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LOW-LEVEL EFFECTS OF VX VAPOR EXPOSURE ON PUPIL SIZE AND CHOLINESTERASE LEVELS IN RATS

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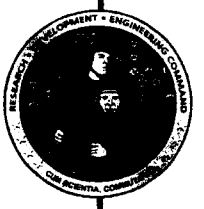

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The effective concentrations (EC_{50}'s) for miosis in male and female rats exposed to VX vapor for 10, 60, and 240 min were estimated using whole body vapor exposures conducted in a 750 L dynamic airflow inhalation chamber. Miosis was defined as at least a 50% reduction in pupil diameter relative to baseline measurements. Contrary to Haber's Rule, median effective dosages (ECT_{50}'s) increased with increasing exposure durations (i.e., the CT for 50% of the exposed population to show miosis was not constant over time). Ordinal regression was used to develop an empirical toxic load model for predicting VX vapor induced miosis by defining the relationship between C and T with a VX specific, toxic load exponent (n) in the equation: $C^n xT=k$. Female rats were more sensitive than male rats to the miotic effects of VX vapor. Statistically significant acetylcholinesterase (AChE) inhibition was detected at the higher concentrations of each exposure time. A VX-G analog assay successfully used rat blood plasma as a biomarker for VX exposure. A general relationship between increasing dose (CT) and increasing amounts of VX-G in the plasma was found.

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Duration	Miosis	ECT_{50}	VX
Concentration	Inhalation	Rats	
Acetylcholinesterase	Low Level	Toxic load	

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PREFACE

The work described in this report was authorized under Project No. 206023, Low Level Toxicology. The work was started in June 2003 and completed in September 2004. The experimental data are contained in laboratory notebook 03-0099. Raw data and the final report from this study are stored in the Toxicology Archives, Building E3150, Aberdeen Proving Ground, MD.

In conducting this study, investigators adhered to the "Guide for the Care and Use of Laboratory Animals," National Institutes of Health Publication No. 86-23, 1985, as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council, Washington, DC. These investigations were also performed in accordance with the requirements of AR 70-18, "Laboratory Animals, Procurement, Transportation, Use, Care, and Public Affairs," and the U.S. Army Edgewood Chemical Biological Center Institutional Animal Care and Use Committee (IACUC), which oversees the use of laboratory animals. This project's assigned IACUC Protocol No. 03-345 was approved on 28 May 2003.

All animals were cared for as stated in this research protocol and as specified in the NIH Publication No. 85-23, 1985 (or updates). Records were maintained in official ECBC Notebooks in the Life Sciences Official Archives (Building E3150) and/or in the Technical Library (Building E3330). Studies were conducted under, and in compliance with, current GLP standards, which were reviewed periodically by the QA Coordinator or his designee.

The performance of this study was consistent with the objectives and standards in "Good Laboratory Practices for Non-clinical Laboratory Studies" (21 CFR 58, Food and Drug Administration, U.S. Department of Health and Human Services, April 1988).

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The authors wish to thank Dr. Diana Scorpio (GEO-CENTERS, INC.) for her support in caring for the animals used in this study and Dennis Johnson (Veterinary Services Team, ECBC) for his guidance with quality assurance issues.

QUALITY ASSURANCE

This study, conducted as described in Protocol 03-345, was examined for compliance with Good Laboratory Practices as published by the U. S. Environmental Protection Agency in 40 CFR Part 792 (effective 17 Aug 1989). The dates of all inspections and the dates the results of those inspections were reported to the Study Director and management were as follows:

Phase Inspected	Date	Reported
Study parameters and exposure	22 Oct 03	22 Oct 03
Data and Final Report	21 Sep 04	21 Sep 04

To the best of my knowledge, the methods described were the methods followed during the study. The report was determined to be an accurate reflection of the raw data obtained.

DENNIS W. JOHNSON
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Obscurants Senior Team
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LOW-LEVEL EFFECTS OF VX VAPOR EXPOSURE ON PUPIL SIZE AND CHOLINESTERASE LEVELS IN RATS

1. INTRODUCTION

O-Ethyl S-[2-(diisopropylamino)ethyl] methylphosphonothiolate (VX) is an organophosphorous (OP) compound that has been the subject of much research for over half a century. It is extremely toxic with an equivalent dose of VX being substantially more toxic than related nerve agents such as sarin (GB), cyclosarin (GF), tabun (GA) and soman (GD). Most of what is known of the effects of VX on whole animals is derived from studies administering VX subcutaneously, percutaneously, intravenously or as an inhaled aerosol (Bide and Risk, 2000; Craig *et al.*, 1977; Gupta *et al.*, 1991; Rickett *et al.*, 1986). However, few studies exist in which reliable toxicity estimates in animals have been established for VX administered as a vapor (Hartman, 2002). Contributing to this lack of information is the difficulty in producing stable vapor concentrations in a controlled environment due to the very low vapor pressure of VX (0.00063 mm Hg @ 25°C compared to 2.9 mm Hg @ 25°C for sarin (GB)).

The available literature addressing the toxicity of VX vapor includes two studies, which dealt with the toxic effects of chemically neutralized VX in rats (Muse *et al.*, 2002; Manthei *et al.*, 1990). There are several studies, which dealt with either aerosolized VX (Bide and Risk, 2000), or VX mixed with other compounds (Weimer and Ballard, 1960; Dimmick *et al.*, 1979). One recent study examined the toxicity of VX vapor inhalation in rats using a "nose-only" exposure design (Bide *et al.*, 1996), but did not address the issue of "first noticeable effect" (FNE) associated with very low concentrations of VX vapor. The concept of FNE is used to define the threshold concentrations of nerve agents below which there are no observable effects and above which more severe measurable effects are produced. Defining the FNE for low-level concentrations of VX vapor is important because of the military implications related to performance degradation and operational readiness. Previous studies in our laboratory using GB (Mioduszewski *et al.*, 2002) and GF (Whalley *et al.*, 2004) vapor have shown miosis to be the FNE resulting from a whole body inhalation exposure. In those two studies and the present study, miosis was defined as a 50% reduction in pupil diameter relative to pre-exposure baseline measurements. This study used VX vapor-induced miosis in rats as the endpoint for the FNE.

Our first objective was to determine the median effective concentrations (EC_{50}'s) of VX vapor that produced miosis in rats at three exposure durations. The second objective was to develop an empirical model for predicting VX vapor toxicity for duration times extending beyond our ability to test directly. Our toxic load model was derived from previous work on the dose-response relationships between concentrations of various chemicals and duration of exposure. The relationship, known as Haber's Rule, is described by the equation $C \times t = k$ (Haber, 1924) where C is equal to the atmospheric concentration of the chemical being tested, t is equal to the duration of exposure, and k is a constant for some effect or response. This equation assigns equal importance to concentration and time in determining the response. Thus, the product of C x t would remain constant regardless of the concentration or exposure time (Figure 1). This assumption proved to be inadequate for many chemicals when attempting to describe cumulative toxicity effects. Thus, the equation was modified to better describe the

relationship between concentration and exposure time for a given chemical (ten Berge *et al.*, 1986). The equation $C^n \times t = k$ includes the exponent n which is an experimentally determined, chemical specific value which helps describe the non-linear relationship between concentration and duration of exposure (Figure 1). Our third objective was to estimate this n value for miosis producing levels of VX vapor. Fourth, we were to determine the degree of cholinesterase inhibition and VX regeneration in whole blood. These data provide important information regarding the relationship between exposure levels, absorption amounts and miosis. The VX regeneration data was particularly important because it more directly related to the internal dose the animal was receiving. Our final objective was to determine if the miotic effects of VX vapor exposure and cholinesterase depression were gender dependent.

Whole body vapor exposures were conducted in a 750 L dynamic airflow inhalation chamber. Rats were exposed for 10, 60, or 240 min. Five concentrations of VX were tested at each exposure duration. Baseline values for cholinesterase and pupil size were established in each rat prior to exposure.

Separate ECT_{50}'s for miosis were established for male and female rats at each exposure duration. The values were derived from pupil measurements taken within 1 hr post exposure. A potency comparison with GB and GF (Table 1) shows that VX is approximately an order of magnitude more potent. There were significant gender differences in the ECT_{50} values for miosis at each exposure duration. An empirical toxic load model was developed and the toxic exponent for miosis (n) in the equation $C^n \times t = k$ was determined to be $n = 1.65$. There was significant AChE depression at the highest concentrations of each exposure duration and detectable levels of VX-G-analog (ethyl methylphosphonofluoridate) were found in blood plasma at the low exposure dosages (CT) used in this study.

This study identified experimental effects that could impact operational readiness and serve as a basis for predictions useful for military Operational Risk Management (ORM) decisions.

2. MATERIALS AND METHODS

2.1 Chemicals.

O-ethyl-S-[2-(diisopropylamino) ethyl] methylphosphonothiolate (VX or EA 1701) was used for all vapor exposures. The structure of VX is shown in Figure 2 with its corresponding physical and chemical properties given in Table 2. The VX was received from the Chemical Transfer Facility at Aberdeen Proving Ground, MD, in individually sealed 5-mL ampoules (Lot #VX-U-1243-CTF-N) and certified as chemical agent standard analytical reagent material (CASARM). Seven iterations of a ^{31}P NMR analysis were performed according to an established method (Brickhouse *et al.*, 1997) to certify the purity of the material as 93.6 ± 0.5 mole percent pure (impurities and their respective percentages are shown in Table 3). A high purity grade of triethylphosphate (99.9%; Aldrich Cat. No.: 24,089-3) was used as the internal

standard for the VX purity assays. All external standards for VX vapor quantitation were prepared daily with isopropanol (IPA) solvent (Burdick & Jackson Cat. No.: 323-4 purity > 99%)

2.2 Inhalation Chamber.

Whole body vapor exposures were conducted in a 750-L dynamic airflow inhalation chamber (Figure 3). The Rochester style chamber was hexagonal and constructed of stainless steel with Plexiglas windows on each of its six sides. The interior of the exposure chamber was maintained under negative pressure (0.25" H_2O) as recorded by a calibrated magnehelix (Dwyer, Michigan City, IN). Room air was drawn through the exposure chamber (570-580 L/min) and measured at the chamber outlet with a calibrated thermoanemometer (Alnor model 8565, Skokie, IL). Temperature and humidity were recorded for every exposure.

2.2.1 Vapor Generation.

The vapor generation system was located at the chamber inlet and was contained within a stainless steel box maintained under negative pressure. Saturated VX vapor streams ($0.00037 - 0.016$ mg/m^3) were generated by a continuous flow of nitrogen carrier gas (8-202 mL/min) through a glass vessel functioning as a multi-pass saturator cell (Glassblowers, Incorporated, Turnersville, NJ) containing 1 mL of liquid VX (Figure 4). The main body of the saturator cell consisted of a 100-mm long, 25-mm outer diameter (o.d.), cylindrical glass tube with two vertical 7-mm o.d. tubes (inlet, outlet) at each end (Figure 4). The main body of the saturator cell contained a porous, hollow, ceramic cylinder, which served to increase the contact area between the liquid VX and the nitrogen carrier gas by absorbing the liquid VX. The saturator cell was fabricated to allow nitrogen gas to make three passes along the surface of the wetted ceramic cylinder (Alundum® fused alumina, Norton Company, Colorado Springs, CO) before exiting the outlet arm of the saturator cell. The saturator cell body was immersed in a constant temperature bath (Thermo NESLAB, Portsmouth, NH) containing mineral oil so that a combination of nitrogen gas flow rate and temperature could regulate the amount of VX vapor entering the inhalation chamber. The bath was maintained at 30-50°C depending upon the required concentration of VX and the outlet arm of the saturator cell was wrapped in heat tape and maintained at 90°C. It was necessary to maintain a continuous flow of VX vapor through the chamber to preserve the passivation of the chamber. This allowed for generation and maintenance of stable chamber concentrations.

2.2.2 Sampling System - Sorbent Tubes.

The solid sorbent tube sampling system consisted of a 20:35 mesh Tenax-TA fast flow sorbent tube (Dynatherm part number AO-06-2717) and a thermal desorption unit (TDU; ACEM-900, Dynatherm Analytical Instruments, Kelton, PA.) coupled to a gas chromatograph with flame photometric detection (GC/FPD). Samples were drawn from the middle of the exposure chamber by inserting a rod containing a sampling tube through small access ports located on the walls of the chamber. The rod was hooked to a vacuum line that drew a sample through the tube at a rate of 3-5 L/min for 1-9 min depending upon the chamber concentration. Sample flow rates were controlled with calibrated mass flow controllers (Matheson Gas

Products, Montgomeryville, PA) and verified before and after sampling with a calibrated flow meter (DryCal, Bios International, Pompton Plains, NJ) connected in-line with the sample stream. The sample tube was transferred to the TDU and prepared for injection onto a Restek RTX-5 column (15m x 0.32mm x 0.5 μm). Temperature and flow programming within the TDU desorbed VX from the sorbent tube directly onto the GC column. Detection was performed with flame photometric detection in the phosphorous mode.

The sampling system was calibrated by direct injection of external standards onto the sorbent tubes prior to insertion into the TDU and analysis with GC/FPD. In this way, injected VX standards were put through the same sampling scheme as the chamber samples. A linear regression fit ($r^2 = 0.999$) of the standard data was used to calculate the VX concentration of each chamber sample.

Concentration uniformity was checked at several locations throughout the chamber, including areas directly above the animal cages. At higher generated agent concentrations, vacuum pumps were used to draw air through glass fiber, filter pads at high flow rates to test for the presence of aerosols. Analysis of the glass fiber pads required isopropanol desorption and liquid extract injection onto a 20:35 mesh Tenax-TA fast flow sorbent tube. The sorbent tube was thermally desorbed and analyzed by GC/FPD.

2.3 Animal Model.

Sexually mature male and female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing between 180 and 300 gm were used in this study. Upon arrival, the animals were identified by tattoo on the tail and segregated according to sex. Rats were housed individually in plastic shoebox cages. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility (Bldg. E-3150). The animals were quarantined for a minimum of 5 days following their arrival. Ambient conditions were maintained at 70 ± 5°F, 30-70% relative humidity with a 12:12 hr light-dark cycle. Rats were provided with certified laboratory rat chow and filtered house water *ad libitum*, except during exposure. All experiments and procedures were approved by the U.S. Army Edgewood Chemical Biological Center (ECBC) Institutional Animal Care and Use Committee, and conducted in accordance with the requirements of Army Regulation 70-18 and the National Research Council's Guide for the Care and Use of Laboratory Animals.

2.4 Blood Sample Collection.

Blood samples were drawn from all test rats and used for the cholinesterase inhibition and VX-G analog regeneration assays. Blood draws were done once before exposure, approximately 60 min post exposure and 7 days post exposure. Approximately 1 mL of blood was taken at each draw. To promote rapid blood flow and collection of samples, the rats were placed in a "shoebox" type holding cage doubling as a warming pen. The shoebox containing the rats was stacked within a second shoebox containing warm water. The heat from the water elevated the rat's body temperature just enough to promote vessel dilation and increased blood flow. The rats were removed from the warming pen after 5 min and approximately 1/8 in. of their tail was removed using sharp scissors. The tail was gently massaged to promote the

collection of blood into Microtainer® tubes (Becton-Dickinson, Franklin Lakes, NJ) containing the anti-coagulant ethylenediaminetetraacetic acid (EDTA). Post collection bleeding was minimal and clotting was facilitated by compression of the incision.

2.4.1 Cholinesterase (ChE) Inhibition Assays.

The method used for measuring whole blood acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities was a modification of the Ellman Reference Method (Ellman *et al.*, 1961).

Approximately 300 µL of blood was collected for determination of whole blood AChE and BChE activities. For each blood sample, a 10 µl aliquot of clot-free whole blood was added to 2 mL distilled water in a 13x75 mm test tube followed by addition of 200 µL of 0.69 mM phosphate buffer at pH 7.4 (EQM Research, Cincinnati, OH). Each tube was then vortexed and 200 µL of the resulting solution from each tube was transferred to individual wells on a 96-well plate. Twenty-five microliters of 30 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB) was added to each well. For determination of AChE activity, 25 µL of a solution containing 10 mM acetylthiocholine and 200 µM 10-(α-diethylaminopropionyl)-phenothiazine, a specific inhibitor of butyrylcholinesterase (EQM Research, Cincinnati, OH), was added to the appropriate wells of the 96-well plate. For determination of BChE activity, 25 µL of a solution containing 20 mM butyrylthiocholine (EQM Research, Cincinnati, OH) was added to the appropriate wells of the 96-well plate. The plate was then read at 450 nm and 37°C using a SpectraMax Plus^{384} microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA) for 10 min, and analyzed using SoftMax Pro LS version 4.3 software (Molecular Devices Corporation, Sunnyvale, CA).

BChE activity values in whole blood were expressed as Units of activity per gram of total plasma protein (U/g TPP). To determine total plasma protein (TPP) concentration in g/mL, whole blood was centrifuged to separate the plasma from the red blood cells (RBCs). Plasma was placed in a refractometer (American Optical Company, Keene, N.H.) and the total protein was read directly from the TPP scale.

AChE activity values in whole blood were expressed as Units of activity per gram of hemoglobin (U/g HGB). Hemoglobin was measured by the Oshiro method (Oshiro *et al.*, 1982). Briefly, 225 µL of hemolysate was added to a 96 well (uncoated) Greiner microplate (Greiner Bio-One, Longwood, FL) along with 25 µL of 2.08 mM sodium lauryl sulfate in a pH 7.2 phosphate buffer (30 mM). The plate was read at 536 nm and 37°C using a SpectraMax Plus^{384} microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA) for 10 min, and analyzed using SoftMax Pro LS version 4.3 software (Molecular Devices Corporation Sunnyvale, CA). All specimens were assayed in duplicate.

2.4.2 VX-G Regeneration Assay.

Several days prior and within 1 hr after inhalation exposure, whole blood from VX exposed male and female rats was collected in capped polyethylene tubes that contained EDTA. The samples were centrifuged at 15000 rpm for 5 min to separate the plasma and red

blood cell fractions. After separation, the plasma samples were frozen at -20°C until analysis and red blood cell samples were refrigerated at 5°C. The plasma samples were analyzed for VX-G (the G refers to the VX analog ethyl methylphosphonofluoridate) by the addition of acetate buffer and fluoride ion (Jakubowski *et al.*, 2001).

