (44, 64 and 56% of the animals in the 0.5-, 1.0- and 2.0-mg/kg groups, respectively). No gross lesions of the gastric mucosa were observed at necropsy, which was 5 days after the administration of the last dose of sulfur mustard.

TABLE 15. Status of Rats in the Teratology Study of Sulfur Mustard

Observation	Dose Level (mg/kg)			
	0	0.5	1.0	2.0
Number of rats dosed	25	25	25	27
Not pregnant	4	5	2	1
Pregnant	21	20	23	26
Number of survivors	24	23	24	25
Not pregnant	4	5	2	1
Pregnant	20	18	22	24

TABLE 16. Observations at Necropsy of Rats in the Teratology Study of Sulfur Mustard

Dose (mg/kg)	Number of Animals	Number of Abnormalities	Gross Observations
Vehicle	25	1	Renal cavitation
		1	Small, red foci in intestine
		3	Lesions from dosing trauma
0.5	25	11	Inflamed mesenteric lymph nodes
		4	Lesions from dosing trauma
1.0	25	16	Inflamed mesenteric lymph nodes
		1	Jejunal evagination
		1	Small inflamed site on stomach
		1	Lesions from dosing trauma
2.0	27	15	Inflamed mesenteric lymph nodes
		1	Intestinal evagination
		1	Inflamed gastric mucosa
		2	Lesions from dosing trauma

A significant dose-dependent decrease in the body weights of pregnant survivors was observed by 9 dg in rats dosed with 1.0 or 2.0 mg/kg and by 12 dg in animals that received 0.5 mg/kg (Table 17). Percentage weight gains from 0 dg (Figure 2), extragestational weights and extragestational gains (Table 18) also displayed these responses. The mean weights of the gravid uteri were significantly decreased in the animals receiving the highest dose of sulfur mustard (Table 18). Hematocrit values for rats dosed with 1.0 and 2.0 mg/kg were significantly lower than control values.

TABLE 17. Body Weights (g, Mean ± SE) of Pregnant Survivors of the Teratology Study of Sulfur Mustard in Rats

Day of Gestation	Dose Level (mg/kg)			
	0	0.5	1.0	2.0
0	232 ± 3	233 ± 4	237 ± 3	239 ± 3
6	266 ± 5	267 ± 4	262 ± 5	266 ± 5
7	266 ± 5	265 ± 4	262 ± 4	262 ± 4
8	269 ± 5	266 ± 4	256 ± 4	260 ± 4
9	275 ± 5[a]	270 ± 4[a,b]	262 ± 4[b,c]	257 ± 3[c]
10	279 ± 5[a]	274 ± 4[a,b]	263 ± 4[b,c]	257 ± 3[c]
11	283 ± 5[a]	275 ± 4[a,b]	268 ± 3[b,c]	260 ± 4[c]
12	290 ± 5[a]	278 ± 4[b]	272 ± 3[b,c]	262 ± 4[c]
13	296 ± 5[a]	281 ± 4[b]	274 ± 4[b,c]	262 ± 4[c]
14	302 ± 5[a]	286 ± 4[b]	278 ± 4[b]	262 ± 4[c]
15	309 ± 5[a]	290 ± 4[b]	282 ± 4[b]	264 ± 4[c]
16	318 ± 5[a]	297 ± 4[b]	289 ± 4[b]	267 ± 4[c]
20	370 ± 6[a]	353 ± 6[b]	351 ± 5[b]	330 ± 6[c]

[a-c]Values that do not share a common supercript letter are significantly different ($P \leq 0.05$) from one another (Duncan's multiple range test).

Eighty-eight percent of the animals surviving the exposure regimen were pregnant. The number of corpora lutea and implantation sites, and the incidence of preimplantation failure and intrauterine mortality, were unaffected by sulfur mustard treatment (Table 19; see Table C2 of Appendix C for individual animals). No significant differences among treatments were noted for the number of live fetuses per litter.

Fetal body weights were significantly decreased in litters exposed to doses of 1.0 and 2.0 mg/kg during development (Table 20). Weights of fetuses of the 0.5-mg/kg group tended to be lower and weights of female fetuses in this group were significantly lower than comparable values for the control group. The percentage of males in the 0.5-mg/kg treatment group was significantly higher than that of the 2.0-mg/kg group. This observation may not be relevant to treatment effects but may have influenced the values for fetal weights when the sexes were combined.

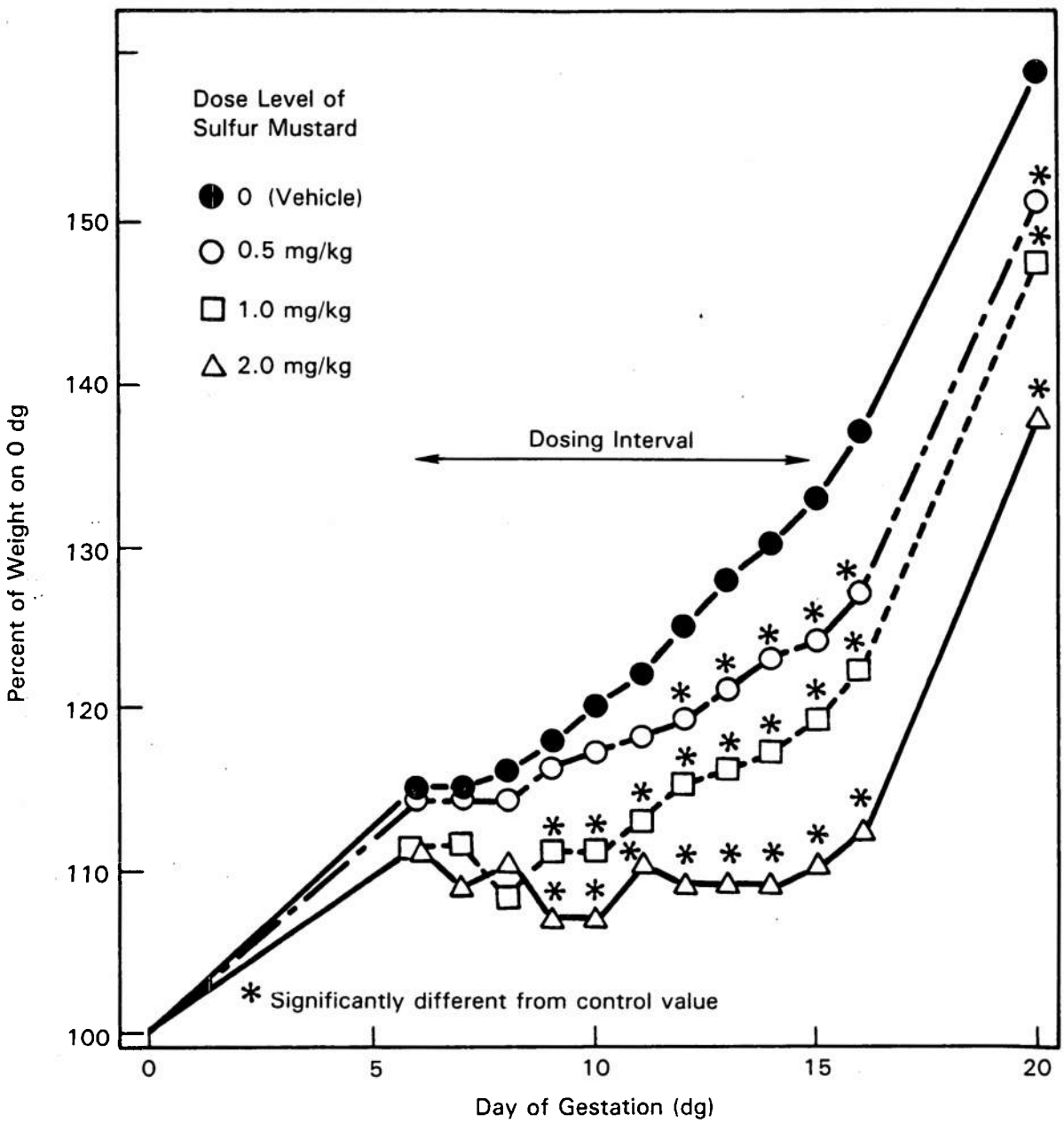


FIGURE 2. Weight Gain of Pregnant Survivors in the Teratology Study of Sulfur Mustard in Rats. (Analyzed by Duncan's multiple range test.)

TABLE 18. Maternal Measures (Mean ± SE) for the Teratology Study of Sulfur Mustard in Rats

Observations	Dose Level (mg/kg)			
	0	0.5	1.0	2.0
Number of rats examined	20	17	22	24
Body weight (g)				
0 dg	232 ± 3.3	233 ± 3.9	237 ± 3.1	239 ± 2.9
20 dg	370 ± 6.3[a]	353 ± 5.5[b]	351 ± 5.2[b]	330 ± 5.6[c]
Extragestational[d]	295 ± 4.7[a]	280 ± 4.2[b]	277 ± 3.8[b,c]	267 ± 4.2[c]
Extragestational gain[d]	63 ± 3.0[a]	47 ± 3.1[b]	39 ± 2.0[b]	27 ± 3.1[c]
Weight of gravid uterus (g)	75 ± 3.8[a]	73 ± 3.9[a]	74 ± 3.1[a]	63 ± 2.5[b]
Hematocrit (%)	37 ± 0.5[a]	36 ± 0.6[a,b]	35 ± 0.5[b]	35 ± 0.5[b]

[a-c]Values that do not share a common superscript letter are significantly different ($P \leq 0.05$) from one another (Duncan's multiple range test).
[d]Extragestational weight = weight on 20 dg minus weight of gravid uterus; extragestational gain = extragestational weight minus weight on 0 dg.