The samples were prepared as follows. To a weighed sample (0.1-0.8 g) of plasma or (0.2-0.3 g) RBC in a 2.0 mL microvial, 1 mL of acetate buffer (pH 3.5), 20 µL/0.1 g sample (for plasma) or 200 µL/0.25 g sample (for RBC) of 6 M potassium fluoride (KF) solution, and 5 µL of $^2H_5$-VX-G (200 pg/µL in ethyl acetate) internal standard were added and vortexed. The RBC reaction mixture was centrifuged at 15000 rpm for 1 min to segregate the insoluble components from the solution. These initial reaction solutions were transferred to C_{18} SPE cartridges (200 mg Sep-Pak, Waters Associates, Millipore Corporation, Milford, MA), which were first conditioned with 1 mL ethyl acetate followed with 1 mL isopropanol and finally with 1 mL acetate buffer. The sample microvials were then washed with a mixture of 750 µL acetate buffer and 20 µL/0.1 g sample (for plasma) or 200 µL/0.25 g sample (for RBC) of KF solution. The RBC microvial solution was centrifuged again. The wash solutions were added to the original reaction mixtures on the SPE columns. Fifteen minutes after the original addition of buffer and KF, the combined reaction mixture was allowed to drain through the conditioned SPE column under a gentle vacuum. After complete draining, the SPE column was dried by using a light vacuum to pull air through the column for 3 min. The regenerated VX-G and deuterated internal standard VX-G were eluted with 1 mL ethyl acetate that was collected and dried over anhydrous sodium sulfate. The ethyl acetate was removed from the collection tube and filtered through a 0.2 µm nylon Acrodisc syringe filter (Pall Gelman Laboratory, Ann Arbor, MI) into a GC autosampler vial. The eluent was concentrated to 50-75 µL total volume using a nitrogen stream directed across the sample surface (Techne Sample Concentrator, Techne, Incorporated, Princeton, NJ).

The regenerated VX-G was analyzed as follows. Injections of 50 µL (twice) of extract were made by autoinjector into the large volume injector port (Agilent Technologies, model PTV, Wilmington, DE) using the following parameters: initial temperature -30°C, initial time 8.1 min, final temperature 225°C, rate 720°C/min (maximum ballistic heating as listed in the Agilent manual), vent time 8.00 min, vent flow 300 mL/min, purge flow 50 mL/min, purge time 11.7 min. The GC (Agilent Technologies model 6890, Wilmington, DE) column used was a HP-5MS (30 m x 0.32 mm x 1.0 µm film thickness) with a flow rate of 3 mL/min (63 cm/s). The GC oven program was as follows: initial temperature was 35°C for 12.3 min to 125°C @15°C/min (0 min hold) to 325°C @30°C/min. Mass spectrometric detection (Agilent Technologies model 5973 MSD, Wilmington, DE) was by chemical ionization with ammonia reagent gas in the positive ion mode using the m/z 144/149 ammonia adduct ion ratio (VX-G/$^2H_5$-VX-G) for quantification and the m/z 161 (VX-G) and 166($^2H_5$-VX-G) ions as qualifiers. Linear internal standard calibration curves for VX-G were generated from 10-1000 pg using standards in ethyl acetate. The Agilent software (Enhanced Chemstation Version D.00.00.38, 2001, Agilent Technologies, Wilmington, DE) provided with the mass spectrometer was used to process and analyze the data. The software allowed automatic analysis of the internal standard method based on the analyte area ratios of the peaks at their respective retention times.

2.5 Photography.

This study utilized a non-invasive method of assessing pupil size whereby projected infrared (IR) light (880 nm) reflected off the animal's retina back through the pupil producing an image of a bright pupil surrounded by a dark iris (Miller *et al.*, 2002, 2003a, and 2003b). The right eye of all rats in the study was digitally photographed on 3 different days prior to exposure, to establish an average baseline pupil size. Pictures were also taken 60 min, 2 hr, 24 hr, 48 hr, and 7 days post exposure. All photographs were taken under low-light conditions (<10 ft-c). All rats were temporarily restrained while photographed. Restraint lasted approximately 30 s per rat and involved immobilizing the head of the rat in a yoke. The photographic equipment included an infrared (IR) light source (SL2420-880100XL24VOLT), a black and white Sony CCD video camera (XC-ST50) and power supply (CD700), a 75 mm/F2.7 video camera lens (LMV7527) and tripod adapter (VCT-ST701) all supplied by Data Science Automation (Canonsburg, PA). Labview and IMAQ software (National Instruments, Austin, TX) were used to write the novel image analysis program and National Instruments also produced the image acquisition computer card (PCI-1411). The central processing unit was a Dell computer (Model WHL; Dell Computer Corporation, Round Rock, TX).

2.6 Experimental Design.

Rats were exposed for 10, 60, or 240 min. Five concentrations of VX were tested at each exposure duration. For each exposure, 10 rats were placed in each compartmentalized stainless steel cage (20" x 14" x 4") with each rat occupying a separate compartment (4" x 7" x 4"). The chamber could accommodate two of these steel cages that were placed on the floor of the exposure chamber prior to the introduction of VX. Each exposure consisted of 10 male and/or 10 female rats exposed to a concentration of VX vapor with an additional 5 male and/or 5 female control rats placed in a control chamber and exposed to air only. The t_{99} (time to attain 99% of the equilibrium concentration within the chamber) ranged from 7.7-8.2 min. Physical parameters monitored during exposure included chamber airflow, nitrogen flow rate through the saturator cell, chamber room temperature and relative humidity. Following exposure, the chamber was purged with air for 10 min prior to removing the rats. After removal from the chamber, the rats were observed for any overt toxic signs of exposure such as tremors, salivation, lacrimation, etc. Clinical signs of exposure were monitored twice daily for up to 7 days post exposure. After 7 days post exposure, surviving rats were euthanized in accordance with the *Report of the American Veterinary Medical Association (AVMA) Panel on Euthanasia* (1993).

Baseline values for cholinesterase activity and pupil size were established in each rat prior to exposure. Within 1 hr after exposure, the right eye of each rat was photographed under low light conditions and a blood sample was drawn from the tail. Additional photographs were taken at 2, 24, and 48 hr post exposure and at 7 days post exposure. For all post exposure photography, the rats were randomized with respect to the order in which they were photographed. On day 7 post exposure, another 1 mL blood sample was drawn from each rat.

Exposures were conducted 1 or 2 days per week and exposure durations were chosen randomly. Due to gender differences in sensitivity to VX (females developed miosis at lower exposure concentrations than males), it was necessary to expose males and females

separately when determining the upper and lower limits of the dosage range for miosis at each exposure duration.

When procuring rats from Charles River Laboratories, Incorporated, the number of rats per shipment was limited to the number sufficient for one exposure each for males and females (exposed and control). The Appendix provides a summary of the exposures and shipments).

2.7 Calculation of Pupil Diameter.

The analysis to determine pupil diameter relative to the baseline value in a given rat (either exposed or control) used an eight-level ratio.

(1) post-exposure pupil radius (1 measurement per rat) -- P_{po}
(2) post-exposure iris radius (1 measurement per rat) -- I_{po}
(3) pre-exposure pupil radius (geometric mean (GM) of 3 measurements per rat) -- P_{pre}
(4) pre-exposure iris radius (GM of 3 measurements per rat) -- I_{pre}
(5) post-exposure (air only) pupil radius (GM of 5 measurements: 1 per rat x 5 control rats of same gender) -- p_{po}
(6) post-exposure (air only) iris radius (GM of 5 measurements: 1 per rat x 5 control rats of same gender) -- i_{po}
(7) pre-exposure pupil radius (GM of 15 measurements: 3 per rat x 5 control rats of same gender) -- p_{pre}
(8) pre-exposure iris radius (GM of 15 measurements: 3 per rat x 5 control rats of same gender) -- i_{pre}

The order of the divisions was: divide pupil radius by iris radius, divide post-exposure value by pre-exposure value, and divide individual rat post/pre ratio value (Π_{ind}) by the geometric mean of the post/pre values for the same-sex control rats (Π_{cntrl}) in the exposure group. The result of this process is the pupil diameter ratio (Π_{ratio}) of post- vs pre-exposure values for an individual rat adjusted for controls. This process is illustrated mathematically in Equations [1] to [3].

$$\Pi_{ind} = \left[\left(\frac{P_{po}}{I_{po}} \right) \Big/ \left(\frac{P_{pre}}{I_{pre}} \right) \right] \quad [1]$$

$$\Pi_{cntrl} = \left[\left(\frac{p_{po}}{i_{po}} \right) \Big/ \left(\frac{p_{pre}}{i_{pre}} \right) \right] \quad [2]$$

$$\Pi_{ratio} = \left[\Pi_{ind} \Big/ \Pi_{cntrl} \right] \quad [3]$$

Calculation of Blood ChE Values.

The analysis to determine the blood AChE or BChE value relative to baseline value in a given rat used a four-level ratio. In the following discussion, (α, a or A) represents variables associated with AChE and (β, b or B) represent variables associated with BChE values:

(1) post-exposure ChE value (1 measurement per rat) – A_{po} or B_{po}
(2) pre-exposure ChE value (1 measurement per rat) – A_{pre} or B_{pre}
(3) post-exposure (air only) control rat ChE value (GM of 5 measurements: 1 per rat x 5 control rats of same gender) – a_{po} or b_{po}
(4) pre-exposure (air only) control rat ChE value (GM of 5 measurements: 1 per rat x 5 control rats of same gender) – a_{pre} or b_{pre}

The order of the divisions was: divide post-exposure value by pre-exposure value, then divide individual rat value (α_{ind} or β_{ind}) by the geometric mean of the values for the same-sex control rats (α_{cntrl} or β_{cntrl}) in the exposure group. The result of this process is the blood ChE ratio (α_{ratio} or β_{ratio}) of post- vs pre-exposure values for an individual rat adjusted for controls. Both AChE and BChE values were reduced with this procedure. This process is illustrated mathematically in Equations [4] to [6] (written in terms of the AChE values).

$$\alpha_{ind} = \left[A_{po} \Big/ A_{pre} \right] \quad [4]$$

$$\alpha_{cntrl} = \left[a_{po} \Big/ a_{pre} \right] \quad [5]$$

$$\alpha_{ratio} = \left[\alpha_{ind} \Big/ \alpha_{cntrl} \right] \quad [6]$$

2.8 Data Analysis.

Minitab®, Version 13 (Minitab, Incorporated, State College, PA) was used for all statistical analyses.

The initial analysis of the reduced data (α_{ratio}, β_{ratio}, and Π_{ratio}) involved the use of Analysis of Variance (ANOVA) (Fox, 1997) to determine if any statistically significant differences existed in the responses between the control and exposed rats. A critical p value of 0.05 was used to assess statistical significance.

For statistically significant pupil constriction and blood ChE depression, a probit analysis (Finney, 1971) was conducted to calculate median effective dosages (ECT_{50}'s). The following equation was used to fit the experimental data:

$$Y_N = (Y_P - 5) = k_C(\log_{10} C) + \sum_{i}^{3} \sum_{j}^{2} k_{i,j} (Time)_i (Gender)_j \quad [7]$$

where Y_N is a normit, Y_P is a probit, the k's are fitted coefficients, C is vapor concentration, *Time* is the exposure durations (treated as a three-level factor), and *Gender* is a two-level factor. The fitted coefficient, k_C, is the estimate for the probit slope for concentration. Y_N equals –1, 0, and 1 at the 16, 50, and 84% response levels, respectively. The binary response to be modeled in Equation [7] is the presence of either miosis or blood ChE depression in an exposed rat, which was defined as at least 50% pupil diameter constriction ($\Pi_{ratio} \leq 0.50$) or 50% ChE depression (α_{ratio} (AChE) or β_{ratio} (BChE) ≤ 0.50), respectively.

Binary and ordinal logistic regressions (with a normit link function) (Finney, 1971; Agresti, 1990; Fox, 1997) were used to fit the toxic load model for probability of effect to the blood ChE depression and pupil diameter datasets, respectively. This approach has been used successfully in several previous mammalian CW agent toxicity studies (Anthony *et al.*, 2004; Hulet *et al.*;* Mioduszewski *et al.*, 2002a, 2002b; Sommerville, 2004;** Whalley *et al.*, 2004). The model used follows:

$$Y_N = k_0 + k_C(\log_{10} C) + k_T(\log_{10} T) + k_S\, Sex \qquad [8]$$

where Sex was coded –1 for female rats and 1 for male rats, k_s is the fitted coefficient for the factor *Sex*, the constants k_C and k_T are the probit slopes for concentration and time, respectively. The exposure duration, T, is treated as a covariate in Equation [8] (in contrast to duration as a factor in Equation [7]). For ordinal regression, k_0 is replaced by $k_1, k_2, \ldots, k_N$, which act as the intercepts for the N levels of response used in the analysis. The significance of the interaction between $\log_{10}T$ and *Sex* was investigated for pupil constriction and ChE depression. In both cases, this interaction was not statistically significant.

The ratio (k_C / k_T) equals the toxic load exponent, n. If this ratio is not different (with statistical significance) from one, then Haber's Rule (Haber, 1924) is appropriate for modeling the toxicity. Otherwise, the classic toxic load model (C^nT) is the proper approach (ten Berge *et al.*, 1986; Sommerville *et al.*, 2004***) assuming there is no significant curvature in the experimental data used to fit the model.

In addition to probability of effect, it is also possible to use Equation [8] to estimate a toxic load model for pupil diameter as described in Whalley *et al.* (2004). The boundaries for the classes used in the ordinal regression correspond to pupil diameter ratios (Section 3.1.3). Thus, it is possible to determine the relationship between Y_N (probability) and Y_N (pupil diameter), as shown in Section 3.1.3.

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*Hulet, S.W.; Sommerville, D.R.; Benton, B.J.; Forster, J.S.; Manthei, J.H.; Miller, D.B.; Scotto, J.A.; Jarvis, J.R.; Way, R.A.; Muse, W.T.; Gaviola, B.; Burnett, D.; Crosier, R.B.; Mioduszewski, R.J.; Thomson, S.A. *Low-Level Sarin Vapor Exposure in the Gottingen Minipig: Effect of Exposure Concentration and Duration on Pupil Size*, Unpublished Data, 2004.

**Sommerville, D.R. Relationship Between the Dose Response Curves for Lethality and Severe Effects for Chemical Warfare Nerve Agents. In *Proceedings of the 2003 Joint Service Scientific Conference on Chemical & Biological Defense Research*, 17-20 November 2003; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, Unpublished Data, 2004.

***Sommerville, D.R.; Park, K.H.; Kierzewski, M.O.; Dunkel, M.D.; Hutton, M.I.; Pinto, N.A. Toxic Load Modeling. In Inhalation Toxicology, Salem, Harry, Ed., Marcel Dekker, NY, Unpublished Data, 2004.

3. RESULTS

This study focused on collecting sufficient data on pupil constriction to estimate ECT_{50}'s for miosis in rats exposed to low-levels of VX vapor for 10, 60, or 240 min. Subsequently, these data were used to formulate a multifactor model to predict dose-response relationships and the probability of incurring VX vapor-induced miosis as a function of exposure concentration and duration. By exposing groups of rats to 5 different concentrations of VX vapor per exposure duration, we were able to establish ECT_{50}'s for miosis for male and female rats at each of the exposure durations. The blood samples collected pre- and post-exposure were analyzed for dosimetric correlations between exposure dosage, whole blood cholinesterase activity and the levels of VX-G found in blood plasma.

The results of the data analysis on the pupil constriction and blood ChE depression measurements are described below. Printouts of the statistical analyses from MINITAB® are in the Appendix.

3.1 Pupil Response.

Figures 5-10 show boxplots of the individual values for pupil diameter ratio of post- vs pre-exposure values (adjusted for controls) (Π_{ratio}). All observations were made 1 hr post-exposure for each combination of gender and exposure duration (10, 60, and 240 min). Each box represents the data from one vapor exposure, with the estimated 10^{th}, 25^{th}, 50^{th}, 75^{th}, and 90^{th} percentiles displayed. However, in one instance, results from two exposures involving male rats exposed for 10 min were combined together into one box (Figure 5) because of the closeness of the two vapor concentrations values (0.00830 and 0.00832 mg/m^3). Percentiles were calculated using the method outlined by Prins (2003).

3.1.1 Pupil Response as a Function of Observation Period.

Of the five post exposure observation periods (1 hr, 2 hr, 24 hr, 48 hr, and 7 days), the lowest Π_{ratio} values (greatest miotic response) were found in the 1 hr post-exposure observations (Figure 11). ANOVAs were done for each observation period. For 1 hr, 2 hr, and 24 hr post-exposure periods, there were statistically significant differences between the ratio values (Π_{ratio}) of exposed and control rats (with over 99.99% confidence). However, for the 48 hr and 7 day post-exposure periods, there was no statistically significant difference between exposed rat Π_{ratio} and control rat Π_{ratio}. Thus, for the range of vapor concentrations and dosages investigated, complete recovery from a miotic response to VX vapor exposure in male and female rats occurred between 24 hr and 48 hr post-exposure.

3.1.2 Probit Analysis of Miotic Response.

The Π_{ratio} values (1-hr post-exposure) were converted into binary data, with ($\Pi_{ratio} \leq 0.50$) being the criteria for miosis. The resulting binary data is listed in Table 4. A probit analysis was then performed (using Equation [7]) on the binary data, and median effective concentrations (EC_{50}) and dosages (ECT_{50}) for miosis were calculated for each gender-exposure

duration combination (Table 1). For comparison, the values for GB (Mioduszewski *et al.*, 2002) and GF (Whalley *et al.*, 2004) are also included in Table 1 and Figure 12.

The probit slope for concentration (k_C) was found to equal 5.2, with a 95% confidence interval of 3.9 to 6.5. The ECT_{50}'s of the male and female rats were statistically significant (with at least 95% confidence) from each other at all exposure durations, with the female rats being more sensitive. The ratio of ECT_{50}'s (male to female) was found to range from 1.4 to 2.2.

3.1.3 Analysis of Time-Dependence of ECT_{50} (Miosis).

The effect of exposure duration on the miotic response was investigated via ordinal logistic regression using Equation [8]. Ternary miosis data (1-hr post-exposure) was generated for the analysis (Table 5) using the following categories:

Score of 0: $\Pi_{ratio} \leq 0.50$	number of exposed rats in group: 90
Score of 1: $0.50 < \Pi_{ratio} \leq 0.84$	number of exposed rats in group: 135
Score of 2: $0.84 < \Pi_{ratio}$	number of exposed rats in group: 75

A ternary scoring system was found to give the best regression fit to the quantal data. The following normit fits (Equation [8]) were obtained for the boundaries between scores 0 to 1 (Equation [9]) and scores 1 to 2 (Equation [10]), respectively.

$$Y_N\{0\} = (5.1107) + (3.5946)(\log_{10} C) + (2.1772)(\log_{10} T) + (-0.2984)\,Sex \quad [9]$$

$$Y_N\{1\} = (6.8141) + (3.5946)(\log_{10} C) + (2.1772)(\log_{10} T) + (-0.2984)\,Sex \quad [10]$$

A plot of Equation [9] is compared to the ECT_{50}'s plotted in Figure 13. The toxic load exponent value (identical for Equations [9] and [10]) equals (3.5946 / 2.1772) or 1.65 $\pm$ 0.092 SE (Table 8). The 95% confidence interval for the exponent value is 1.47 to 1.83. Since this interval does not overlap 1, the toxic load exponent for miosis is different from 1 (with at least 95% statistical significance). Therefore, a toxic load model better describes the time-dependence of the probability of miosis than does Haber's Rule. Also, gender (*Sex*) was found to be statistically significant with the female rats being more sensitive by a factor of 1.46.