TABLE 19. Reproductive Measures (Mean ± SE) for the Teratology Study of Sulfur Mustard in Rats

Observations	Dose Level (mg/kg)			
	0	0.5	1.0	2.0
Number of:				
Rats examined	20	17	22	24
Corpora lutea/dam	17 ± 0.5	16 ± 0.7	17 ± 0.5	16 ± 0.5
Implantations/dam	14 ± 0.8	14 ± 0.6	15 ± 0.5	14 ± 0.6
Implantations/corpus luteum (%)	85.6 ± 4.5	90.3 ± 2.7	88.5 ± 2.4	85.5 ± 2.9
Resorptions/litter (%)				
Early	2.72 ± 0.83	6.87 ± 3.15	5.30 ± 1.67	8.27 ± 2.42
Late	0.36 ± 0.36	0.69 ± 0.47	0.25 ± 0.25	0
Total	3.08 ± 0.84	7.56 ± 3.17	5.55 ± 1.81	8.27 ± 2.42
Litters with resorptions (%)	40	53	45	50
Resorptions in litters with resorptions (%)	6.85 ± 0.73	16.1 ± 5.41	12.2 ± 2.79	16.5 ± 3.48
Number of:				
Dead fetuses/litter	0	0	0	0
Live fetuses/litter	13.6 ± 0.69	13.5 ± 0.72	14.2 ± 0.56	12.4 ± 0.55

The depressed fetal body weights were not accompanied by a corresponding decrease in crown-rump length. Placental weights tended to be lower in the rats dosed with 1.0 mg/kg and were significantly lower in the 2.0-mg/kg group.

Major malformations were observed in three fetuses of the high-dose group; but the incidence of malformations was not statistically significant (Table 21). The number of minor anomalies (most commonly, misaligned sternebrae) was significantly

TABLE 20. Fetal Measures (Mean ± SE) for the Teratology Study of Sulfur Mustard in Rats

Observation	Dose Level (mg/kg)			
	0	0.5	1.0	2.0
Number of:				
Litters examined	20	17	22	24
Fetuses examined	272	229	315	299
Body weight (g)				
Female	3.67 ± 0.06[a]	3.50 ± 0.07[b]	3.41 ± 0.04[b]	3.41 ± 0.05[b]
Male	3.81 ± 0.07[a]	3.73 ± 0.08[a,b]	3.58 ± 0.05[b]	3.59 ± 0.06[b]
Both sexes	3.74 ± 0.06[a]	3.62 ± 0.07[a,b]	3.49 ± 0.04[b]	3.50 ± 0.06[b]
Crown-rump length (mm)				
Female	37 ± 0.2	37 ± 0.3	37 ± 0.2	37 ± 0.2
Male	38 ± 0.3	38 ± 0.3	37 ± 0.2	37 ± 0.2
Both sexes	37 ± 0.3	38 ± 0.3	37 ± 0.2	37 ± 0.2
Sex ratio (% males)	51.0 ± 2.9[a,b]	57.3 ± 3.7[a]	50.0 ± 2.9[a,b]	46.2 ± 2.8[b]
Placental weight (mg)	416 ± 11[a]	420 ± 13[a]	403 ± 11[a,b]	381 ± 11[b]

[a-b]Values that do not share a common superscript letter are significantly different ($P \leq 0.05$) from one another (Duncan's multiple range test).

increased in the 2.0 mg/kg dose group. Hydroureter, infrequently accompanied by renal pelvic cavitation, occurred in a significant number of fetuses in the 0.5- and 1.0-mg/kg groups but not in the litters exposed to 2.0 mg/kg. Supernumerary ribs were observed in 9 of the 15 fetuses of one litter of the high-dose group. The incidence of reduced ossification of the vertebrae and/or sternebrae in all groups treated with sulfur mustard was significantly higher than that observed in control fetuses.

It should be noted that significant differences in the incidence of all morphologic alterations, except for the reduced vertebral ossification in the 0.5-mg/kg group, were based on comparisons of individual pups and not on litter comparisons (Table 21). When mean values for the percentage of morphologic alterations per litter were calculated, and the data were transformed ($2 \arcsin \sqrt{p}$, where p = proportion of alterations) and an analysis of variance was performed, many of the significant differences were no longer apparent (Table 22). The mean value for sternebral anomalies of fetuses in the high-dose group was significantly different from the control value but the mean for total minor anomalies in this treatment group was not significantly higher ($P \geqq 0.05$) than the control mean. Values for renal variations in the 0.5- and 1.0-mg/kg group were significantly different from the means for the high-dose group, but were not different from the control values. The percentage of fetuses per litter with reduced skeletal ossification was similar in all treatment groups.

Data from one dam of the 0.5-mg/kg-dose group have been excluded from all summaries because a perforated esophagus and evidence of tissue damage in the axilla and thorax were noted at scheduled sacrifice. Clinical observations of this animal indicated that dosing trauma probably occurred on 10 dg. At necropsy, a diaphragmatic hernia was observed in one fetus of this litter; two pups had renal anomalies (hypoplasia and an ectopic kidney); rib abnormalities (rudimentary and missing) were observed in eight fetuses; one of the fetuses had a misaligned sterne-

TABLE 21. Incidence of Morphologic Alterations in Rat Fetuses Exposed to Sulfur Mustard

Observations	Dose Level (mg/kg)			
	0	0.5	1.0	2.0
Number of fetuses/litters examined	272/20	229/17	315/22	299/24
Malformations				
Multiple[a]	--	--	--	1/1
Diaphragmatic hernia	--	--	--	1/1
Umbilical hernia	--	--	--	1/1
Number of malformed fetuses/litters	0	0	0	3/3
Minor Anomalies				
Renal agenesis (unilateral)	--	--	--	1/1
Cardiovascular				
Missing innominate artery	2/2	--	2/1	--
Retroesophageal right subclavian artery	--	--	--	1/1
Sternebra				
Misaligned	1/1	--	3/2	7[b]/5
Fused	--	--	--	1/1
Number of fetuses/litters with anomalies	3/3	--	5/3	10[b]/8
Morphologic Variations				
Renal				
Hydroureter	5/5	16[b]/7	17[b]/10	3/3
Renal pelvic cavitation		3/2[c]	4/3[c]	--
Skeletal				
Ribs				
Supernumerary ribs	--	--	--	9[b]/1
Ossification at first lumbar	1/1	2/1	1/1	5/2
Reduced Ossification				
Vertebra	28/10	44[b]/14[d]	53[b]/15	40/16
Sternebra[e]	16/10	6/6	28/10	42[b]/15
Skull	--	--	--	1/1
Ribs	--	1/1	--	4/1
Pelvis	--	--	--	1/1
Number with reduced ossification	42/14	51[b]/15	76[b]/19	72[b]/19

[a]Fetus 13 in litter 151 had multiple malformations, including cleft palate, pulmonary artery stenosis and a septal defect of the ventricle.
[b]Significantly different ($P \leq 0.05$) from control fetuses, using a chi-square test for each comparison.
[c]Renal pelvic cavitation with hydroureter
[d]Significantly different ($P \leq 0.05$) from control litters, using a one-tailed Fisher's Exact Test for each comparison.
[e]Excluding sternebrae 5 and 6

bra, and reduced ossification occurred in all 12 fetuses. These morphologic alterations may have been due to a "parenteral injection" of the sulfur mustard or to the extreme stress on the dam imposed by the injection of a foreign material.

DOSE-RANGE STUDY IN RABBITS

Mortality and reproductive data for the rabbits in this study are summarized in Table 23. Time of death, number of sulfur mustard doses administered and observations at necropsy for individual animals are listed in Table B3 of Appendix B. The dosing solution was inadvertently delivered to the lungs of three rabbits (Table 23). Gastric hemorrhage was observed at necropsy in one rabbit following only one dose of the agent (2.5 mg/kg). "Stress" was designated as the probable cause of death when the animal became cyanotic, went into convulsions and died during handling. At necropsy, these animals presented no evidence of injury or of foreign material in the trachea, lungs or thoracic cavity. Gastric hemorrhage was observed in two of the three animals that died from stress. Treatment-related effects, such as gastric lesions or intestinal and respiratory infections, were observed in all but one of the

TABLE 22. Percentage of Fetuses (Mean ± SE) with Morphologic Alterations in the Teratology Study of Sulfur Mustard in Rats

Observation	Dose Level (mg/kg)			
	0	0.5	1.0	2.0
Malformations	0	0	0	1.00 ± 0.55
Minor anomalies	1.05 ± 0.58[a,b]	0[a]	2.24 ± 1.33[a,b]	3.47 ± 1.16[b]
Renal	0	0	0	0.26 ± 0.26
Cardiovascular	0.74 ± 0.51	0	1.14 ± 1.14	0.32 ± 0.32
Sternebral	0.31 ± 0.31[a]	0[a]	1.10 ± 0.77[a,b]	2.89 ± 1.15[b]
Physiologic variations				
Renal	2.44 ± 1.07[ab]	8.32 ± 2.95[b]	7.67 ± 2.74[b]	1.04 ± 0.58[a]
Rib	0.38 ± 0.38	0.98 ± 0.98	0.35 ± 0.35	6.13 ± 4.07
Reduced ossification of skeleton	15.5 ± 4.14	22.6 ± 4.78	24.3 ± 4.61	23.6 ± 4.73

[a-b]Values that do not share a common superscript letter are significantly different ($P \leq 0.05$) from one another (Duncan's multiple range test following arcsin transformation of the data).

animals that died or were euthanized in the groups that received 1.0, 2.0 or 2.5 mg of sulfur mustard/kg. Clinical signs and observations of these animals at necropsy indicated that the progression of lesions was: gastric hemorrhage on 7 to 9 dg; gastric ulceration by 10 dg; onset of diarrhea in some animals by 11 dg; and intestinal and respiratory infections from 13 to 24 dg. At scheduled sacrifice (30 dg), the gross abnormalities observed in the survivors were limited to enlarged mesenteric lymph nodes and inflamed or enlarged Peyer's patches.

TABLE 23. Status of Rabbits in the Dose-Range Study of Sulfur Mustard

Observation	Dose Level (mg/kg)				
	0	0.5	1.0	2.0	2.5
Number of rabbits dosed	8	8	8	8	7
Number of survivors	7	7	4	3	2
Probable cause of death/morbidity:					
Lung dose	0	1	1	0	1[a]
Stress	1	0	0	2[a]	0
Infection	0	0	3	3	4
Number of rabbits:					
Without corpora lutea	1	1	0	0	3
With corpora lutea/no implantations	0	3	2	0	2
Aborting	0	0	0	2[b]	0
Pregnant	7	4	6	8	2
Pregnant survivors	6	4	4	3[b]	0

[a]Gastric hemorrhage or ulceration evident
[b]Rabbit #6607 aborted on 24 days of gestation but survived until necropsy.

Changes in body weights of all pregnant animals (survivors and nonsurvivors) during the experimental period are shown in Figure 3. The low dose (0.5-mg/kg) had no apparent effect on maternal weights, but weight gains of all other treatment groups tended to be depressed and values of animals that received 2.0 mg/kg were significantly decreased from 9 dg until sacrifice. None of the pregnant animals

in the group exposed to 2.5 mg/kg survived until sacrifice. When body weights of pregnant rabbits that survived until sacrifice were examined (Table 24), similar relationships among the dose groups were evident, but the differences between values for the 2.0-mg/kg group and the controls were not apparent until 15 dg. These effects on body weight can be correlated with qualitative observations of anorexia in the rabbits that received the higher dose levels of sulfur mustard (Figure 4).

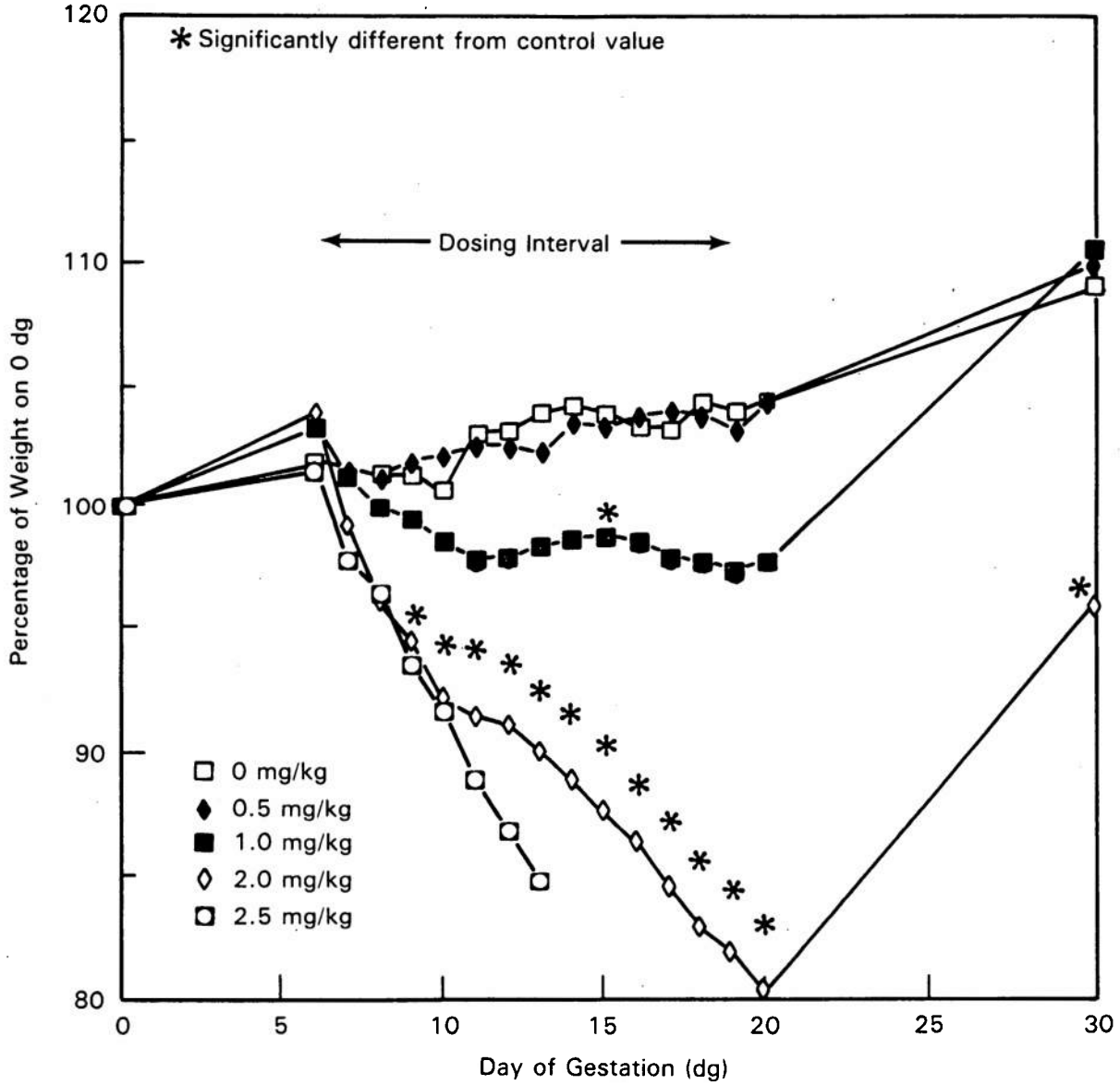


FIGURE 3. Body Weight Change in all Pregnant Rabbits (Survivors and Nonsurvivors) of the Dose-Range Study of Sulfur Mustard. (Analyzed by Duncan's multiple range test.)

Because of the small number of pregnant survivors in the 2.0-mg/kg group, maternal weight measures, with the exception of the sacrifice weight, were not significantly different from control values; however, extragestational body weights

TABLE 24. Body Weights (kg, Mean ± SE) of Pregnant Survivors in the Dose-Range Study of Sulfur Mustard in Rabbits

Day of Gestation	Vehicle N = 6	Dose Level (mg/kg)		
		0.5 N = 4	1.0 N = 4	2.0 N = 2
Pre-	3.73 ± 0.10	3.73 ± 0.17	3.49 ± 0.13	3.76 ± 0.14
0	3.90 ± 0.12	3.87 ± 0.13	3.64 ± 0.14	3.89 ± 0.14
6	3.99 ± 0.10	4.02 ± 0.17	3.77 ± 0.09	4.10 ± 0.13
7	3.99 ± 0.09	3.94 ± 0.19	3.68 ± 0.09	3.92 ± 0.07
8	4.01 ± 0.11	3.92 ± 0.19	3.62 ± 0.13	3.87 ± 0.03
9	4.01 ± 0.10	3.96 ± 0.19	3.63 ± 0.15	3.76 ± 0.01
10	4.00 ± 0.13	3.97 ± 0.18	3.58 ± 0.16	3.69 ± 0.01
11	4.02 ± 0.13	3.98 ± 0.17	3.56 ± 0.15	3.62 ± 0.03
12	4.02 ± 0.12	3.98 ± 0.17	3.56 ± 0.16	3.64 ± 0.03
13	4.04 ± 0.11	3.97 ± 0.18	3.61 ± 0.16	3.61 ± 0.01
14	4.06 ± 0.11	4.01 ± 0.19[a]	3.62 ± 0.16	3.55 ± 0.01
15	4.05 ± 010[a]	4.01 ± 0.16[a]	3.64 ± 0.13[a,b]	3.51 ± 0.06[b]
16	4.03 ± 0.10[a]	4.03 ± 0.19[a]	3.64 ± 0.13[a,b]	3.48 ± 0.11[b]
17	4.02 ± 0.11[a]	4.05 ± 0.19[a]	3.64 ± 0.13[a,b]	3.41 ± 0.10[b]
18	4.06 ± 0.11[a]	4.03 ± 0.21[a]	3.65 ± 0.14[a,b]	3.36 ± 0.07[b]
19	4.05 ± 0.12[a]	4.01 ± 0.21[a]	3.64 ± 0.16[a,b]	3.31 ± 0.05[b]
20	4.06 ± 0.12[a]	4.06 ± 0.21[a]	3.69 ± 0.15[a,b]	3.25 ± 0.02[b]
30	4.25 ± 0.08[a]	4.27 ± 0.19[a]	4.02 ± 0.08[a,b]	3.74 ± 0.23[b]

[a-b]Values that do not share a common superscript letter are significantly different ($P \leq 0.05$) from one another (Duncan's multiple range test).

and weight gains tended to be lower in animals that received 1.0 and 2.0 mg/kg (Table 25). No differences among groups in weights of gravid uteri or hematocrit values were observed.