Potential lack of fit for the toxic load model was tested by adding the term $(\log_{10}T)^2$ to Equation [8] to test for curvature. It was found that this term was statistically significant (with 99.7% confidence). Thus, there is significant curvature, and the toxic load model does not completely explain the time dependency of the miotic response relationship. However, the toxic load model is still a better alternative for explaining the data than Haber's Rule.

3.1.4 Toxic Load Model for Pupil Diameter.

The ordinal regression approach to the analysis of the pupil diameter data (Section 3.1.3) allowed for development of a model for pupil diameter as well as a model for the fraction of rats with miosis (Whalley *et al.*, 2004). Equation [11] determines the combinations of exposure concentration and duration that yield the pupil diameter for the median rat:

$$Y_N\{\Pi_{Ratio}\} = (-3.0003) - (2.1103)(\log_{10} C) - (1.2781)(\log_{10} T) + (0.1752)\,Sex \qquad [11]$$

Equation [11] is the result of dividing the constants in Equation [9] by (-1.7034) (which is derived from the constants of Equations [9] and [10], or [{-1} x {6.8141 – 5.1107}]). The (-1) reflects the fact that as the concentration of agent increases, producing larger fractions of rats with miosis, the pupil diameter gets increasingly smaller.

3.2 Blood ChE Response.

Figures 14-16 show boxplots of the individual values for the AChE ratio of post- vs pre-exposure values (adjusted for controls) (α_{ratio}) for observations made 1 hr post-exposure for each exposure duration (10, 60 and 240 min). Each box represents the data from one vapor exposure, with the estimated 10^{th}, 25^{th}, 50^{th}, 75^{th} and 90^{th} percentiles displayed. However, in one instance, results from two exposures involving rats exposed for 10 min were combined together into one box (Figure 14) because of the closeness of the two vapor concentrations values (0.00830 and 0.00832 mg/m^3). Percentiles were calculated using the method outlined by Prins (2003).

Boxplots for the BChE ratio of post- vs pre-exposure values (adjusted for controls) (β_{ratio}) for observations made 1 hr post-exposure are shown in Figure 17 for all three exposure durations combined. In several instances results from two or three exposures were combined together into one box because of the closeness of the vapor concentrations.

3.2.1 Analysis of Variance for Blood ChE Response.

ANOVAs were performed separately on the AChE and BChE 1-hr post-exposure data. In the case of the BChE data, no statistically significant difference was found between the ratio values (β_{ratio}) of the exposed and control rats at any of the exposure durations (Figure 17). For the AChE data, there were statistically significant differences between the ratio values (α_{ratio}) of exposed and control rats (with over 99.9% confidence) at each of the three exposure durations (10, 60, and 240 min). The significant depression occurred at the highest vapor concentrations within each duration (Figures 14-16).

3.2.2 Probit Analysis of AChE Response.

The α_{ratio} values (1-hr post-exposure) were converted into binary data, with ($\alpha_{ratio} \leq 0.50$) being the classification for the existence of AChE depression. The resulting binary data are listed in Table 4. A probit analysis was then performed (using Equation [7]) on the binary data, and median effective concentrations (EC_{50}) and dosages (ECT_{50}) for AChE depression were

calculated for each exposure duration (Table 6). The ECT_{50}'s for AChE depression from VX are shown in Figure 18 as a function of exposure duration.

The probit slope for concentration (k_C) was found to equal 3.7, with a 95% confidence interval of 2.3 to 5.1. The ECT_{50}'s of the male and female rats were not statistically different from each other at each of the three exposure durations (10, 60, and 240 min).

3.2.3 Analysis of Time-Dependence of ECT_{50} (AChE Depression).

The effect of exposure duration on the AChE depression response in rats exposed to VX vapor was investigated via binary logistic regression using Equation [8]. The binary data (Table 4) were used in the analysis. The following normit fit was obtained:

$$Y_N = (3.5240) + (3.2388)(\log_{10} C) + (2.0605)(\log_{10} T) + (-0.2140)\,Sex \qquad [12]$$

Equation [12] has been plotted in Figure 18 along with the ECT_{50}'s for AChE depression. The toxic load exponent value equals (3.2388 / 2.0605) or 1.57, with a standard error of 0.14 (Table 8). Thus, the 95% confidence interval for the exponent value is the range from 1.29 to 1.85. Since this interval does not overlap 1, the toxic load exponent for AChE depression is significantly different (with at least 95% statistical significance) from 1. Therefore, the toxic load model better describes the time-dependence of the probability of AChE depression than Haber's Rule.

Potential lack of fit for the toxic load model was tested by adding the term $(\log_{10}T)^2$ to Equation [8] to test for curvature. It was found that this term and its interaction with *Sex* were not statistically significant. Thus, there is no significant curvature and the toxic load model adequately explains the time dependency of the AChE response relationship.

3.2.4 Gender Differences in AChE Depression.

The p-value for *Sex* in Equation [12] equals 0.066, or in other words, the null hypothesis (i.e., no difference in AChE depression exists between the genders) can be rejected with 93.4% confidence. If exposure duration is treated as a factor instead of a covariate in Equation [12], the p-value for *Sex* drops to 0.049 (or 95.1% confidence for rejecting the null hypothesis). From the probit analysis results (Section 3.2.2), the p-value was not close to being < 0.05. So, the statistical significance of the *Sex* term in general is marginal, but it is enough to support the reporting of separate ECT_{50}'s for the two genders.

3.3 Comparison of Miotic and AChE Responses.

A comparison is shown in Figure 19 of the toxic load relationships for miosis and AChE depression (Equations [9] and [11], respectively) in rats exposed to VX vapor. The toxic load exponents 1.65 (miosis) and 1.57 (AChE depression) are represented by the slopes of the lines in Figure 19. The lines are nearly parallel and the toxic load exponents, therefore, are not significantly different from each other. Also, the ECT_{50}'s for miosis in male and female rats are lower in value than those for AChE depression (Tables 1 and 6). To illustrate miosis as the FNE

of VX exposure, a contingency table (Table 7) was prepared, to investigate through non-parametric means which endpoint is likely to be observed first in rats. Each of the 300 exposed rats were divided into four categories based upon their paired binary responses for miosis ($\Pi_{ratio} \leq 0.50$) and AChE depression ($\alpha_{ratio} \leq 0.50$):

(1) Rat shows neither miosis nor AChE depression (203 rats)
(2) Rat shows miosis but not AChE depression (62 rats)
(3) Rat does not show miosis but does show AChE depression (7 rats)
(4) Rat shows miosis and AChE depression (28 rats)

Of particular interest are Categories (2) and (3) containing 69 rats total--rats only showing one of the two possible signs. There were 62 rats showing miosis without AChE depression and 7 rats with AChE depression without miosis. Therefore, miosis occurred without AChE depression 90% of the time in those rats showing only one sign or the other.

3.4 Fluoride Ion Generated VX-G Analog in Blood Plasma.

Figures 20-22 summarize the results of the VX-G analog assay of the blood plasma from exposed rats. Levels of fluoride ion-generated, nerve agent biomarkers in rat plasma are typically one to two orders of magnitude greater than in the RBC fraction. The VX-G analog was not seen in the initial RBC samples tested. Since sample volume from rats is limited, miosis level biomarker studies are problematic for the RBC fraction. Some samples were pooled at the lowest exposure concentrations to have a large enough sample to quantify. Because we were attempting to quantify the VX-G in the RBC samples on the periphery of our detection limits and had limited sample volumes we halted further testing of the RBC fraction of the samples.

4. DISCUSSION

Over the past several years much of the work in our laboratory has focused on establishing ECT_{50}'s as a function of exposure duration for miosis-producing levels of GB (Mioduszewski *et al.*, 2002a; Hulet *et al.*[*]) and GF (Whalley *et al.*, 2004) vapor. The results of the current study on low-level VX vapor exposures add to this database and directly establish ECT_{50}'s for VX miosis in rats (Table 1). These newly established ECT_{50}'s for VX may reduce the need for relative potency analysis using other nerve agents such as GB to establish toxicity levels for VX inhalation exposures (Hartmann, 2002). Table 1 and Figure 12 offer a direct potency comparison for VX, GB and GF at exposure durations of 10, 60, and 240 min. When comparing the ECT_{50}'s for these 3 chemical warfare (CW) agents, it must be noted that the GF (Whalley *et al.*, 2004) and present study used infrared pupillometry to assess pupil diameter whereas the miosis level GB study (Mioduszewski *et al.*, 2002a) used a different methodology.

[*]Hulet, S.W.; Sommerville, D.R.; Benton, B.J.; Forster, J.S.; Manthei, J.H.; Miller, D.B.; Scotto, J.A.; Jarvis, J.R.; Way, R.A.; Muse, W.T.; Gaviola, B.; Burnett, D.; Crosier, R.B.; Mioduszewski, R.J.; Thomson, S.A. *Low-Level Sarin Vapor Exposure in the Gottingen Minipig: Effect of Exposure Concentration and Duration on Pupil Size*, Unpublished Data, 2004.

VX potency for male rats at all exposure durations - ranges between 7.9 and 13.0 times greater than GB. For female rats, VX potency ranges between 9.3 and 11.3 times greater than GB. Similarly, for comparisons of VX to GF in male rats, VX potency ranges 11.0 to 18.1 times more potent than GF, while for females, VX potency ranges between 10.9 and 15.5 times more potent than GF.

In addition to potency comparisons based upon similar exposure duration, comparison of the toxic load exponents offers insight into whether relative potency between two agents is constant with respect to duration. If there is no statistically significant difference between a pair of exponent values, then the relative potency is constant. The miosis toxic load exponent values corresponding to the studies in Table 1 as well as Hulet *et al.*,* are presented in Table 9. Among the rat miosis studies, the exponent values (n_{all}) of GB and VX are significantly different from each other. Therefore, VX is increasingly more potent than GB as exposure duration increases. The exponent value for GF lies in between those of GB and VX and its value is not significantly different from either that of GB or VX.

The need to develop toxic load exponents (n) in the equation ($C^n \times t = k$) arose because Haber's Rule failed to adequately describe the relationship between concentration and time and the net effect this interaction had on cumulative toxicity levels. It should be recognized that the toxic load relationship is based more on empirical observations than on basic biological theories (Fairhurst and Turner, 1993; Griffiths, 1991; Sommerville *et al.*, 2004**). It should not be a surprise for an empirical model to provide a poor fit when extended over too wide a range. In all of the ECBC miosis studies performed to date (Mioduszewski *et al.*, 2002a; Hulet *et al.;* Whalley *et al.*, 2004; and the present study), significant upward curvature was found in the relationship between effective median dosages (ECT_{50}'s) and exposure duration as demonstrated in Figure 12. Thus, the toxic load model does not adequately explain the time dependency of the miotic response relationship (Section 3.1.3). There are three broad categories of possible explanations for the curvature in the ECT_{50} vs exposure duration relationships found in the GB, GF and VX rat miosis studies. First, such curvature is the natural relationship for the species-agent-endpoint systems under consideration. The next two categories presuppose that the toxic load model is the proper model and that any observed curvature from the model must be due to factors whose effect on miosis levels is not constant with respect to exposure duration. For instance, certain behavioral and physiological factors (i.e., changes in activity, sleep, reduced minute volume, closed eyelids, etc.) are likely to have more pronounced effects on the observed levels of miosis in longer duration exposures. Lastly, the same duration-dependent effect could possibly result from differences in pupil size measurement methodologies (single measurement, 1 hr post exposure (ECBC rat studies) vs continuous real-time measurements (ECBC GB minipig study, Hulet *et al.*)*. In the rat studies, there was potential for recovery from miosis

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*Hulet, S.W.; Sommerville, D.R.; Benton, B.J.; Forster, J.S.; Manthei, J.H.; Miller, D.B.; Scotto, J.A.; Jarvis, J.R.; Way, R.A.; Muse, W.T.; Gaviola, B.; Burnett, D.; Crosier, R.B.; Mioduszewski, R.J.; Thomson, S.A. *Low-Level Sarin Vapor Exposure in the Gottingen Minipig: Effect of Exposure Concentration and Duration on Pupil Size*, Unpublished Data, 2004.

**Sommerville, D.R. Relationship Between the Dose Response Curves for Lethality and Severe Effects for Chemical Warfare Nerve Agents. In *Proceedings of the 2003 Joint Service Scientific Conference on Chemical & Biological Defense Research*, 17-20 November 2003; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, Unpublished Data, 2004.

during the time following the conclusion of the CW agent exposures until the rats' eyes were photographed for the presence of miosis (< 60 min post exposure).

These last two categories have important implications for risk assessment applications of the ECBC studies. Figure 23 illustrates this point with plots of two ECT_{50} extrapolations based upon the female rat VX miosis data (taken from Figure 12). The extrapolation based on the 10- and 60-min ECT_{50} values produces lower (more conservative) ECT_{50} estimates at longer durations than a similar extrapolation that is based upon the 60- and 240-min ECT_{50} values. From a risk assessment perspective, the former has an implicit built-in safety factor. Expanding this approach, a second set of toxic load exponents (n_{short}) was calculated for GB, GF and VX miosis (Table 9) based on a reanalysis of the original data with exposure durations greater than 100 min excluded. Thus, with the uncertainty about why the curvature exists, more weight should be given to using the more conservative n_{short} values rather than the n_{all} values when attempting to establish a human toxic load exponent for miosis based upon the data in Table 9. Moreover, estimates for a lethality toxic load exponent value should not be based upon the results of experimental miosis studies, since it can be expected that with different endpoints would come differing degrees of curvature.

The need to establish separate ECT_{50}'s for males and females at each exposure duration is the result of the female rats being significantly more sensitive to VX vapor. The differential sensitivity to this OP was consistent with similar findings from recent GB (Mioduszewski *et al.,* 2002a,b) and GF (Whalley *et al.,* 2004) rat studies. In addition, there are numerous studies which show that the actions of a variety of other drugs such as amobarbital and nicotine (Holck *et* al., 1937), and strychnine and picrotoxin (Kato *et al*, 1962a,b), to name a few, are more pronounced and/or persist longer in female rats than in male rats (Kato, 1974). In many instances, gender differences in drug sensitivity appear to be mediated at least in part by androgens present in male rats that can increase 2 to 3 times the activities of drug-metabolizing enzymes in liver microsomes (Booth and Gillette, 1962). The present study was tracking low-level VX vapor-induced miosis, considered the result of a localized depression of AChE. This localized effect might preclude involvement of liver microsome mediated changes in VX toxicity but there are also other gender differences related to the eye that may indirectly alter the local response to CW agents. There is evidence of structural dimorphism of the lacrimal gland (Sullivan *et al.*, 1990) as well as differences in the quantities and activity levels of various enzymes associated with the lens of the rat eye (Bours *et al.,* 1988). These gender differences in rats are well documented and mentioned here as a means of emphasizing the point that males and females of any mammalian species but especially rats, cannot be assumed to have the same thresholds for response when documenting biological endpoints. Whatever the reason(s) for the increased sensitivity of female rats to VX vapor, the larger issue is whether these gender differences in the rat are relevant to humans.

The finding that exposure to the highest concentrations of VX vapor at the three exposure durations produced significant whole blood AChE depression (Figures 14-16) was divergent from previous results obtained with miosis-producing levels of GB (Mioduszewski *et al.,* 2002a) and GF (Whalley *et al.,* 2004) in rats. In those studies, there was not any significant depression of AChE, carboxylesterase (CaE), or BChE following exposure to GB or GF. A possible explanation for AChE depression after exposure to miosis producing levels

of VX vapor is that VX has a higher binding affinity for AChE than does GB or GF. In a study looking at the effectiveness of CaE protection against the toxicity of OP compounds such as VX, soman, sarin, and tabun, Maxwell (1992) found VX was the most specific *in vitro* inhibitor of AChE while showing very little affinity for CaE. In the present study, gender differences in the degree of AChE depression were minimal but may have been masked by the large variability in baseline AChE activity levels within and between individual rats. Also, the fact that significant depression only occurred at the highest dosages of VX used in this study limited the number of groups available for statistical comparison.

Since this study used whole body inhalation exposures, a potential confounding effect of testing a low volatility compound such as VX was the possibility of delayed toxicity effects due to percutaneous absorption. To account for agent deposition on the surface of the animal, a pilot study was performed in which two groups of rats (8-9 rats per group) were exposed to whole-body VX vapor concentrations of 0.08-0.09 mg/m^3 for 240 min. These conditions represented the maximal output for VX from a "saturator cell" type generator (Figure 4). After exposure, surviving rats were euthanized and VX was extracted from the surface of the rat using whole-body immersion into jars containing isopropanol. The average amount of VX recovered per rat from the combined results of groups 1 and 2 for whole-body extraction was 13.8 ± 3.2 µg as determined by GC-MS. This amount of surface deposition of VX represents < 1/10 of 1% of the entire exposure dose. This exposure dose represented a "worst case" scenario. The highest exposure dose actually used in this study was 0.003 mg/m^3 for 240 min (0.72 $mg\text{-}min/m^3$). From post exposure observations of all exposed rats in this study, we have concluded that there were no observable delayed toxicity effects such as tremors, salivation or convulsions. Figure 11 illustrates our findings that any delayed miotic effects were minimal. In addition, statistical analysis of all 5 post exposure observation periods revealed that the greatest miotic response occurred at the 1 hr post exposure observation period. Also, for the range of vapor concentrations and dosages investigated, complete recovery from a miotic response to VX vapor exposure in male and female rats occurred between 1 and 2 days post-exposure.