The effects of the sulfur mustard regimens on reproductive measures (Table 26; see Table C3 of Appendic C for values for individual animals) were not significant except for an increase in the percentage of late resorptions in litters of the 1.0-mg/kg group, in comparison with the 0.5-mg/kg values; however, the small numbers of pregnant survivors that were examined may have contributed to the variability observed in the data. In the 0.5-mg/kg dose group, only one nidation site was detected by staining the uterus in one of the four does and undoubtedly accounted for the high value for early and total resorptions/litter in this dose group. In the 2.0-mg/kg dose group, two of the does aborted on 24 dg; one of these rabbits died, and one survived until scheduled sacrifice. Resorptions in two surviving animals that retained their litters until sacrifice amounted to 30 and 50%, respectively.

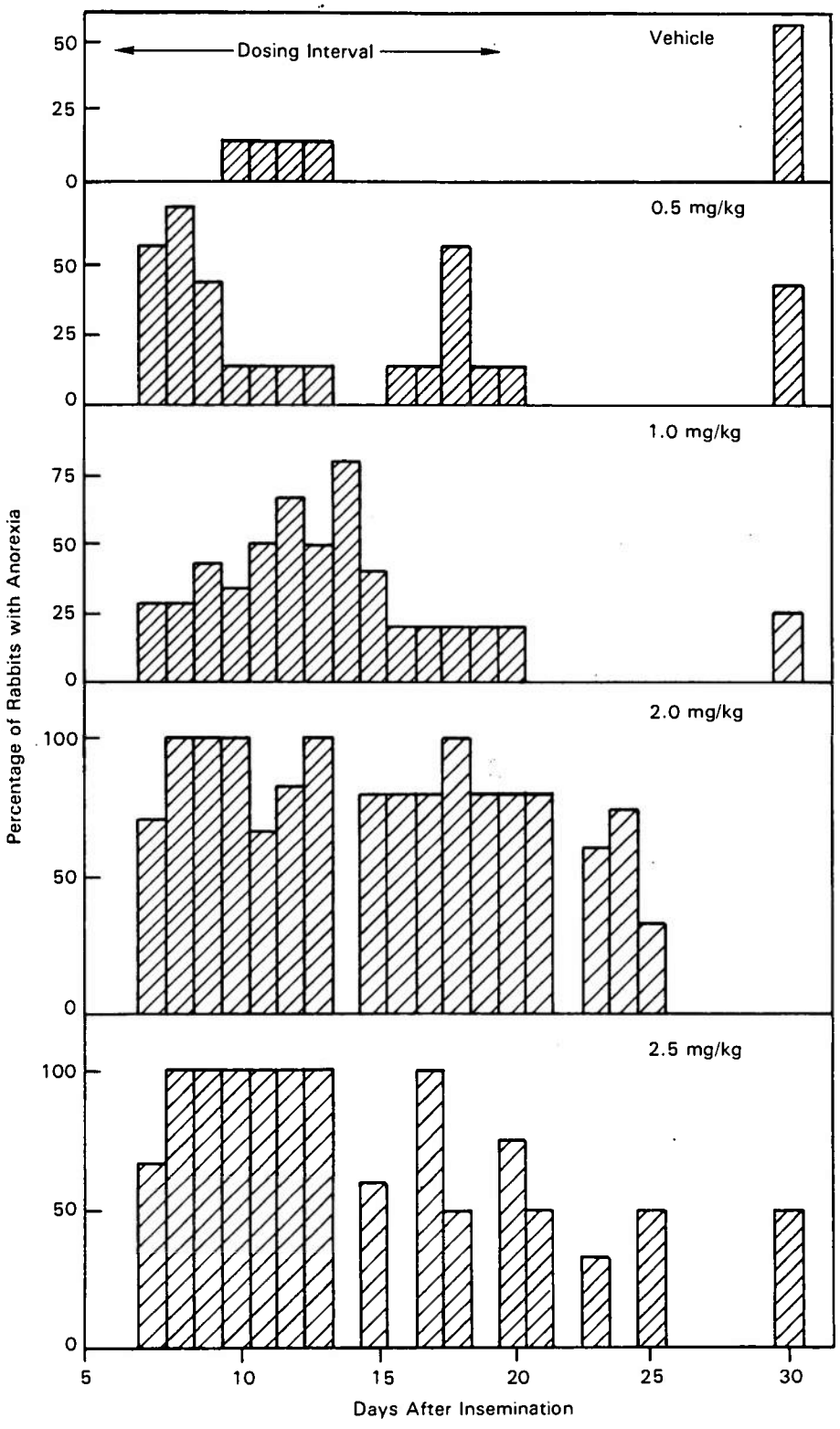


FIGURE 4. Percentage of Pregnant and Nonpregnant Anorectic Rabbits in the Dose-Range Study of Sulfur Mustard

TABLE 25. Maternal Measures (Mean ± SE) for the Dose-Range Study of Sulfur Mustard in Rabbits

Observation	Dose Level (mg/kg)[a]			
	0	0.5	1.0	2.0
Number of pregnant survivors	6	4	4	2
Body weight (kg)				
0 dg	3.90 ± 0.12	3.87 ± 0.13	3.64 ± 0.14	3.89 ± 0.14
30 dg	4.25 ± 0.08[b]	4.27 ± 0.19[b]	4.02 ± 0.08[b,c]	3.74 ± 0.23[c]
Extragestational[d]	3.84 ± 0.13	3.91 ± 0.24	3.49 ± 0.04	3.46 ± 0.29
Extragestational gain[d]	-0.06 ± 0.11	0.04 ± 0.15	-0.15 ± 0.11	-0.43 ± 0.15
Weight of gravid uterus (kg)	0.41 ± 0.06	0.36 ± 0.12	0.53 ± 0.05	0.28 ± 0.07
Hematocrit (%)	44 ± 2.5	40 ± 1.0	41 ± 0.6	36 ± 6.5

[a]There were no pregnant survivors in the group dosed with 2.5 mg/kg.
[b-c]Values that do not share a common superscript letter are significantly different ($P \leq 0.05$) from one another (Duncan's multiple range test).
[d]Extragestational weight = weight at 30 dg minus weight of gravid uterus; extragestational gain = extragestational weight minus weight at 0 dg.

TABLE 26. Reproductive Measures (Mean ± SE) for the Dose-Range Study of Sulfur Mustard in Rabbits

Observation	Dose Level (mg/kg)[a]			
	0	0.5	1.0	2.0
Number of:				
Pregnant survivors	6	4	4	2
Corpora lutea/doe	9.3 ± 1.7	9.3 ± 3.1	13.3 ± 0.9	11.5 ± 1.5
Implantations/doe	7.2 ± 1.6	6.5 ± 2.2	11.3 ± 1.5	10.0 ± 0
Implantations/corpus luteum (%)	73.8 ± 7.1	76.3 ± 8.7	91.9 ± 4.8	88.5 ± 11.5
Resorptions/litter (%)				
Early	0	27.3 ± 24.3[b]	2.1 ± 2.1	25.0 ± 25.0
Mid	0	2.3 ± 2.3	6.7 ± 4.7	10.0 ± 10.0
Late	4.2 ± 2.8[c,d]	0[c]	11.0 ± 1.1[d]	5.0 ± 5.0[c,d]
Total	4.2 ± 2.8	29.5 ± 23.9[b]	19.8 ± 3.7	40.0 ± 10.0
Litters with resorptions (%)	33	50	100	50
Resorptions in litters with resorptions (%)	12.7 ± 2.7	59.1 ± 40.9	19.8 ± 3.7	40.0 ± 10.0
Number of:				
Dead fetuses/litter	0	0	0	0
Live fetuses/litter	6.67 ± 1.33	5.75 ± 2.14	9.00 ± 1.22	6.00 ± 1.00

[a]There were no pregnant survivors in the group dosed with 2.5 mg/kg.
[b]One implantation site detected by uterine stain in rabbit #6631
[c-d]Values that do not share a common superscript letter are significantly different ($P \leq 0.05$) from one another (Duncan's multiple range test).