Previous studies in our laboratory with GB (Mioduszewski *et al.*, 2002a) and GF (Whalley *et al.*, 2004) have shown miosis to be the FNE resulting from a whole body inhalation exposure. In these studies, there was no significant ChE depression in either the plasma or RBC components of the blood. In contrast, the current study with VX has identified significant AChE depression at the higher dosage levels (CT) of each exposure duration. To determine if AChE depression could be expected to occur prior to miosis, we used Equation [7] (Section 2.8) to perform a probit analysis and calculated ECT_{50}'s for AChE depression of whole blood (Section 3.2.2). Tables 1 and 6 list the ECT_{50}'s for miosis and AChE depression, respectively. ECT_{50} values for miosis are approximately 1.5 to 2 times lower than those for AChE depression. Therefore, miosis would be expected to occur first. Also, Table 7 lists the total number of VX-exposed rats and the numbers that had miosis and AChE depression. Thirty percent of all exposed rats were miotic while 12% of all exposed rats had AChE depression. Twenty one percent of all the exposed rats had miosis without AChE depression while 2% of all the exposed rats had AChE depression without miosis. Miosis would, therefore, likely be the FNE at low dosages of VX.

There were quantifiable amounts of bound VX found in the blood plasma. Since VX has a very low binding affinity for CaE, the most likely sources of bound VX in the plasma are AChE (Silver, 1974) and non-acetylcholinesterase binding sites such as BChE. Even though the levels of BChE in the whole blood did not show any significant depression (Figure 17), the greater detection limits of the VX-G assay can account for the levels of VX-G found in the plasma.

Figures 20-22 demonstrate a general increase in VX-G analog in the plasma with increasing dose. This was especially noticeable in the 240-min exposure data (Figure 22). Scatter was seen at the lower exposure durations (10 and 60 min) and concentrations (Figures 20 and 21), possibly due to being close to the detection limit for quantification. It is also possible that the scatter seen in the lower exposure concentrations is a function of the variable nature of VX absorption during short exposure durations. A detailed account of the results of the VX-G assay can be found in Jakubowski *et al.*[*]

5. CONCLUSIONS

This study filled some of the gaps in our understanding of the threshold toxic effects of low-level VX vapor exposure. The ECT_{50}'s were calculated for miosis and blood AChE inhibition resulting from 10, 60, or 240 min. whole-body vapor exposures. In contrast to that predicted using Haber's Rule, the ECT_{50}'s associated with miosis and AChE inhibition were not constant over time. Ordinal regression was used to develop empirical toxic load models ($C^{1.65}xT=k$ for miosis and $C^{1.57}xT=k$ for AChE inhibition) to describe the threshold effects of VX vapor dosage over time. Although female rats were more sensitive to the miotic effects of VX vapor than males in this study, the applicability of gender differences in the sensitivity to OP's in human populations is unresolved. Lastly, the VX-G analog assay successfully used rat blood plasma as a biomarker for VX exposure, finding a general relationship between increasing dosage (CT) and increasing amounts of VX-G in the plasma. Insofar as effects such as miosis may impact operational effectiveness, the results of the current study are critical to operational risk management. Data derived from this study are also essential for determining lower detection levels, how "dirty is clean enough" following decontamination, and when it is safe to come out of protective posture. The results of this study pertain only to miosis levels of VX exposure. Estimates for a lethality toxic load exponent value should not be based upon the results of experimental miosis studies, since it can be expected that with lethal concentrations of VX vapor, percutaneous effects would have a greater influence on the dose-response relationship.

[*]Jakubowski, E.M.; Benton, B.J.; Whalley, C.E.; Anthony, J.S.; Haley, M.V.; Manthei, J.H.; Way, R.A.; Burnett, D.C.; Gaviola, B.P.; Scotto, J.A.; Sommerville, D.R.; Crosier, R.B.; Edwards, J.L.; Evans, R.A.; McGuire, J.M.; Crouse, C.L.; Matson, K.L.; Mioduszewski, R.J.; Thomson, S.A. *The Inhalation Toxicity Testing of VX Vapor in Rats at Miosis Levels: VX Surface Contamination Analysis and Fluoride Ion Generated Product Determination*, Unpublished Data, 2004.

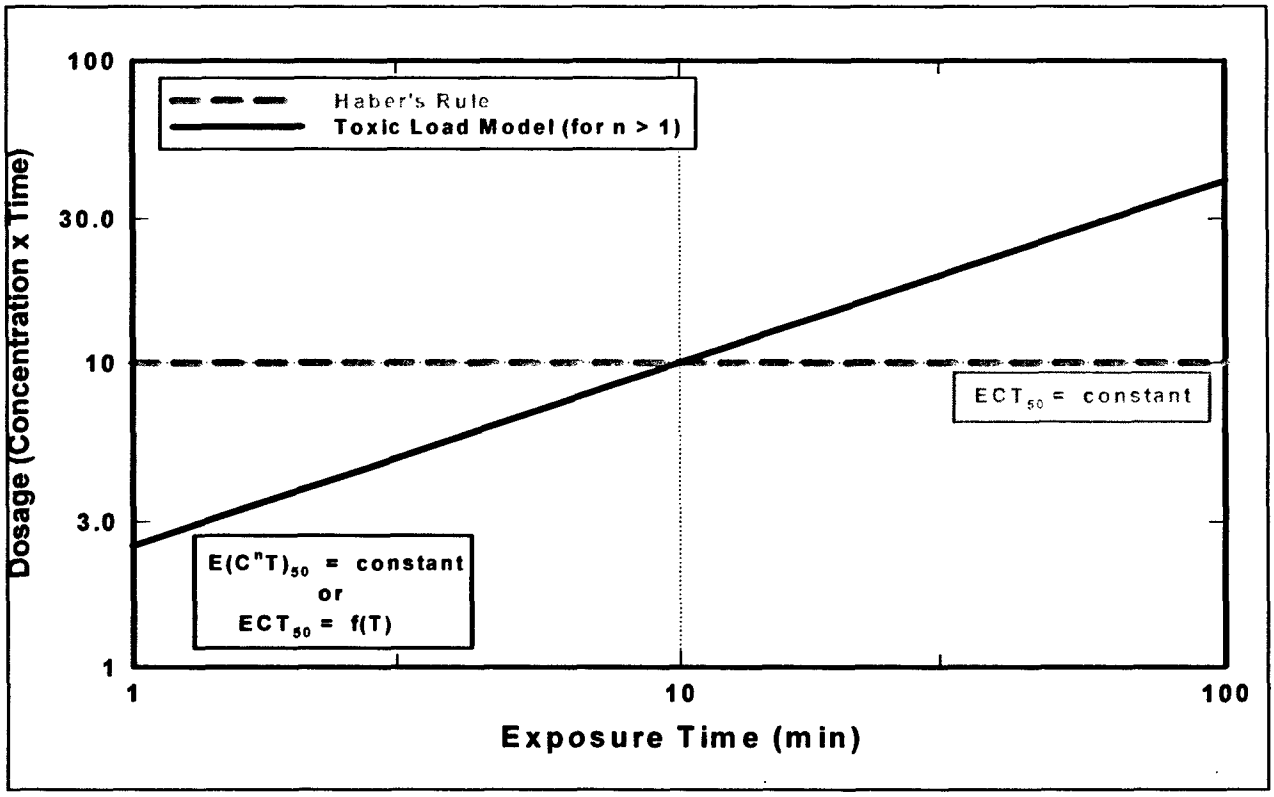
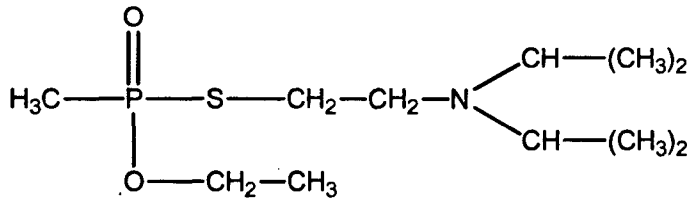


Figure 1. Comparison of Haber's Rule and Toxic Load Models for Toxicity Time Dependence



Mol. Wt. 267 g mol^{-1}

$C_{11}H_{26}NO_2PS$

(CAS Registry Numbers: 50782-69-9, 51848-47-6, 53800-40-1, 70938-84-0)

Figure 2. Structure of VX

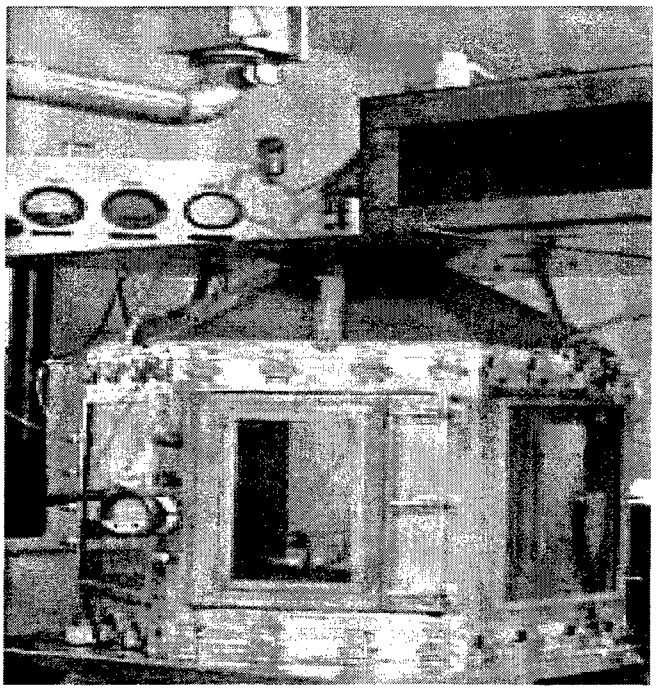


Figure 3. 750-L Rochester-Style Exposure Chamber

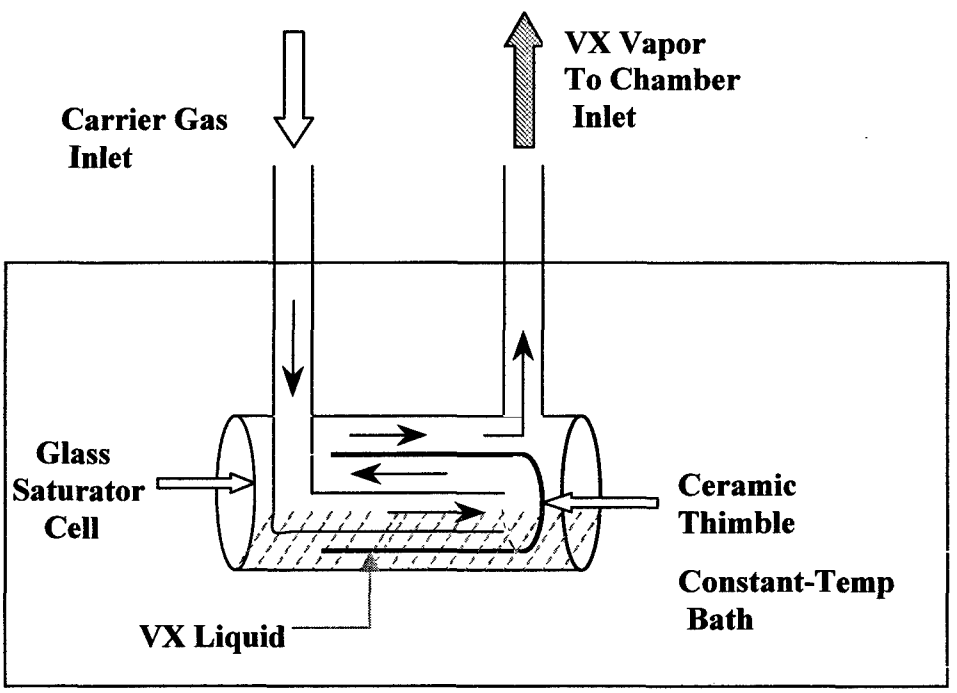


Figure 4. VX Vapor Generation Using a Saturator Cell

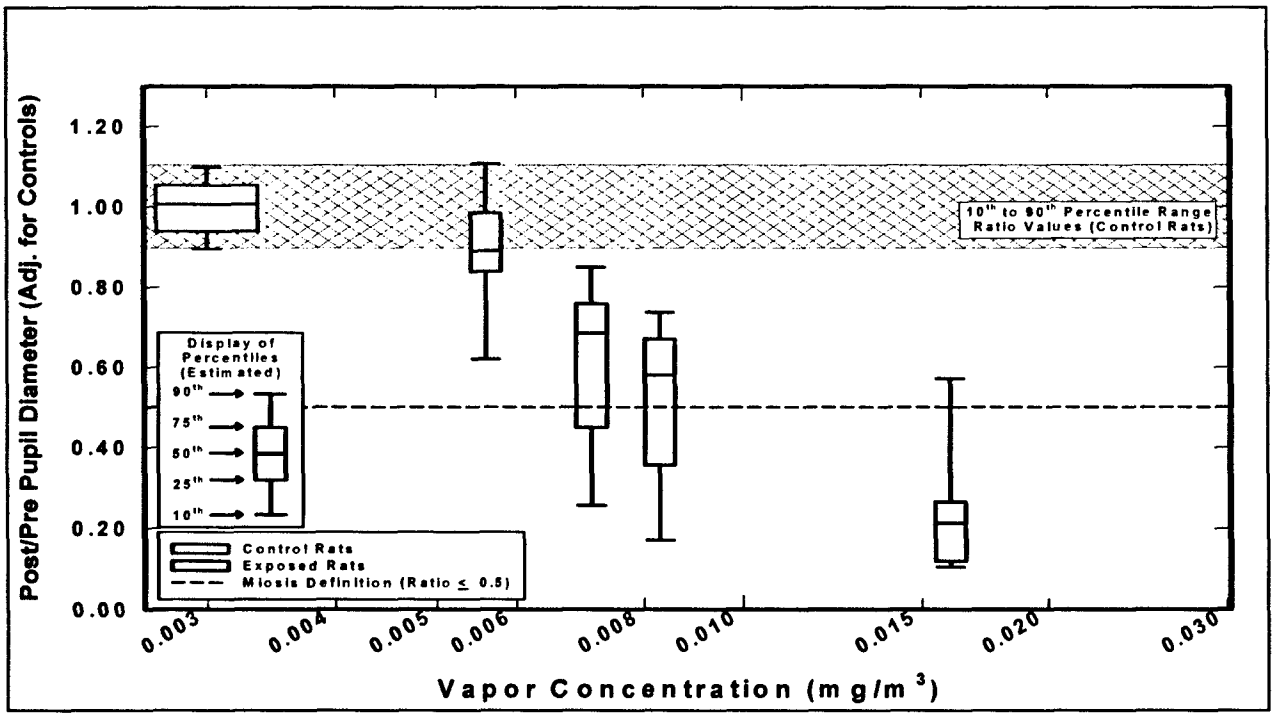


Figure 5. Box Plots of Ratio Values of Post (1 hr) to Pre-Exposure Pupil Diameter (Adjusted for Controls) in Male Rats Exposed to Fixed Concentrations of VX Vapor for 10 Min

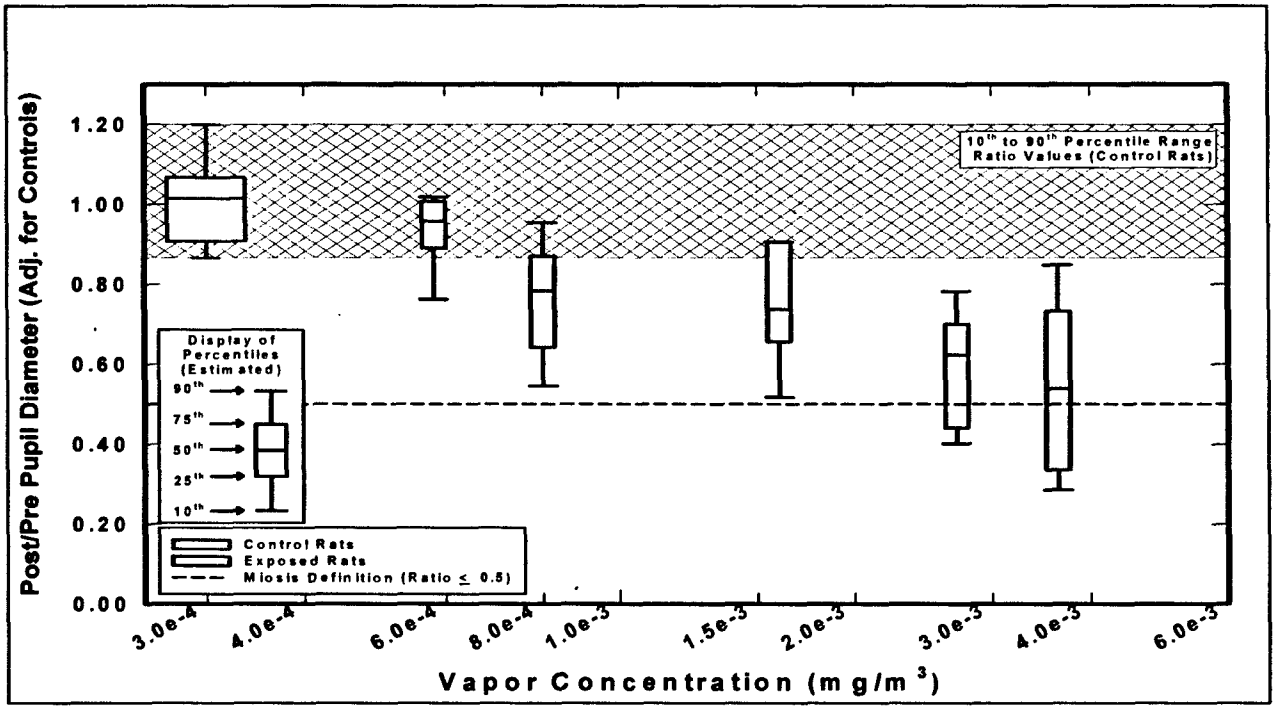


Figure 6. Box Plots of Ratio Values of Post (1 hr) to Pre-Exposure Pupil Diameter (Adjusted for Controls) in Male Rats Exposed to Fixed Concentrations of VX Vapor for 60 Min