Body weights for both male and female fetuses of the 2.0-mg/kg dose group were significantly lower than values for all other treatment groups (Table 27). Fore-

limb flexure was observed in one fetus of the lowest-dose group; all other malformations were confined to one of the two surviving litters of the 2.0-mg/kg group. Of the seven live fetuses in this litter, one had forelimb flexure only, and five fetuses had incomplete closure of the sagittal suture, one accompanied by spina bifida and one with forelimb flexure.

TABLE 27. Fetal Measures (Mean ± SE) for the Dose-Range Study of Sulfur Mustard in Rabbits

Observation	Dose Level (mg/kg)[a]			
	0	0.5	1.0	2.0
Number of:				
Litters examined	6	3	4	2
Fetuses examined	40	23	36	12
Body weight (g)				
Female	47.1 ± 3.4[b]	45.4 ± 1.8[b]	41.5 ± 3.4[b]	28.8 ± 2.8[c]
Male	48.1 ± 3.6[b]	47.7 ± 2.4[b]	43.1 ± 4.1[b]	30.0 ± 0.1[c]
Both sexes	47.5 ± 3.5[b]	46.4 ± 2.0[b]	42.4 ± 3.7[b]	29.6 ± 1.2[c]
Sex ratio (% males)	45 ± 6.5	43 ± 1.5	55 ± 6.3	51 ± 8.6
Gross observations (fetuses/litters):				
Forelimb flexure	0	1/1	0	2/1[d]
Incomplete closure of sagittal suture	0	0	0	5/1[d]
Spina bifida	0	0	0	1/1[d]

[a]No fetuses were available for examination from the group dosed with 2.5 mg/kg.
[b-c]Values that do not share a common superscript letter are significantly dif-ferent ($P \leq 0.05$) from one another (Duncan's multiple range test).
[d]Litter from one doe, #6644

Results from this study indicated that a dose level of 0.5 mg/kg caused no apparent maternal or fetal effects and that exposure to 1.0 mg/kg induced maternal mortality and tended to decrease body weights and weight gains of survivors. Therefore, the dose levels selected for the subsequent teratology study included two doses between these levels (0.6 and 0.8 mg/kg) and one dose (0.4 mg/kg) just below the low dose of this preliminary range-finding study.

TERATOLOGY STUDY IN RABBITS

Gastric ulcers were observed in four experimental animals, three of which died: one in the 0.4-mg/kg group and two in the 0.8-mg/kg group (Table 28). A gastric ulcer was also evident in one of the animals in the high-dose group that survived until sacrifice. No abortions were observed in this study, but the death of one animal in the 0.6-mg/kg group was ascribed to toxemia, since no lesions other than the resorbing conceptus were observed at necropsy. Deaths attributed to dosing injuries (including lung doses) and "stress" (dyspnea, cyanosis and convulsions, with no evidence of injury or misapplication of the dose) occurred in all treatment groups but were more commonly observed in the vehicle control and low-dose groups. Rabbits in these groups were alert and active throughout the experimental period and were more inclined to vigorously resist the insertion of the dosing tube than were the animals of the two higher-dose groups.

TABLE 28. Status of Rabbits in the Teratology Study of Sulfur Mustard

Observation	Dose Level (mg/kg)			
	0	0.4	0.6	0.8
Number of rabbits dosed	19	18	18	18
Number of survivors	15	15	16	15
Probable cause of death/morbidity[a]				
Dosing injury	1	3[b]	1	1
Stress	3	0	0	0
Toxemia	0	0	1	0
Gastric ulcer	0	0	0	2

[a]Observations and findings at necropsy are listed in Table B4 of Appendix B.
[b]Gastric ulcer observed at necropsy of rabbit #7149.

A comparison of the body weights of surviving pregnant rabbits shows that the values tended to be depressed in all groups exposed to sulfur mustard and, with the exception of values for 15 dg, were significantly decreased in the high-dose group from 11 through 20 dg (Table 29). No differences in weights were observed among treatment groups at sacrifice. When these data were calculated as the percentage of the body weight on 0 dg, which was a measure of body weight gain, values for the 0.8-mg/kg group were significantly lower from 9 through 30 dg, the weights of the 0.6-mg/kg dose level tended to be lower than control values, and the values for the 0.4-mg/kg group appeared to be unaffected by treatment (Table 30; Figure 5). A compilation of maternal weight measures, expressed in absolute values (Table 31), shows that all values for weight parameters for the 0.8-mg/kg group tended to be lower than control values, although the differences were not significant; the difference among treatments was significant only when extragestational gains of the low and high dose groups were compared. Some of these differences in the expression of the results may be attributed to the variability within the group means, introduced by the removal of data for nonpregnant animals from groups in which members were randomly selected on a weight basis. Since mature females were used in this study, detectable weight gains over the 30-day period would not be anticipated.

Anorexia was most commonly observed in rabbits of the 0.8-mg/kg dose group (Figure 6). The onset of anorexia appeared to be earlier in the 0.6-mg/kg group than in the 0.4-mg/kg dose level. The hematocrit value for maternal animals tended to be lower in the 0.6-mg/kg treatment group and was significantly lower in rabbits that received 0.8 mg/kg when compared with values for control and 0.4-mg/kg animals (Table 31).

Of the 73 rabbits inseminated for this study, 52 (71%) were pregnant. Reproductive measures, such as pre- and postimplantation losses and live fetuses per litter were not significantly affected by sulfur mustard treatment (Table 32; see Table C4 of Appendix C for individual values). Mean values for the percentage of resorptions and resorptions in litters with resorptions tended to be higher in the 0.6- and 0.8-mg/kg dose groups but were not significantly higher than control values.

TABLE 29. Body Weights (kg, Mean ± SE) of Pregnant Survivors in the Teratology Study of Sulfur Mustard in Rabbits

Day of Gestation	Dose Level (mg/kg)			
	0 N = 11	0.4 N = 10	0.6 N = 14	0.8 N = 8
Pre-Study	3.66 ± 0.08	3.51 ± 0.08	3.68 ± 0.07	3.66 ± 0.08
0	3.75 ± 0.09	3.60 ± 0.07	3.71 ± 0.07	3.69 ± 0.08
6	3.89 ± 0.08	3.75 ± 0.09	3.87 ± 0.05	3.81 ± 0.08
7	3.77 ± 0.11	3.73 ± 0.09	3.84 ± 0.06	3.72 ± 0.08
8	3.84 ± 0.08	3.70 ± 0.09	3.80 ± 0.07	3.65 ± 0.08
9	3.84 ± 0.08	3.70 ± 0.09	3.80 ± 0.08	3.57 ± 0.09
10	3.86 ± 0.08	3.71 ± 0.10	3.77 ± 0.09	3.58 ± 0.09
11	3.87 ± 0.08[a]	3.71 ± 0.10[a,b]	3.76 ± 0.09[a,b]	3.53 ± 0.09[b]
12	3.89 ± 0.09[a]	3.70 ± 0.10[a,b]	3.76 ± 0.09[a,b]	3.54 ± 0.09[b]
13	3.88 ± 0.09[a]	3.70 ± 0.11[a,b]	3.75 ± 0.10[a,b]	3.56 ± 0.09[b]
14	3.90 ± 0.09[a]	3.71 ± 0.09[a,b]	3.76 ± 0.10[a,b]	3.59 ± 0.09[b]
15	3.94 ± 0.09	3.70 ± 0.08	3.72 ± 0.11	3.61 ± 0.11
16	3.94 ± 0.09[a]	3.72 ± 0.08[a,b]	3.71 ± 0.12[a,b]	3.58 ± 0.11[b]
17	3.94 ± 0.09[a]	3.72 ± 0.09[a,b]	3.70 ± 0.12[a,b]	3.57 ± 0.11[b]
18	3.97 ± 0.10[a]	3.73 ± 0.09[a,b]	3.72 ± 0.12[a,b]	3.56 ± 0.13[b]
19	3.97 ± 0.10[a]	3.76 ± 0.09[a,b]	3.72 ± 0.12[a,b]	3.55 ± 0.13[b]
20	3.99 ± 0.10[a]	3.78 ± 0.09[a,b]	3.72 ± 0.13[a,b]	3.58 ± 0.13[b]
30	4.10 ± 0.10	3.95 ± 0.10	3.95 ± 0.11	3.75 ± 0.19

[a-b]Values that do not share a common superscript letter are significantly different ($P \leq 0.05$) from one another (Duncan's multiple range test).

Exposure to sulfur mustard during development had no effect on fetal body weights, crown-rump lengths or sex ratios (Table 33). No differences in placental weights were observed among treatment groups. Multiple malformations were evident in two fetuses (Table 34). One fetus from the vehicle control group had anemia, hepatic jaundice, thoracic edema and an enlarged heart. One kit from litter 7476 of the low-dose group had cardiovascular, pulmonary, renal and sternebral defects. Two additional fetuses from litter 7476 exhibited rib anomalies. Fetuses with supernumerary ribs and reduced sternebral ossification were commonly observed in all treatment groups.

TABLE 30. Body Weights (Percentage of Weight on 0 Days of Gestation, Mean ± SE) of Pregnant Rabbits in the Teratology Study of Sulfur Mustard

Day of Gestation	Dose Level (mg/kg)			
	0	0.4	0.6	0.8
6	103.9 ± 0.8	103.9 ± 0.9	104.6 ± 0.9	103.3 ± 1.2
7	100.9 ± 2.1	103.3 ± 1.0	103.7 ± 1.0	100.9 ± 1.0
8	102.5 ± 0.9	102.6 ± 0.8	102.5 ± 1.2	99.0 ± 0.9
9	102.5 ± 1.0[a]	102.7 ± 1.0[a]	101.9 ± 1.4[a]	96.8 ± 0.7[b]
10	103.1 ± 0.9[a]	102.9 ± 1.3[a]	101.7 ± 1.4[a]	97.1 ± 1.0[b]
11	103.5 ± 0.9[a]	102.9 ± 1.3[a]	101.5 ± 1.6[a]	95.8 ± 1.2[b]
12	104.0 ± 0.9[a]	102.7 ± 1.6[a]	101.3 ± 1.7[a]	96.1 ± 1.4[b]
13	103.6 ± 1.0[a]	102.6 ± 1.5[a]	101.0 ± 2.0[a,b]	96.6 ± 1.5[b]
14	104.3 ± 1.0[a]	103.0 ± 1.2[a]	101.2 ± 2.0[a,b]	97.4 ± 1.7[b]
15	105.3 ± 1.0[a]	102.8 ± 1.3[a,b]	100.0 ± 2.3[a,b]	97.9 ± 1.9[b]
16	105.4 ± 1.0[a]	103.2 ± 1.5[a]	99.8 ± 2.4[a,b]	96.9 ± 1.8[b]
17	105.4 ± 1.3[a]	103.3 ± 1.8[a]	99.6 ± 2.4[a,b]	96.8 ± 2.1[b]
18	106.1 ± 1.5[a]	103.7 ± 1.8[a]	100.0 ± 2.3[a,b]	96.5 ± 2.3[b]
19	106.3 ± 1.6[a]	104.4 ± 1.8[a]	100.3 ± 2.5[a,b]	96.2 ± 2.4[b]
20	106.6 ± 1.6[a]	105.1 ± 1.9[a]	100.1 ± 2.6[a,b]	97.0 ± 2.6[b]
30	109.7 ± 2.1[a]	109.8 ± 1.9[a]	106.3 ± 2.3[a,b]	101.5 ± 4.0[b]

[a-b]Values that do not share a common superscript letter are significantly different ($P \leq 0.05$) from one another (Duncan's multiple range test).

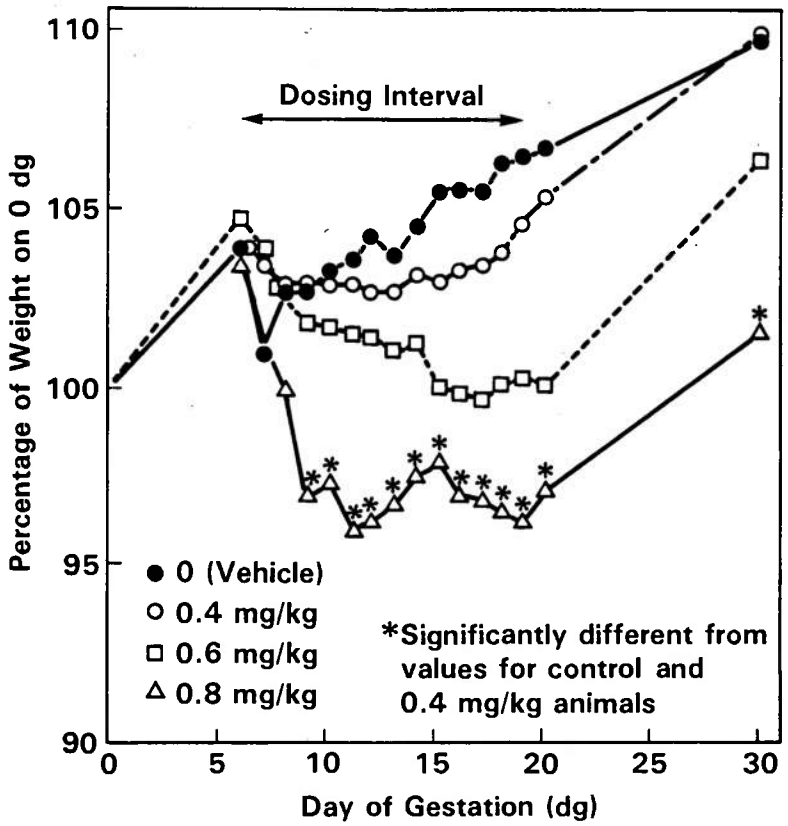


FIGURE 5. Body Weights (g) of Pregnant Rabbits in the Teratology Study of Sulfur Mustard. (Analyzed by Duncan's multiple range test.)

TABLE 31. Maternal Measures (Mean ± SE) for the Teratology Study of Sulfur Mustard in Rabbits

Observations	Dose Level (mg/kg)			
	0	0.4	0.6	0.8
Number of pregnant survivors	11	10	14	8
Body weight (kg):				
0 dg	3.75 ± 0.09	3.60 ± 0.07	3.71 ± 0.07	3.69 ± 0.08
30 dg	4.10 ± 0.10	3.95 ± 0.10	3.95 ± 0.11	3.75 ± 0.19
Extragestational[a]	3.63 ± 0.09	3.51 ± 0.09	3.53 ± 0.08	3.35 ± 0.16
Extragestational gain[b]	-0.11 ± 0.08[c,d]	-0.09 ± 0.06[c]	-0.18 ± 0.06[c,d]	-0.33 ± 0.11[d]
Weight of gravid uterus (kg)	0.47 ± 0.04	0.44 ± 0.04	0.42 ± 0.04	0.40 ± 0.08
Hematocrit (%)	42.8 ± 0.7[c]	42.4 ± 1.3[c]	41.6 ± 0.7[c,d]	38.9 ± 1.5[d]

[a]Extragestational body weight = body weight at 30 dg - weight of gravid uterus.
[b]Extragestational weight gain = extragestational body weight - body weight at 0 dg.
[c-d]Values that do not share a common letter are significantly different ($P \leq 0.05$) from one another (Duncan's multiple range test).

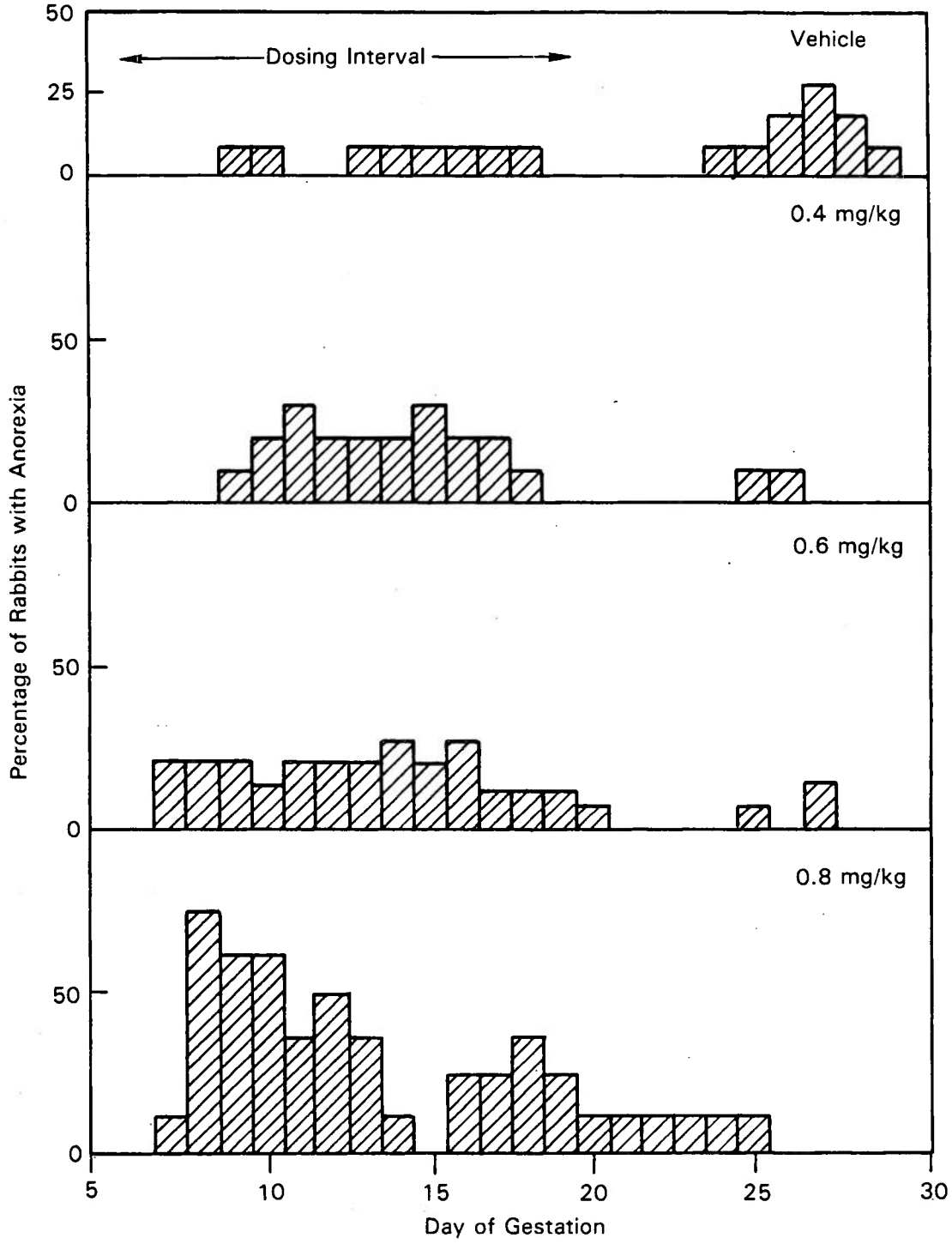


FIGURE 6. Percentage of Anorectic Rabbits in the Teratology Study of Sulfur Mustard