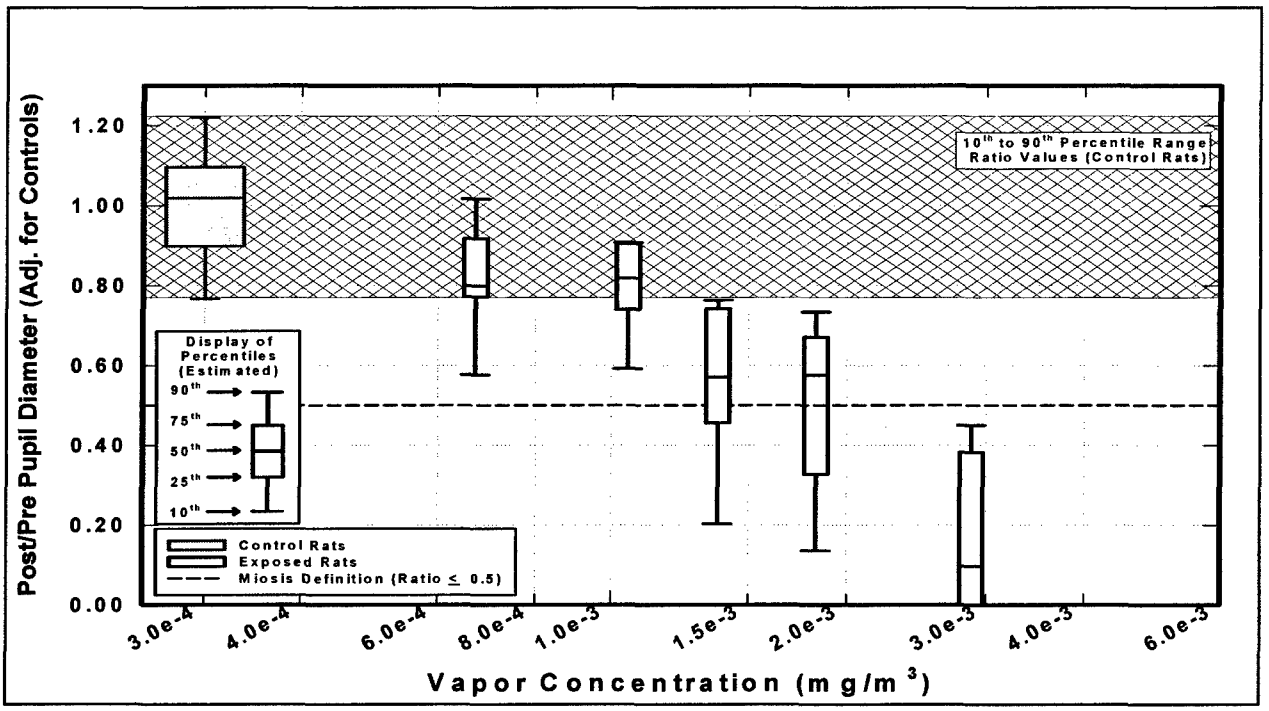


Figure 7. Box Plots of Ratio Values of Post (1 hr) to Pre-Exposure Pupil Diameter (Adjusted for Controls) in Male Rats Exposed to Fixed Concentrations of VX Vapor for 240 Min

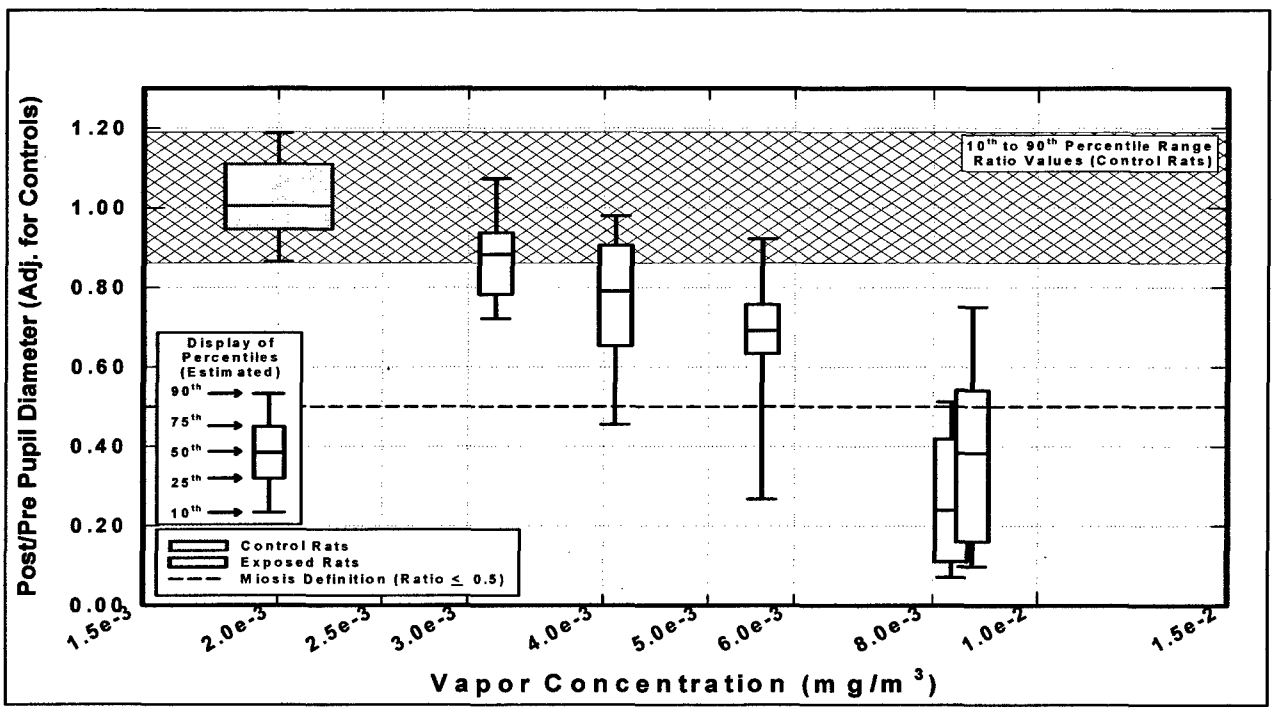


Figure 8. Box Plots of Ratio Values of Post (1 hr) to Pre-Exposure Pupil Diameter (Adjusted for Controls) in Female Rats Exposed to Fixed Concentrations of VX Vapor for 10 Min

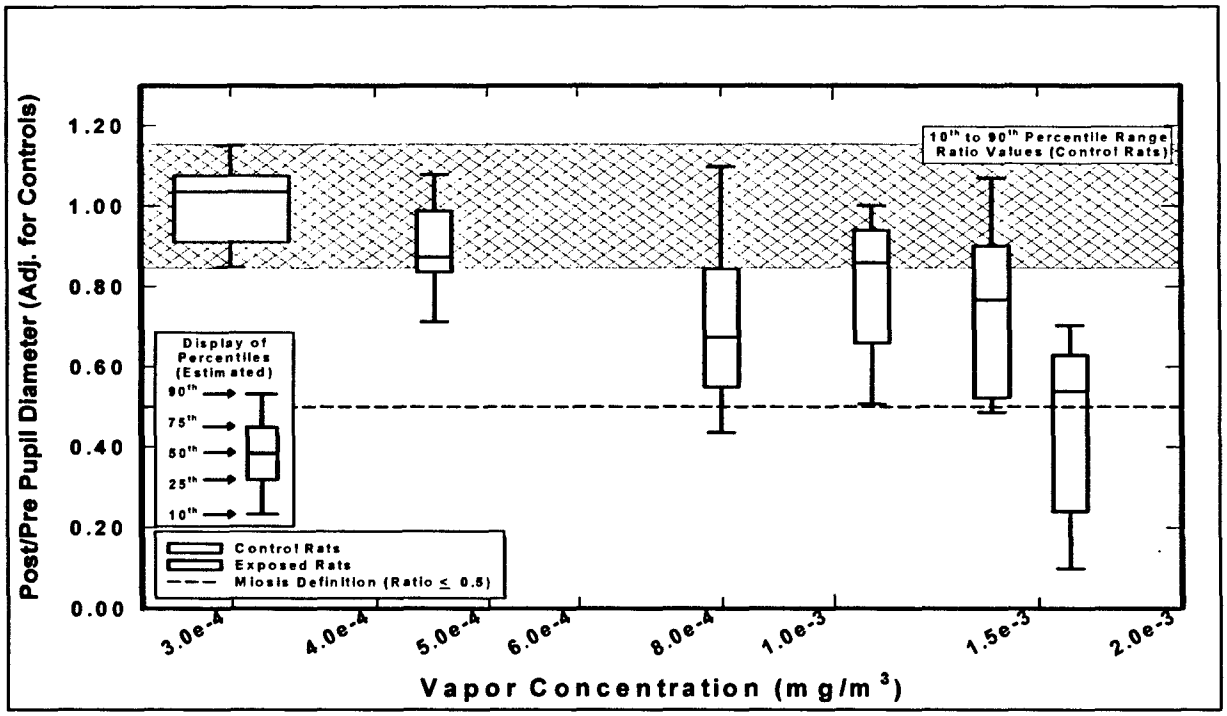


Figure 9. Box Plots of Ratio Values of Post (1 hr) to Pre-Exposure Pupil Diameter (Adjusted for Controls) in Female Rats Exposed to Fixed Concentrations of VX Vapor for 60 Min

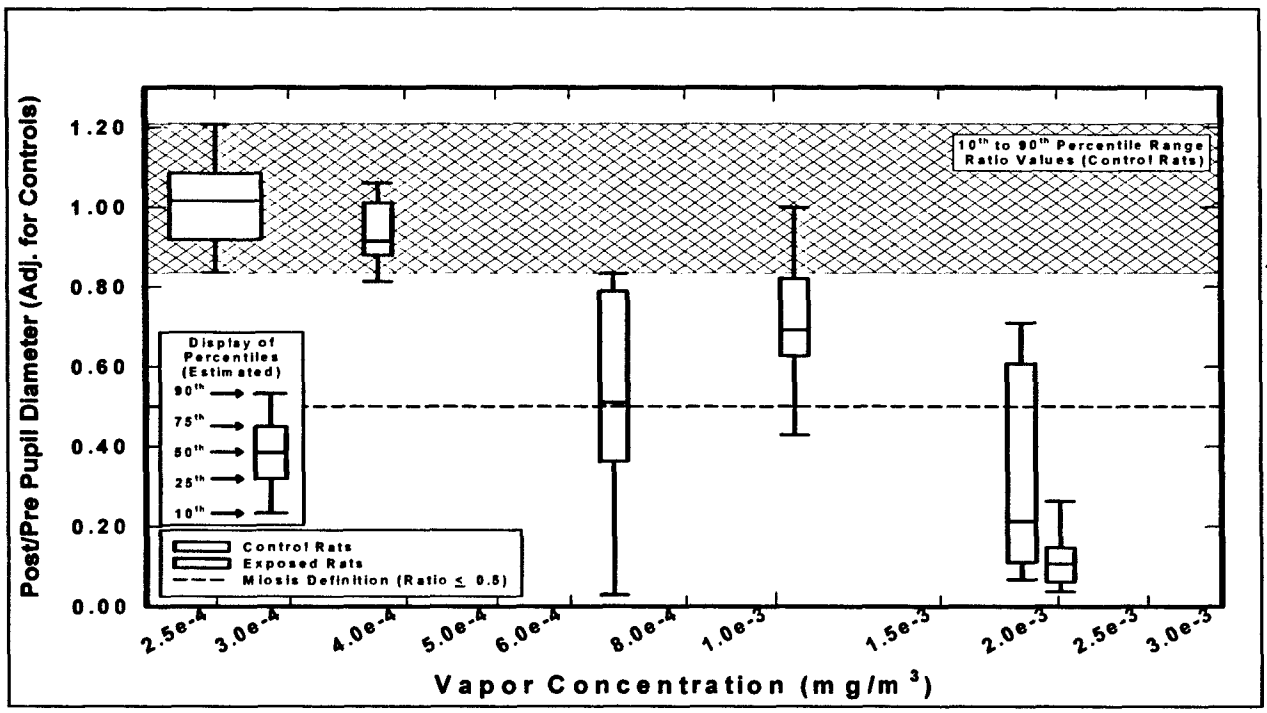


Figure 10. Box Plots of Ratio Values of Post (1 hr) to Pre-Exposure Pupil Diameter (Adjusted for Controls) in Female Rats Exposed to Fixed Concentrations of VX Vapor for 240 Min

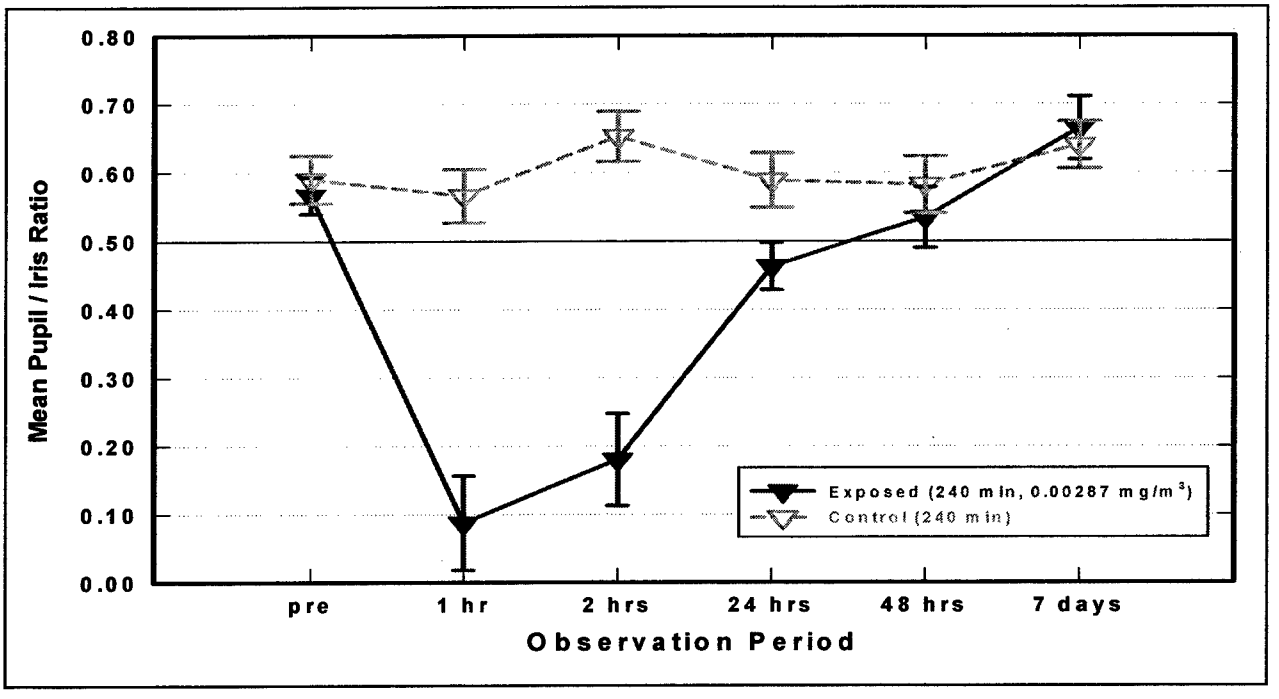


Figure 11. Representative Graph Showing Mean Pupil to Iris Ratio Values for Male Rats Exposed to VX Vapor for 240 Min

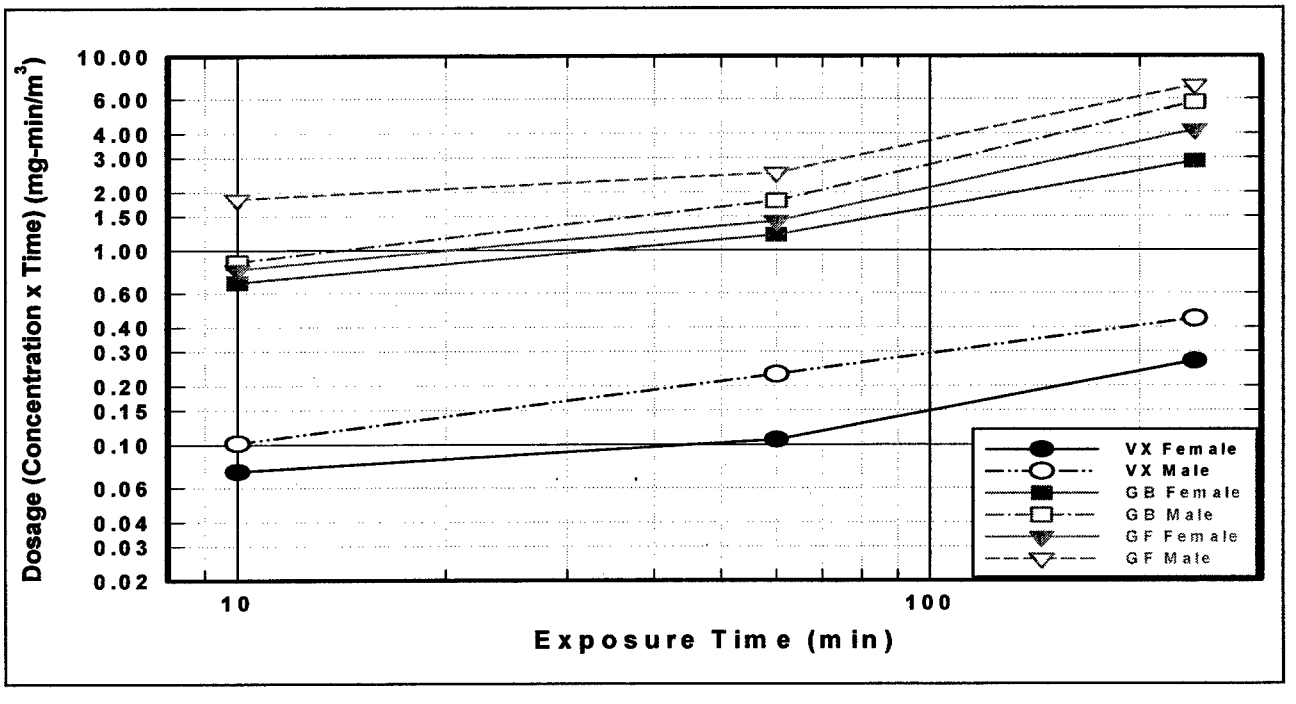


Figure 12. Comparison of ECT_{50} Estimates for Miosis in Rats for VX, GB, (Mioduszewski *et al.*, 2002) and GF (Whalley *et al.*, 2004) as a Function of Exposure Duration

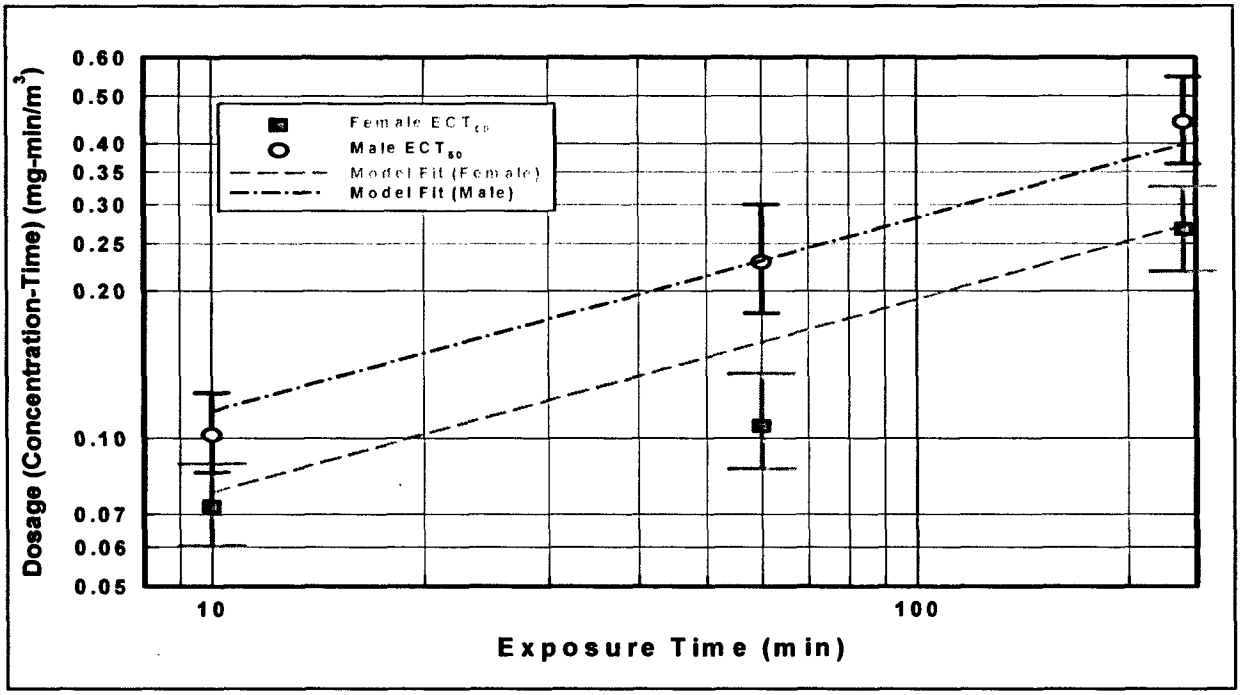


Figure 13. Comparison of Toxic Load Model Fit (Equation [9]) with VX ECT_{50} Miosis Estimates for Male and Female Rats (Equation [7]) as a Function of Exposure Duration

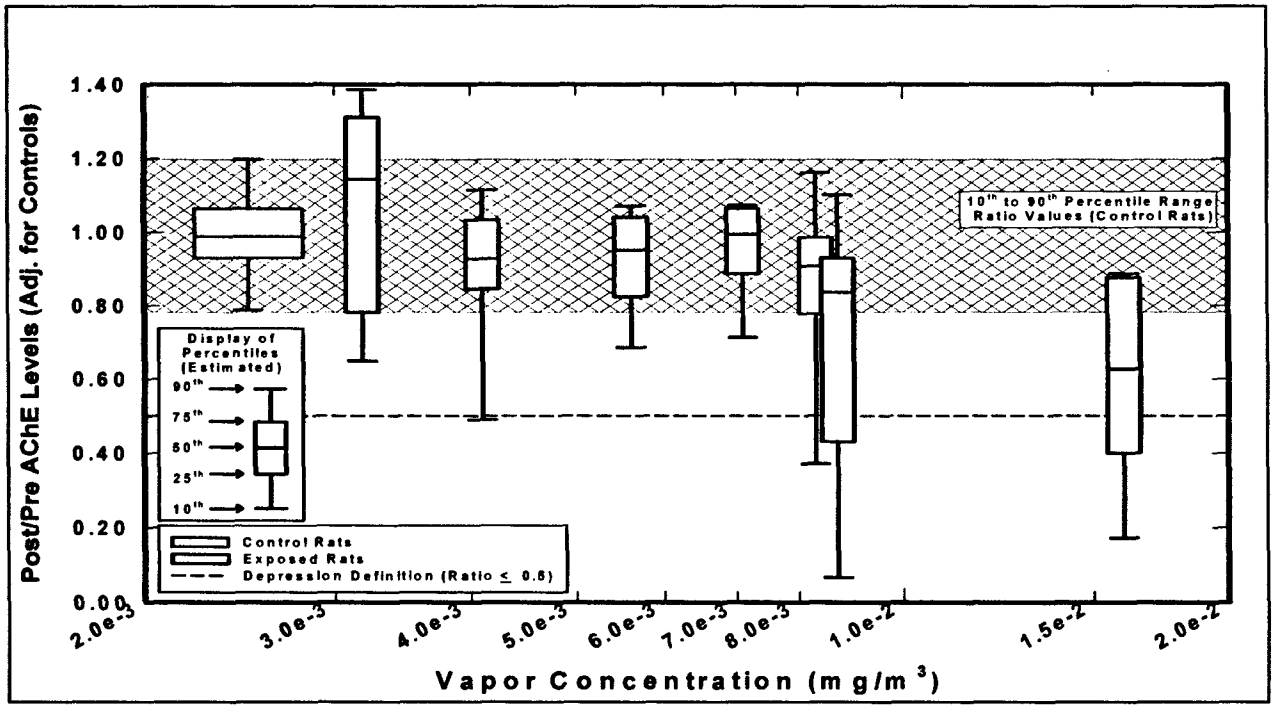


Figure 14. Box Plots of Ratio Values of Post (1 hr) to Pre-Exposure Blood AChE Levels (Adjusted for Controls) in Male and Female Rats Exposed to Fixed Concentrations of VX Vapor for 10 Min

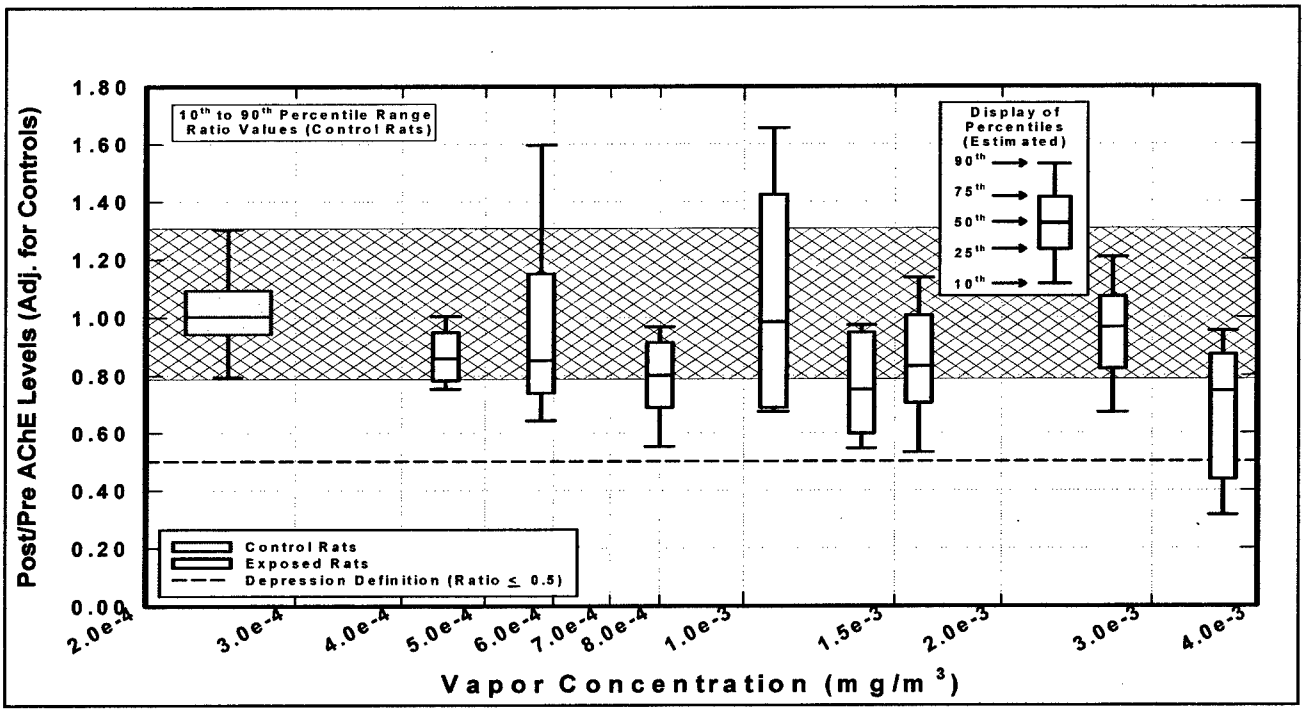


Figure 15. Box Plots of Ratio Values of Post (1 hr) to Pre-Exposure Blood AChE Levels (Adjusted for Controls) in Male and Female Rats Exposed to Fixed Concentrations of VX Vapor for 60 Min

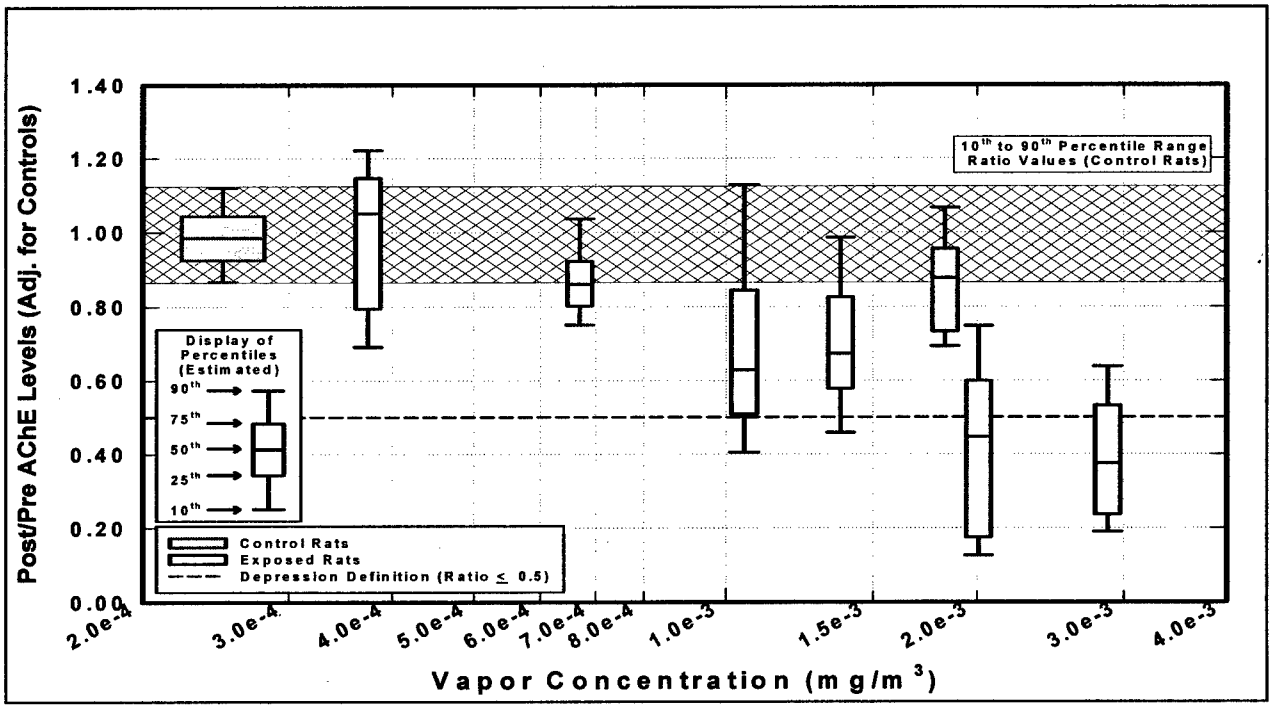


Figure 16. Box Plots of Ratio Values of Post (1 hr) to Pre-Exposure Blood AChE Levels (Adjusted for Controls) in Male and Female Rats Exposed to Fixed Concentrations of VX Vapor for 240 Min

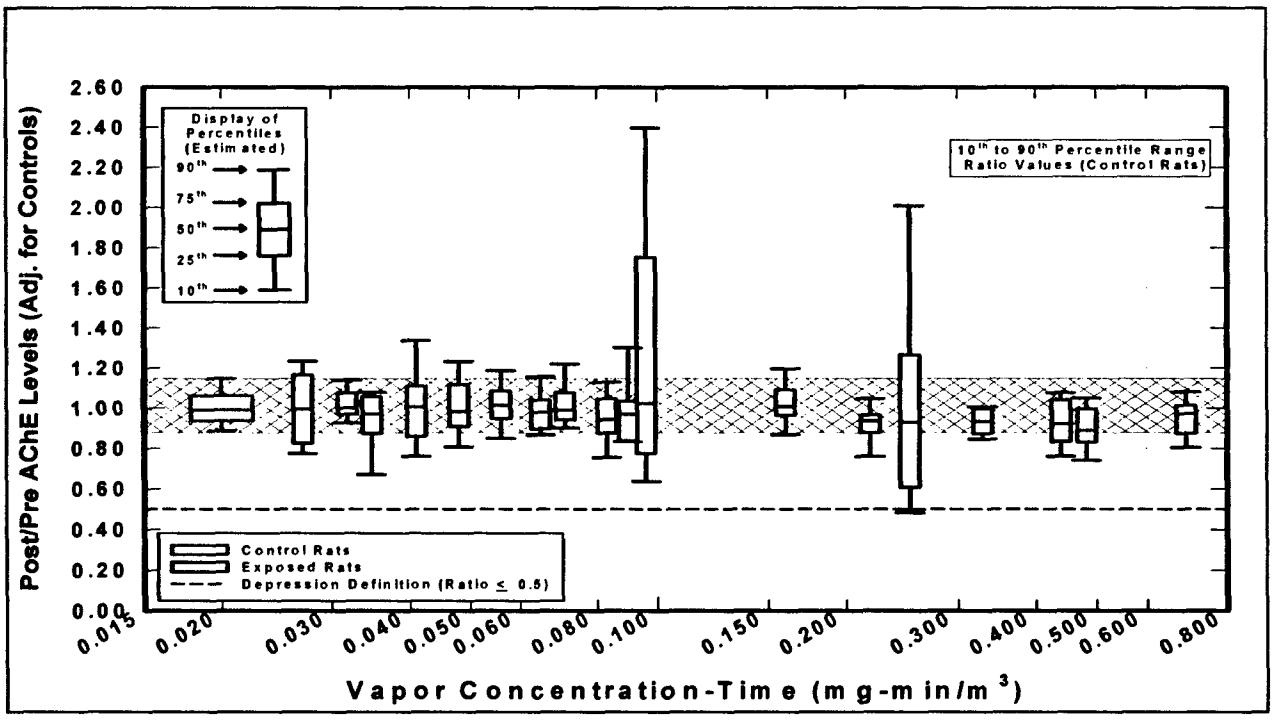


Figure 17. Box Plots of Ratio Values of Post (1 hr) to Pre-Exposure Blood AChE Levels (Adjusted for Controls) in Male and Female Rats Exposed to Fixed Concentrations of VX Vapor for 10, 60, and 240 Min

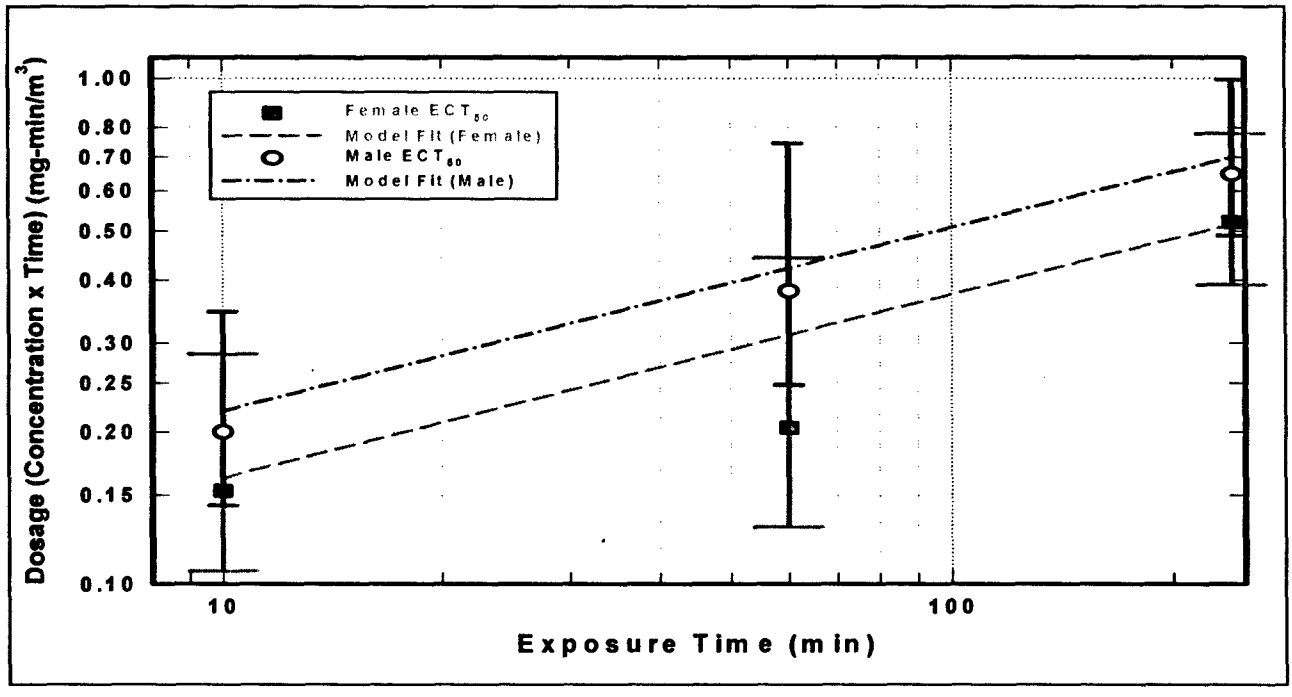


Figure 18. Comparison of Toxic Load Model Fit with ECT_{50} Estimates for AChE Depression for Male and Female Rats Exposed to VX as a Function of Exposure Duration