TABLE 32. Reproductive Measures (Mean ± SE)[a] for the Teratology Study of Sulfur Mustard in Rabbits

Observation	Dose Level (mg/kg)			
	0	0.4	0.6	0.8
Number of rabbits:				
Without corpora lutea	4	1	1	6
With corpora lutea/no implantations	1[b]	5	1	2
Pregnant	14	12	16	10
Pregnant survivors	11	10	14	8
Nonpregnant survivors	4	5	2	7
Number of:				
Corpora lutea/doe	10.8 ± 0.8	10.6 ± 0.8	10.8 ± 0.6	10.3 ± 0.6
Implantations/doe	8.7 ± 0.8	8.4 ± 0.9	8.9 ± 0.4	8.3 ± 1.0
Implantations/corpus luteum (%)	82.2 ± 6.0	78.5 ± 4.6	85.1 ± 4.6	81.2 ± 10.3
Resorptions (%)				
Early	4.6 ± 2.6	2.3 ± 1.6	11.6 ± 7.1	13.6 ± 12.4
Mid	4.8 ± 2.0	7.5 ± 4.3	5.0 ± 5.0	1.1 ± 1.1
Late	3.0 ± 1.6	2.8 ± 1.9	6.4 ± 2.2	5.6 ± 3.6
Total	12.4 ± 4.8	12.6 ± 4.5	23.0 ± 8.0	20.3 ± 12.0
Litters with resorptions (%)	55	60	64	50
Resorptions in litters with resorptions (%)	22.8 ± 6.0	21.0 ± 5.1	35.9 ± 10.3	40.7 ± 19.8
Number of:				
Dead fetuses/litter	0	0	0	0
Live fetuses/litter	7.64 ± 0.83	7.00 ± 0.68	6.93 ± 0.79	6.25 ± 1.26

[a]Analyzed by Duncan's multiple range test.
[b]Rabbit #7495 died at 6 days of gestation; implantation may not have been evident.

TABLE 33. Fetal Measures (Mean ± SE)[a] for the Teratology Study of Sulfur Mustard in Rabbits

Observation	Dose Level (mg/kg)			
	0	0.4	0.6	0.8
Number of:				
Litters examined	11	10	13	7
Fetuses examined	84	70	97	50
Body weight (g)				
Female	44.8 ± 1.9	46.4 ± 1.9	43.7 ± 1.5	47.5 ± 1.3
Male	47.4 ± 2.1	46.8 ± 2.0	44.2 ± 1.3	47.2 ± 2.1
Both sexes	46.3 ± 2.0	46.7 ± 1.9	43.7 ± 1.3	47.3 ± 1.5
Crown-rump length (mm)				
Female	99.6 ± 1.6	100.6 ± 1.3	97.5 ± 1.0	100.3 ± 1.6
Male	100.6 ± 1.4	100.9 ± 1.7	98.7 ± 1.1	100.5 ± 2.7
Both sexes	99.9 ± 1.4	100.9 ± 1.4	98.2 ± 0.7	100.1 ± 2.1
Sex ratio (% males)	53 ± 6	49 ± 3	56 ± 4	47 ± 9
Placental weight (g)				
Female	5.01 ± 0.22	5.03 ± 0.18	4.96 ± 0.19	5.19 ± 0.26
Male	5.53 ± 0.26	5.16 ± 0.23	5.12 ± 0.16	5.00 ± 0.23
Both sexes	5.31 ± 0.23	5.09 ± 0.19	5.01 ± 0.16	5.18 ± 0.26

[a]Analyzed by Duncan's multiple range test.

TABLE 34. Incidence of Morphologic Alterations in Rabbit Fetuses Exposed to Sulfur Mustard

Observations	Dose Level (mg/kg)			
	0	0.4	0.6	0.8
Number of fetuses/litters examined	84/11	70/10	97/13	50/7
Malformations				
Multiple	1/1[a]	1/1[b]	--	--
Minor Anomalies				
Rudimentary left carotid artery	--	--	1/1	--
Ribs				
Branched	--	1/1	--	--
Fused	1/1[c]	1/1[d]	--	1/1
Agenesis	--	1/1[d]	--	--
Vertebra misaligned	1/1[c]	--	--	--
Sternebra misaligned	--	1/1	--	--
Forelimb flexure	1/1	2/1	--	--
Morphologic Variations				
Misshapen cardiac apex	1/1	--	--	--
Gallbladder bifurcation	--	1/1	--	1/1
Ribs - supernumerary	39/9	25/7	30/10	25/7
Vertebrae - extra ossification site	1/1	--	--	--
Reduced Ossification				
Vertebra	1/1[c]	1/1[d]	--	--
Sternebra	22/7	21/8	17/8	12/5

[a]Fetus 12 in litter 7456 had anemia, hepatic jaundice, thoracic edema and an enlarged heart.
[b]Fetus 4 in litter 7476 had edema, pulmonary hypoplasia, ballooned pulmonary artery, cardiac levorotation, unilateral kidney/ureter agenesis and fused, misaligned sternebrae.
[c]Fetus 9 in litter 7237
[d]Fetus 6 in litter 7476

DISCUSSION

Table 35 summarizes the effects of sulfur mustard treatment and compares the lowest dose at which significant observations were detected in each species of animals in each study.

TABLE 35. Lowest Dose Level at Which Significant Observations Attributed to Sulfur Mustard Treatment were Detected

	Rat Studies		Rabbit Studies	
	Dose-Range	Teratology	Dose-Range	Teratology
Dose Levels (mg/kg)	0.2, 0.4, 0.8, 1.6, 2.0, 2.5	0.5, 1.0, 2.0	0.5, 1.0, 2.0, 2.5	0.4, 0.6, 0.8
Maternal Effects				
Mortality	2.5	--	1.0	0.8
Gross lesions				
Major[a]	2.0	--	1.0	0.4
Minor[b]	0.4	0.5	0.5	0.4
Decreased weight				
Body	1.6	0.5	2.0	0.8
Extragestational	1.6	0.5	--	--
Extragestational gain	0.4	0.5	--	0.8[c]
Gravid uterus	--	2.0	--	--
Hematocrit	0.8	1.0	--	0.8
Reproductive Effects				
Resorptions in litters with resorptions	0.4[d]	--	--	--
Placental and Fetal Effects				
Lower weights				
Female fetuses	--	0.5	2.0	--
Male fetuses	--	1.0	2.0	--
Placenta	--	2.0	--	--
Morphologic alterations				
Misaligned sternebrae	--	2.0[e]	--	--
Supernumerary ribs	--	2.0[e]	--	--
Reduced ossification			--	
Vertebrae	--	0.5[d,f]	--	--
Sternebrae	--	2.0[e]	--	--
Hydroureter	--	0.5[d,e]	--	--

[a]Major--gastric lesions or infections
[b]Minor--inflamed mesenteric lymph nodes in rats; enlarged Peyer's patches in rabbits
[c]Significantly different the from value for lowest dose group, but not from the control value (Duncan's multiple range test)
[d]Not significant in highest dose group (Duncan's multiple range test)
[e]Significance based on fetal unit (chi-square test)
[f]Significance based on litter unit (Fisher's exact test)

Gastric lesions (edema, hemorrhage or sloughing of the mucosa and ulceration) and intestinal or respiratory infections were observed at necropsy of rats and rabbits that died prior to the scheduled sacrifice. In all cases, respiratory infections were secondary to gastrointestinal infections. These findings suggested that, in maternal animals, the primary toxic effects of the sulfur mustard regimen were localized in the gastrointestinal tract. The lowest administered dose of sulfur mustard that produced these lesions was 0.4 mg/kg in the rabbit and 2.0 mg/kg in the rat. The minimal dose levels that resulted in maternal mortality bear a similar

relationship, 0.8 mg/kg and 2.5 mg/kg for the rabbit and rat, respectively. These results do not necessarily imply that the rabbit is more sensitive to localized damage of the gastric mucosa but rather that these lesions may be a consequence of the higher concentration of sulfur mustard in the dose solutions administered to rabbits than in those used for the rats.