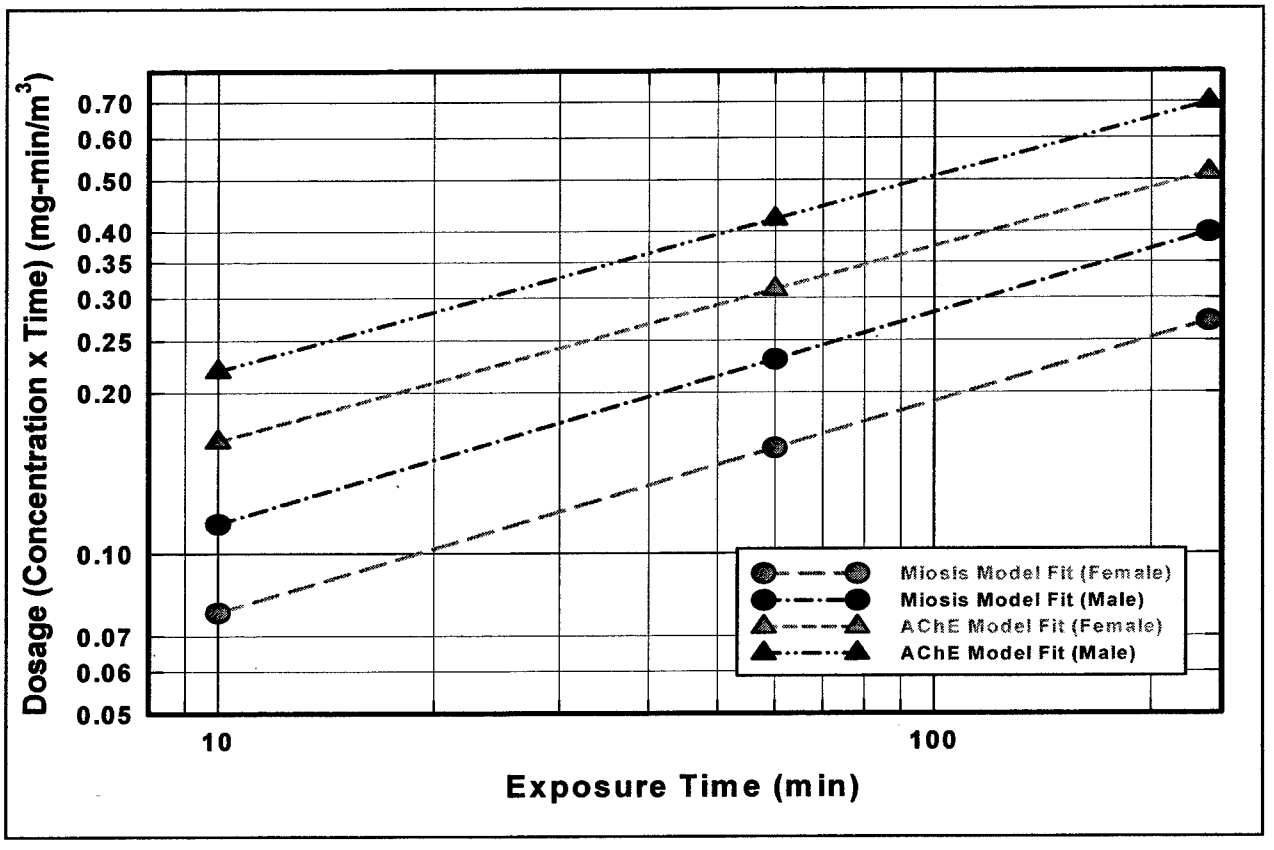


Figure 19. Comparison of Toxic Load Model Fits for Miosis and AChE Depression in Rats Exposed to VX Vapor as a Function of Exposure Duration

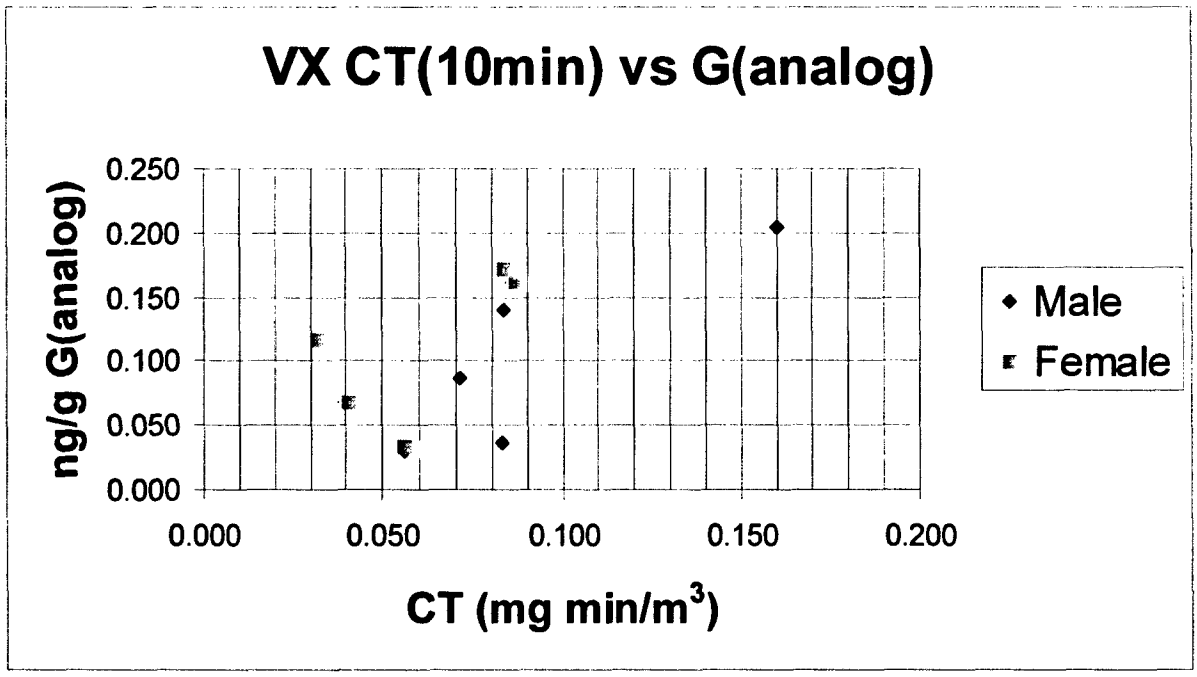


Figure 20. Average G-Analog Found in the Blood Plasma of Rats Exposed to Miosis Levels of VX for 10 Min

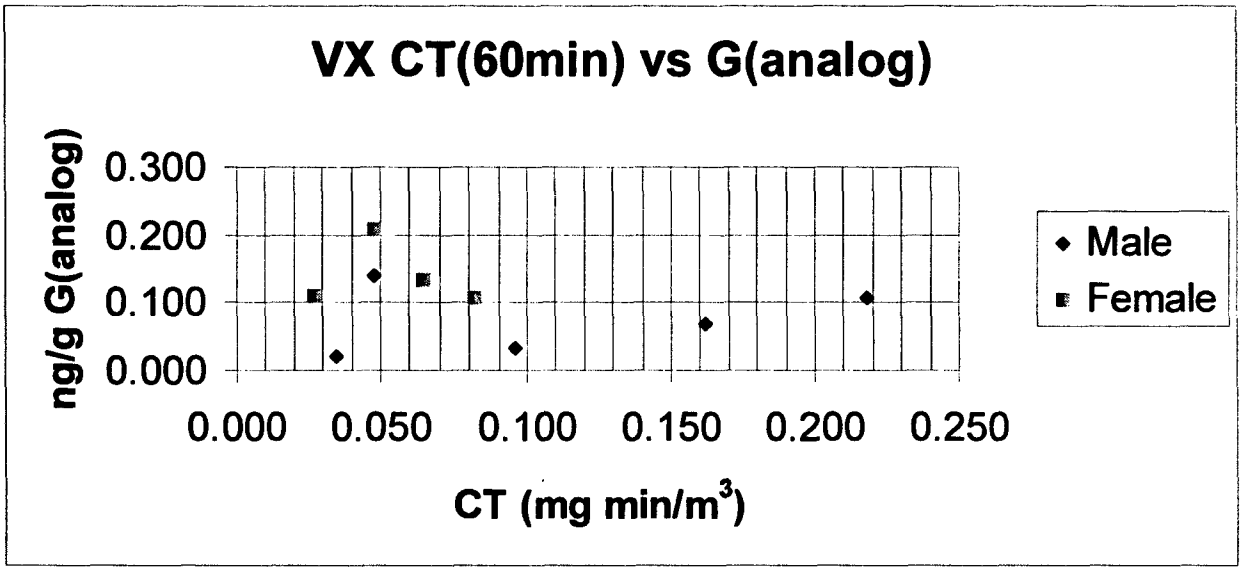


Figure 21. Average G-Analog Found in the Blood Plasma of Rats Exposed to Miosis Levels of VX for 60 Min

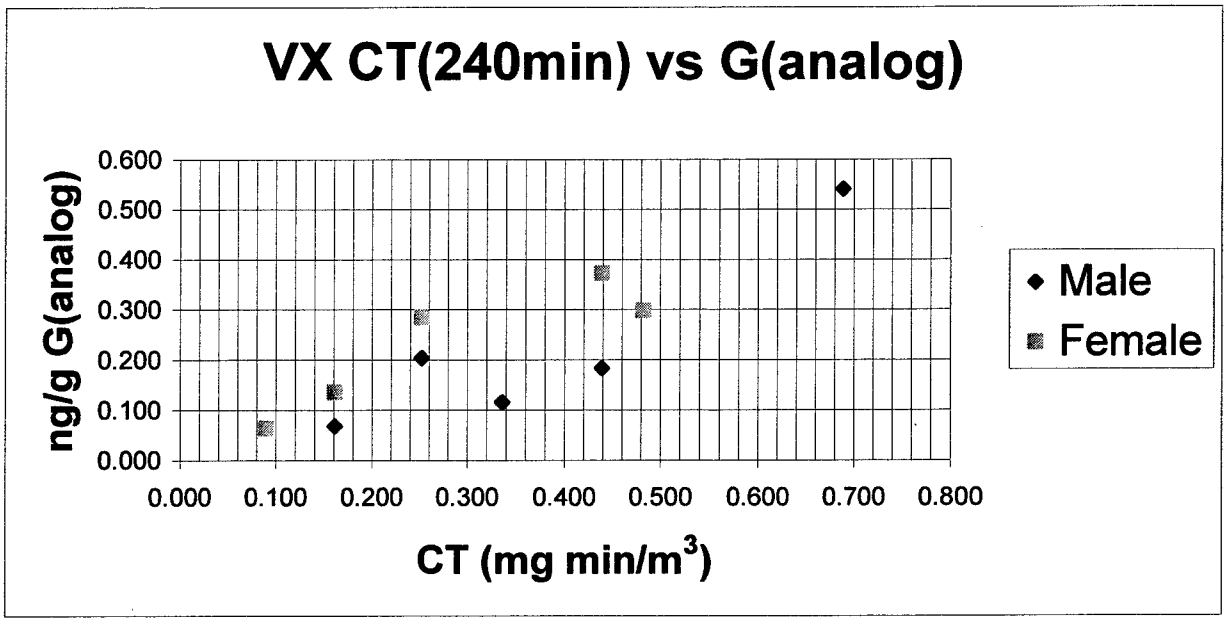


Figure 22. Average G-Analog Found in the Blood Plasma of Rats Exposed to Miosis Levels of VX for 240 Min

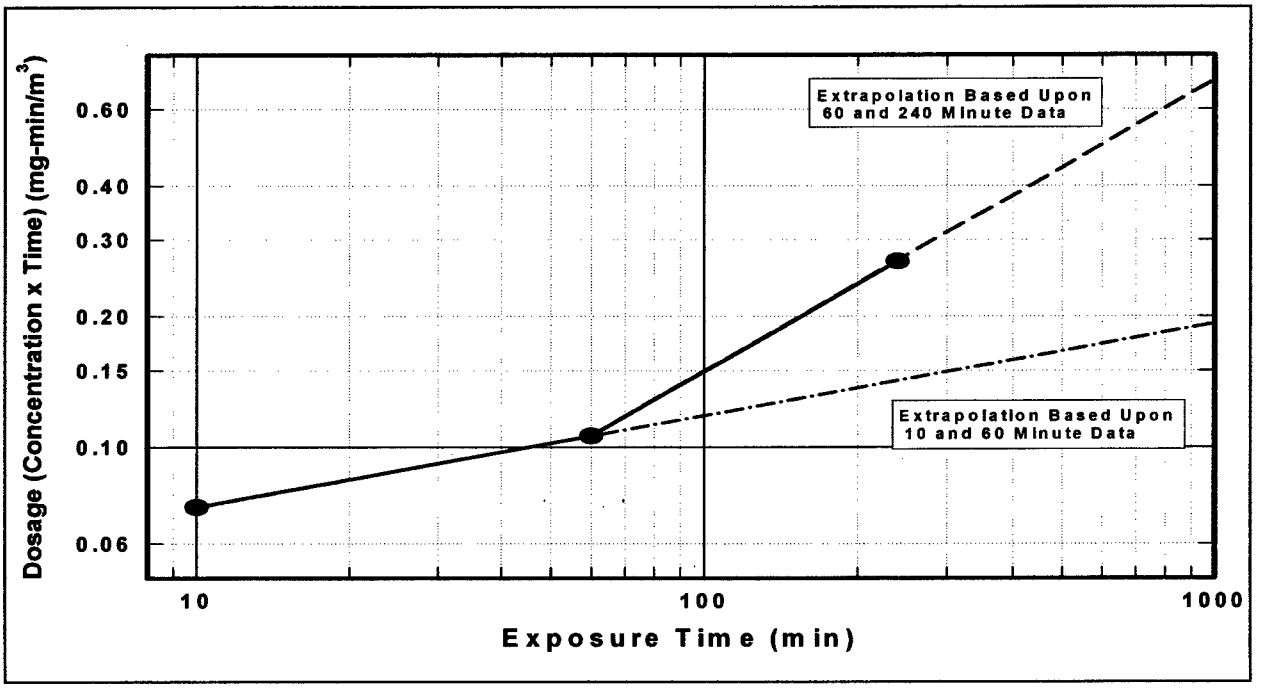


Figure 23. Comparison of Different Extrapolations of the Median Effective Dosage — Exposure Duration Relationship Using the Female Rat VX Miosis ECT_{50} Data from Present Study

Table 1. Miosis Level EC_{50} and ECT_{50} Values for VX, GB, and GF

VX			95% Fiducial Interval			95% Fiducial Interval	
Sex	Time (min)	**EC_{50} mg/m^3**	Lower Limit	Upper Limit	**ECT_{50} mg-min/m^3**	Lower Limit	Upper Limit
m	10	**0.01**	0.0085	0.0124	**0.102**	0.085	0.124
m	60	**0.004**	0.0030	0.0050	**0.229**	0.180	0.300
m	240	**0.002**	0.0015	0.0023	**0.443**	0.363	0.547
f	10	**0.007**	0.0060	0.0089	**0.073**	0.060	0.089
f	60	**0.002**	0.0014	0.0023	**0.106**	0.087	0.136
f	240	**0.001**	0.0009	0.0014	**0.268**	0.219	0.326

GB			95% Fiducial Interval			95% Fiducial Interval	
Sex	Time (min)	**EC_{50} mg/m^3**	Lower Limit	Upper Limit	**ECT_{50} mg-min/m^3**	Lower Limit	Upper Limit
m	10	**0.087**	0.076	0.099	**0.87**	0.76	0.99
m	60	**0.030**	0.022	0.043	**1.80**	1.34	2.58
m	240	**0.024**	0.016	0.044	**5.76**	3.84	10.56
f	10	**0.068**	0.059	0.078	**0.68**	0.59	0.78
f	60	**0.020**	0.014	0.027	**1.20**	0.84	1.62
f	240	**0.012**	0.006	0.019	**2.88**	1.44	4.56

GF			95% Fiducial Interval			95% Fiducial Interval	
Sex	Time (min)	**EC_{50} mg/m^3**	Lower Limit	Upper Limit	**ECT_{50} mg-min/m^3**	Lower Limit	Upper Limit
m	10	**0.184**	0.146	0.239	**1.843**	1.46	2.39
m	60	**0.042**	0.031	0.059	**2.511**	1.86	3.56
m	240	**0.029**	0.023	0.038	**7.031**	5.41	9.19
f	10	**0.080**	0.063	0.099	**0.796**	0.63	0.99
f	60	**0.024**	0.018	0.031	**1.413**	1.13	1.84
f	240	**0.017**	0.014	0.022	**4.155**	3.27	5.25

Table 2. Physical and Chemical Properties of VX (VX MSDS, 2003)

Boiling Point @ 760 mm Hg	568°F (298°C)
Vapor Pressure	0.00063 mm Hg @ 25°C
Vapor Density (Air = 1 STP)	9.2 @ 25°C
Solubility (g/100g solvent)	5.0 @ 21.5°C and 3.0 @ 25°C in water. Soluble in organic solvents
Specific Gravity (H_20=1g/mL)	1.0113 @ 25°C
Freezing/Melting Point (°C)	-50°C
Liquid Density (g/mL)	1.0083 @ 25°C
Volatility (mg/m^3)	8.9 @ 25°C
Appearance and Odor	Colorless to straw colored liquid and odorless, similar in appearance to motor oil.

Table 3. Impurites Present in VX (Lot # VX-U-1243-CTF-N)

Compound	Mole %
VX	93.6
Diisopropylaminoethane thiol	2.1
HCN/H^+	1.2
Diethyl methylphosphonate	1.0
Diethyl dimethyldiphosphonate (VX pyro)	0.8
Phosphonic acids/esters (δ 20-39)	0.4
Other ^{1}H impurities	0.31
Unsymmetrical VX Pyro	0.11
Chloroform	0.1
Other phosphorus impurities	0.27

Table 4. Fraction of Exposed Male and Female Rats that Developed Miosis (Pupil Constriction ≥50%) and at Least 50% Blood AChE Depression per each Combination of VX Vapor Concentration (C) and Time (T)

Date	T (min)	C (mg/m^3)	CT ($mg\text{-}min/m^3$)	Miosis		AChE Depression	
				Female	Male	Female	Male
24 Nov 03	10	0.00318	0.0318	0/10	*	0/10	*
15 Dec 03	10	0.00410	0.0410	1/10	*	1/10	*
12 Nov 03	10	0.00560	0.0560	1/10	0/10	0/10	0/10
15 Dec 03	10	0.00710	0.0710	*	2/10	*	0/10
24 Nov 03	10	0.00830	0.0830	*	2/10	*	0/10
14 Oct 03	10	0.00832	0.0832	9/10	5/10	1/10	2/10
21 Jan 04	10	0.00870	0.0870	6/10	*	2/10	*
13 Jan 04	10	0.01600	0.1600	*	9/10	*	4/10
4 Nov 03	60	0.00045	0.0270	0/10	*	0/10	*
2 Dec 03	60	0.00058	0.0348	*	0/10	*	0/10
23 Sep 03	60	0.00080	0.0480	2/10	0/10	1/10	0/10
2 Dec 03	60	0.00108	0.0648	1/10	*	0/10	*
5 Jan 04	60	0.00137	0.0822	1/10	*	0/10	*
22 Oct 03	60	0.00160	0.0960	4/10	0/10	1/10	0/10
5 Nov 03	60	0.00270	0.162	*	3/10	*	0/10
5 Jan 04	60	0.00364	0.218	*	4/10	*	3/10
8 Dec 03	240	0.00037	0.0888	0/10	*	0/10	*
30 Sep 03	240	0.00067	0.161	5/10	0/10	0/10	0/10
27 Oct 03	240	0.00105	0.252	1/10	0/10	2/10	2/10
9 Dec 03	240	0.00137	0.329	*	3/10	*	1/10
18 Nov 03	240	0.00183	0.439	7/10	4/10	1/10	0/10
20 Jan 04	240	0.00201	0.482	10/10	*	7/10	*
12 Jan 04	240	0.00287	0.689	*	10/10	*	7/10

*Single sex exposed at VX vapor concentration listed.