Inflamed mesenteric lymph nodes were consistently observed at the scheduled necropsy of rats exposed to sulfur mustard, but enlarged Peyer's patches were more frequently encountered in the exposed rabbits. These effects may have been due to a difference in inflammatory response in the two species or to the fact that the interval between cessation of sulfur mustard exposure and sacrifice was shorter (5 days) for the rat than for the rabbit (11 days).

Other signs of maternal toxicity included decreased body weights and depressed weight gains during gestation in both rats and rabbits. During the dosing interval, significantly decreased body weights occurred in rats at a lower dose level (0.5 mg/kg) than in rabbits (0.8 mg/kg). This apparent difference in response may be partially due to the fact that we could detect smaller differences in rats because of the greater uniformity of their body weights. On the other hand, the response to sulfur mustard exposure in the younger, still-growing rats may have been greater than that of the more mature rabbits. In either case, these toxic signs were probably attributable to anorexia. While food consumption was not measured, daily consumption was readily ascertained for rabbits since they normally ate a large portion of the pelleted food that was supplied. Reliable estimates of rat food intake were not obtained because of their more limited intake of the cubed feed.

The lower hematocrit values obtained for animals that received dose levels of 0.8 mg/kg or higher may be an additional indicator of the cytotoxic effects of sulfur mustard. Fox and Scott (1980) observed cytotoxic effects of alkylating agents in rapidly renewing tissues such as lymphoid and gastrointestinal tissues and bone marrow. However, these values may have been influenced by the changes in plasma volume and erythropoiesis that occurred during pregnancy (Eastman and Hellman, 1966) or by the prolonged periods of anorexia observed in some of the animals.

No clear signs were evident of a significant effect of the agent on reproductive measures. Intrauterine mortality tended to be higher in treated groups than in control animals but was not significant except in one instance (resorptions in litters with resorptions were higher in rats at the 0.5-mg/kg dose level). Abortions were observed in two of eight rabbits at the 2.0-mg/kg dose level of the dose-range study; in the teratology study, all fetuses of one rabbit of the 0.6-mg/kg dose group died. The fact that there is no consistent evidence in reproductive measures for a direct dose response to sulfur mustard suggests that these effects may have been due to maternal toxicity.

Fetal body weights were depressed in litters of rats dosed with 0.5 or 1.0 mg/kg of sulfur mustard, but weights of rabbit fetuses were not affected in the highest dose group (0.8 mg/kg) of the teratology study. Fetal growth depression was observed, however, in the dose-range study in which the rabbits were dosed with 2.0 mg/kg during gestation.

No consistent, significant patterns in structural changes or dose responses were observed with regard to the incidence of major fetal malformations. Anatomical changes considered to be incompatible with survival were detected in three rat fetuses of the high dose group (2.0 mg/kg) and in two rabbit fetuses, one in the con-

trol group and one in the 0.5-mg/kg dose group. In addition, severe defects were observed during gross examinations in one rabbit litter of the 2.0-mg/kg group of the range-finding study.

The litter, rather than the fetus, was defined as the experimental unit for determining the significance of observations for fetal morphologic alterations (Kalter, 1974; Haseman and Hogan, 1975). Using this constraint, an increased incidence of reduced vertebral ossification in rat fetuses of the lowest dose group (0.5 mg/kg) was the only statistically significant finding in the teratology studies of both the rat and rabbit. When significant increases in incidence were based on fetal units, retarded fetal development was also indicated by reduction in vertebral and sternebral ossification in the 1.0- and 2.0-mg/kg dose groups of the rat study. These findings tend to correspond with fetal body-weight depression but were not exactly correlated, since the weights in the lowest dose group were not significantly depressed. According to Khera (1981), reduced ossification, particularly of the sternum, may occur as a consequence of maternal weight-gain depression during gestation, or because of fetal failure to gain weight or secondary fetal immaturity as a result of large litter size. Retarded sternal ossification was common (61%) in rabbit fetuses from all experimental groups and has been observed in 65% of the litters from this strain used in previous studies in this laboratory. Since there appears to be a marked degree of variation in skeletal ossification in near-term fetuses, Aliverti et al. (1979) have suggested that rat fetuses be evaluated for retarded development on 21 dg, rather than on 19 or 20 dg.

Another sternebral anomaly, misalignment, was evident in rat fetuses of the high dose group, but the increased incidence of this aberration was not significant on a litter basis. Khera (1985) has reviewed a number of teratology studies to examine the contributory role of maternal toxicity in the induction of fetal deaths and malformations. He concluded that certain malformations, including sternebral anomalies, were associated with maternal toxicity in rats and rabbits. Kimmel and Wilson (1973) had previously reported that sternebral variations were of doubtful value in assessing the teratogenic potential of an agent in rats, but that an increased incidence in supernumerary ribs might be a useful indicator. Recently, Kavlock et al. (1985) determined that the incidence of supernumerary ribs increased in response to a nonspecific maternal toxicity induced by an exposure of CD-1 mice on 8 dg to a variety of chemicals. In our sulfur mustard studies, the majority of fetuses from only one litter of rats in the highest dose group presented supernumerary ribs. This finding was not significant on a litter basis. Supernumerary ribs commonly occur in rabbits (Green, 1939; Khera, 1981) and were observed in 80% of the litters of all treatment groups of the teratology study. In previous studies in this laboratory, we have found that supernumerary ribs occur in 82% of the litters of the rabbits from this source.

The incidence of hydroureter, which is commonly observed in rats (Perraud, 1976), tended to be higher in the low- and mid-range dose groups of the teratology study in rats but was not accompanied by either mild or severe renal pelvic cavitation in a significant number of fetuses.

In the teratology studies, there was considerable evidence of maternal toxicity in rats at dose levels of 0.5 mg/kg and higher. Although intrauterine mortality was not significantly increased, fetal growth was retarded in female fetuses of the 0.5-mg/kg dose group and in all fetuses exposed to doses in excess of this level. This growth retardation and the trends observed in reduced skeletal ossification, sterne-

bral defects and rib anomalies suggest that, in the rat, fetal responses may be attributed to maternal toxicity.

In rabbits, maternal weight gains were depressed following administration of 0.8 mg/kg, and gastric lesions were observed in one doe that received 0.4 mg/kg. Despite these overt signs of maternal toxicity, no significant effects on intrauterine death or fetal growth and development were detected. We may therefore conclude that, under the conditions of this study, sulfur mustard is not teratogenic in rabbits.

Definitive values for "no observable effect levels" (NOEL) were not obtained in these studies; however, specific limits of dose ranges for NOEL can be estimated from the results of the teratology studies. Furthermore, some inferences may be made with data from dose range studies that encompassed larger dose ranges with smaller sample sizes so that the performance of reliable statistical comparisons was limited. Estimates of dose ranges for NOEL in rats are based on reductions of maternal body weights and significant retardation of fetal development following exposure to 0.5 mg/kg, the lowest dose level administered in the teratology study; however, no abnormalities, including inflamed mesenteric lymph nodes, were observed in maternal rats of the 0.2-mg/kg dose group of the dose range study. Gastric lesions and enlarged Peyer's patches were evident in maternal rabbits that received the lowest dose level (0.4 mg/kg), but doses of 0.8 mg/kg were required to reduce body weights. In rabbit fetuses, no significant effects were evident in litters exposed to the highest dose level of the teratology study (0.8 mg/kg), but in the dose range study, effects were apparent following exposure to 2.0 mg/kg. Using these criteria, estimates for dose ranges for NOEL for sulfur mustard may be summarized as follows:

	Dose Range (mg/kg)	
	Rats	Rabbits
Maternal	<0.5	<0.4
Fetal	<0.5	>0.8

During the performance of these studies, no problems that would effect the integrity of the results were encountered.

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GLOSSARY

AI	= Artificial insemination
ALS	= Automatic liquid sample
AV	= Artificial vagina
dg	= Days of gestation
FDA	= Food and Drug Administration
GC	= Gas chromatography
GLP	= Good Laboratory Practices
HD	= 2,2'-dichlorodiethyl sulfide, or distilled mustard
HP	= Hewlett-Packard
IG	= Intragastric
IP	= Intraperitoneal
ISTD	= Internal standard
IV	= Intravenous
KRV	= Kilham rat virus
LD_{50}	= Median lethal dose
LSL	= Life Sciences Laboratory
NBF	= Neutral buffered formalin
NMR	= Nuclear magnetic resonance
NOEL	= No observable effect level
PC	= Percutaneous
PNL	= Pacific Northwest Laboratory
PVM	= Pneumonia virus of mice
r^2	= Coefficient of determination
RCV/SDA	= Rat corona virus/sialodacryoadenitis virus
RH	= Relative humidity
SC	= Subcutaneous
SD	= Standard deviation
SE	= Standard error
SOP	= Standard operating procedure
USABRDL	= U.S. Army Biomedical Research and Development Laboratory
USAMRICD	= U.S. Army Medical Research Institute of Chemical Defense

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