Table 5. Fraction of Exposed Male and Female Rats Belonging to each Pupil Size Category per each Combination of VX Vapor Concentration (C) and Time (T)

Date	T (min)	CT (mg-min/m^3)	Score 2		Score 1		Score 0	
			Female	Male	Female	Male	Female	Male
24 Nov 03	10	0.0318	7/10	*	3/10	*	0/10	*
15 Dec 03	10	0.0410	4/10	*	5/10	*	1/10	*
12 Nov 03	10	0.0560	1/10	8/10	8/10	2/10	1/10	0/10
15 Dec 03	10	0.0710	*	1/10	*	7/10	*	2/10
24 Nov 03	10	0.0830	*	1/10	*	7/10	*	2/10
14 Oct 03	10	0.0832	0/10	0/10	1/10	5/10	9/10	5/10
21 Jan 04	10	0.0870	0/10	*	4/10	*	6/10	*
13 Jan 04	10	0.1600	*	0/10	*	1/10	*	9/10
4 Nov 03	60	0.0270	7/10	*	3/10	*	0/10	*
2 Dec 03	60	0.0348	*	8/10	*	2/10	*	0/10
23 Sep 03	60	0.0480	2/10	4/10	6/10	6/10	2/10	0/10
2 Dec 03	60	0.0648	6/10	*	3/10	*	1/10	*
5 Jan 04	60	0.0822	4/10	*	5/10	*	1/10	*
22 Oct 03	60	0.0960	0/10	3/10	6/10	7/10	4/10	0/10
5 Nov 03	60	0.162	*	0/10	*	7/10	*	3/10
5 Jan 04	60	0.218	*	1/10	*	5/10	*	4/10
8 Dec 03	240	0.0888	9/10	*	1/10	*	0/10	*
30 Sep 03	240	0.161	0/10	3/10	5/10	7/10	5/10	0/10
27 Oct 03	240	0.252	2/10	4/10	7/10	6/10	1/10	0/10
9 Dec 03	240	0.329	*	0/10	*	7/10	*	3/10
18 Nov 03	240	0.439	0/10	0/10	3/10	6/10	7/10	4/10
20 Jan 04	240	0.482	0/10	*	0/10	*	10/10	*
12 Jan 04	240	0.689	*	0/10	*	0/10	*	10/10

*Single sex exposed at VX vapor concentration listed.

Table 6. Blood AChE Depression EC_{50} and ECT_{50} Values for Rats Exposed to VX Vapor

Gender	Time (min)	EC_{50} mg/m^3	95% Fiducial Limits		ECT_{50} mg-min/m^3	95% Fiducial Limits	
			Lower	Upper		Lower	Upper
Male	10	0.0200	0.0143	0.0346	0.200	0.143	0.346
	60	0.00634	0.00413	0.0124	0.380	0.248	0.744
	240	0.00270	0.00204	0.00414	0.648	0.490	0.994
Female	10	0.0153	0.0106	0.0285	0.153	0.106	0.285
	60	0.00340	0.00216	0.00737	0.204	0.130	0.442
	240	0.00217	0.00163	0.00324	0.521	0.391	0.778

Table 7. Contingency Table Showing Numbers of Rats with Miosis and/or AChE Depression Following Exposure to VX Vapor. All measurements taken 1 hr post-exposure and all exposure durations combined.

		50% AChE Present		Miosis Totals
		No	Yes	
Miosis Present	No	203	7	210
	Yes	62	28	90
AChE Totals		265	35	

Table 8. Probit Slopes and Toxic Load Exponents (n) Obtained from Various Ordinal (Miosis) and Binary (AChE) Logistic Regression Model Fits

Endpoint	Terms in Model	k_C	SE(C)	k_T	SE(T)	n	SE(n)
Miosis	LogC (Time)(Sex)	5.24	0.63	---	---	---	---
Miosis	LogC LogT Sex	3.59	0.32	2.18	0.23	1.65	0.09
AChE Depression	LogC (Time)(Sex)	3.72	0.71	---	---	---	---
AChE Depression	LogC LogT Sex	3.24	0.58	2.06	0.35	1.57	0.14

Table 9. Miosis Toxic Load Exponents (n) Obtained from ECBC Low Level Toxicology Program

Agent	Species	Toxic Load Exponents (n)			$(n_{all} - n_{short})$	Source
		Whole Dataset		T < 100 min		
		n_{all}	95% CI	n_{short}		
GB	Minipig	1.33	1.13 to 1.53	1.08	0.25	Hulet *et al.* (unpublished data)
GB	Rat	2.33	1.85 to 2.81	1.62	0.71	Mioduszewski *et al.* (2002a)
GF	Rat	1.98	1.70 to 2.26	1.30	0.68	Whalley *et al.* (2004)
VX	Rat	1.65	1.47 to 1.83	1.35	0.30	present study

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LITERATURE CITED

Agresti, A. Categorical Data Analysis. John Wiley & Sons, New York (1990).

Anthony, J.S., Haley, M., Manthei, J., Way, R., Burnett, D., Gaviola, B., Sommerville, D., Crosier, R., Mioduszewski, R., Thomson, S., Crouse, C., and Matson, K. Inhalation Toxicity of Cyclosarin (GF) Vapor in Rats as a Function of Exposure Concentration and Duration: Potency Comparison to Sarin (GB). Inhalation Toxicology 16: 103-111, 2004.

Bide, R.W., and Risk, D.J. Inhalation Toxicity of Aerosolized Nerve Agents. 1. VX Revisited. Defence Research Establishment Suffield. Technical Report DRES TR 2000-063, 2000 (UNCLASSIFIED Report).

Bide, R.W., Risk, D.J., and Beifus, D.G. A Functional Small Volume Inhalation System for Highly Toxic Materials. In Proceedings of the 1996 ERDEC Scientific Conference on Chemical and Biological Defense Research, 19-22 November 1996, AD-A334 105, pp 25-32.

Booth, J., and Gillette, J.R. The Effect of Anabolic Steroids on Drug Metabolism by Microsomal Enzymes in Rat Liver. J. Pharmacol. Exp. Ther. Sep; 137:374-9 (1962).

Bours, J., Fink, H., and Hockwin, O. The Quantification of Eight Enzymes from the Aging Rat Lens, with Respect to Sex Differences and Special Reference to Aldolase. Curr. Eye Res. May 7(5):449-55 (1988)

Brickhouse, M.D., Rees, M.S., O'Connor, R.J., and Durst, H.D. Nuclear Magnetic Resonance (NMR) Analysis of G-class and VX Nerve Agents and Reaction Masses Produced by Their Chemical Neutralization. ERDEC-TR-449, U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 1997 (UNCLASSIFIED Report) (AD-A339 308).

Craig, F.N., Cummings, E.G., and Sim, V.M. Environmental Temperature and the Percutaneous Absorption of a Cholinesterase Inhibitor, VX. J. Invest. Dermatol. 68(6), 357-361 (1977).

Dimmick, R.L., Jr., Owens, E.J., and Farrand, R.L. The Acute Inhalation Toxicity of QL in the Rat and Guinea Pig. ARCSL-TR-79031, 1979 (UNCLASSIFIED Report).

Ellman, G.L., Courtney, K.D., Andres, V., Jr., and Featherstone, R.M. A New Rapid Colorimetric Determination of Acetylcholinesterase Activity. Biochem. Pharm. 7:88-95 (1961).

Fairhurst, S., and Turner R.M. Toxicological Assessments in Relation to Major Hazards. J. Hazard. Mater. 33: 215-227 (1993).

Finney, D.J. Probit Analysis. 3rd Edition, Cambridge University Press, Cambridge (1971).

Fox, J. Applied Regression Analysis, Linear Models, and Related Methods. SAGE Publications, Inc., Thousand Oaks, CA (1997).

Griffiths, R.F. The Use of Probit Expressions in the Assessment of Acute Population Impact of Toxic Releases. J. Loss Prev. Process Ind. 4: 49-57 (1991).

Gupta, R.C., Patterson, G.T., and Dettbarn, W.D. Comparison of Cholinergic and Neuromuscular Toxicity Following Acute Exposure to Sarin and VX in Rat. Fundam. Appli. Toxicol. 16(3), 449-458 (1991).

Haber, F.R. Zur geschichte des gaskrieges. In Funf Vortrage aus Jahren 1920-1923, Spinger, Berlin, 1924.

Hartmann, H.M. Evaluation of Risk Assessment Guideline Levels for the Chemical Warfare Agents Mustard, GB, and VX. Regul. Toxicol. and Pharmacol. 35, 347-356 (2002).

Holck, H.G.O., Kanan, M.A., Mills, L.M., and Smith, E.L. Studies Upon the Sex-Difference in Rats in Tolerance to Certain Barbiturates and to Nicotine. J. Pharmacol. Exp. Ther. 60, 323 (1937).

Jakubowski, E.M., Heykamp, L. S., Durst, H.D., and Thomson, S.A. Preliminary Studies in the Formation of Ethyl Methylphosphonofluoridate from Rat and Human Serum Exposed to VX and Treated with Fluoride Ion. Anal. Lett. 34(5): 727-737 (2001).

Kato, R. Sex-Related Differences in Drug Metabolism. Drug Metab. Rev. 3(1), 1-32 (1974).

Kato, R., Chiesara, E., and Vassanelli, P. Metabolic Differences of Strychnine in the Rat in Relation to Sex. Jpn J. Pharmacol. Jun;12:26-33 (1962a).

Kato, R., Chiesara, E., and Vassanelli, P. Increased Activity of Microsomal Strychnine-Metabolizing Enzyme Induced by Phenobarbital and Other Drugs. Biochem. Pharmacol. Oct;11:913-22 (1962b).

Manthei, J.H., Beckett, E.L., Heitkamp, D.H., James, J.T., Cameron, K.P., Bona, D.M., Moore, R.D., and Vickers, E.L. Toxic Hazard Evaluation of Decontaminated/Neutralized Chemical Surety Materials Waste at the U.S. Army Chemical Research, Development and Engineering Center. CRDEC-TR-146, 1990 (UNCLASSIFIED Report).

Maxwell, D.M. The Specificity of Carboxylesterase Protection Against the Toxicity of Organophosphorus Compounds. Toxicol. Appl. Pharmacol. 114, 306-312 (1992).

Miller, D.B., Benton, B.J., Hulet, S.W., Mioduszewski, R.J., Whalley, C.E., Carpin, J.C., and Thomson, S.A. An automated infrared image acquisition and analysis method for quantifying optical responses to chemical agent vapor exposure. 29th Annual IEEE Northeast Bioengineering Conference, 22-23 March 2003, New Jersey Institute of Technology, Newark, NJ, 2003a.

Miller, D.B., Benton, B.J., Hulet, S.W., Mioduszewski, R.J., Whalley, C.E., Carpin, J.C., and Thomson, S.A. An image analysis method for quantifying elliptical and partially obstructed pupil areas in response to chemical agent vapor exposure. 29th Annual IEEE Northeast Bioengineering Conference, 22-23 March 2003, New Jersey Institute of Technology, Newark, NJ, 2003b.

Miller, D.B., Benton, B.J., Hulet, S. W., Mioduszewski, R.J., Whalley, C.E., Carpin, J.C., and Thomson, S.A. An infrared image acquisition and analysis method for quantifying optical responses to chemical agent vapor exposure. 2002 Joint Services Scientific Conference on Chemical & Biological Defense, 19-21 November 2002, Hunt Valley, MD.

Mioduszewski, R.J., Manthei, J.H., Way, R.A., Burnett, D.C., Gaviola, B.I., Muse, W.T., Jr., Matson, K.L., Sommerville, D.R., Crosier, R.B., Scotto, J.A., McCaskey, D.A., Crouse, C.L., and Thomson, S.A. Low-Level Sarin Vapor Exposure in Rats: Effect of Exposure Concentration and Duration on Pupil Size. ECBC-TR-235, U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, May 2002a (UNCLASSIFIED Report).

Mioduszewski, R.J., Manthei, J.H., Way, R.A., Burnett, D.C., Gaviola, B.P., Muse, W.T., Thomson, S.A., Sommerville, D.R., and Crosier, R.B., Interaction of Exposure Concentration and Duration in Determining Acute Toxic Effects of Sarin Vapor in Rats. Toxicol. Sci. 66: 176-184 (2002b).

Muse, W.T., Jr., Anthony, J.S., Thomson, S.A., Crouse, C.L., and Crouse, L.C.B. Acute Inhalation Toxicity of Chemically Neutralized/Hydrolyzed VX in Rats. SCWO Effluent Prior to Evaporation SCWO Effluent Post-Evaporation. ECBC-TR-219, 2002 (UNCLASSIFIED Report).

Oshiro, I., Takenaka, T., and Maeda, J. New Method for Hemoglobin Determination by Using Sodium Lauryl Sulfate (SLS). Clin. Biochem. 15(1) 83-88 (1982).

Prins, J., "Product and Process Comparison," Chapter 7. In Croarkin, C., and Tobias, P., Eds., NIST/SEMATECH e-Handbook of Statistical Methods. http://www.itl.nist.gov/div898/handbook/. Last revised November 2003, National Institute of Standards and Technology, Washington, DC, 8 July 2004.

Report of the American Veterinary Medical Association (AVMA) Panel on Euthanasia. JAVMA, Vol. 202, No. 2, 15 January 1993.

Rickett, D.L., Glenn, J.F., and Beers, E.T. Central Respiratory Effects VX. Neuromuscular Actions of Nerve Agents. Neurotoxicology 7(1), 225-236 (1986).

Silver, A. The Biology of Cholinesterases: Frontiers of Biology 36, Elsevier/North-Holland, Amsterdam (1974).

Sullivan, D.A., Hann, L.E., Yee, L., and Allansmith, M.R. Age- and Gender-Related Influence on the Lacrimal Gland and Tears. Acta Ophthalmol (Copenh) Apr 68(2):188-94 (1990)

ten Berge, W.F., Zwart, A., and Appelman, L.M., Concentration-Time Mortality Response Relationship of Irritant and Systemically Acting Vapours and Gases. J. Hazard. Mater. 13:301-309 (1986).

VX; Materials Safety Data Sheet. https://cbnet.apgea.army.mil/org/rda/od/RISK/safety/main/DOCS/vx.doc, 2003.

Weimer, J.T., and Ballard, T.A. Note on the Influence of Inhaled SNA on the Inhalation Toxicity of VX to Rats. CWL-TM-24-36, 1960 (UNCLASSIFIED Report).

Whalley, C.E., Benton, B.J., Manthei, J.H., Way, R.A., Jakubowski, E.M., Jr., Burnett, D.C., Gaviola, B.I., Crosier, R.B., Sommerville, D.R., Muse, W.T., Forster, J.S., Mioduszewski, R.J., Thomson, S.A., Scotto, J.A., Miller, D.B., Crouse, C.L., Matson, K.L., and Edwards, J.L. Low-Level Cycolosarin (GF) Vapor Exposure in Rats: Effect of Exposure Concentration and Duration on Pupil Size. ECBC-TR-407, U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2004 (UNCLASSIFIED Report).

APPENDIX

PROBIT ANALYSIS AND ORDINAL LOGISTIC REGRESSION PRINTOUTS FROM MINITAB®

A1. INTRODUCTION

Three types of statistical analyses were used in MINITAB® on the total dataset (genders and all three exposure durations) for each of the three measured responses (changes is pupil size, AChE levels and BChE levels): general linear model (or ANOVA), traditional probit analysis and ordinal logistic regression with a normit link function. The printouts from these analyses are included in this appendix.

Comments by the analyst about the printouts are preceded by [RBC] or [DRS].

Nomenclature

Conc	Concentration of VX vapor in milligrams per cubic meter
T	Exposure duration (in min)
logC	Log base 10 of vapor concentration
logT	Log base 10 of exposure duration
Control:	Indicates whether the rat is a control rat or an exposed rat (0 for exposed and 1 for control)
Group:	Exposure run ID/gender combination—a factor of 30 levels (ex. 13m refers to Run 13, male rats). Table A1 lists Run ID's vs exposure conditions.
Miosis@1hr:	Miosis as determined by the ratio of pupil diameter at 1 hr post-exposure to pre-exposure pupil diameter, adjusted for the same-sex control rats in the exposure group; a ratio of 0.5 or less indicates miosis
Sex:	Male or female
Sex&T	Factor that accounts for sex and exposure duration with six levels M10—male rats for 10 min M60—male rats for 60 min M240—male rats for 240 min F10—female rats for 10 min F60—female rats for 60 min F240—female rats for 240 min

SizeScore Classification of the ratio of pupil diameter at 1 hr post-exposure to pre-exposure pupil diameter, adjusted for the same ratio of same-sex control rats in the exposure group.

SizeScore = 0 if ratio is < or equal to 0.5
SizeScore = 1 if 0.5 < ratio < 0.8413
SizeScore = 2 if 0.8413 < ratio.

A ratio: Ratio of post-exposure AChE to pre-exposure AChE, adjusted for the ratio of the same-sex control rats in the exposure group (also known as AChE depression)

B ratio: Ratio of post-exposure BChE to pre-exposure BChE, adjusted for the ratio of the same-sex control rats in the exposure group

Z: Normit (Z = 0 for 50% response, -1 for 16% response and 1 for 84% response)

SE: Standard error of coefficient

n: Toxic load exponent

Table. Summary of Exposure Runs for Rat VX Miosis Study

Date	Shipment ID	Exposure Run ID	t (min)	C (mg/m^3)	CT ($mg\text{-}min/m^3$)	Gender(s) Exposed in Run	
23-Sep-03	1	13	60	0.0008	0.048	f	m
30-Sep-03	2	14	240	0.00067	0.161	f	m
14-Oct-03	3	15	10	0.00832	0.0832	f	m
22-Oct-03	4	16	60	0.0016	0.096	f	m
27-Oct-03	5	17	240	0.00105	0.252	f	m
4-Nov-03	6	18a	60	0.00045	0.027	f	*
5-Nov-03	6	18b	60	0.0027	0.162	*	m
12-Nov-03	7	19	10	0.0056	0.056	f	m
18-Nov-03	8	20	240	0.00183	0.439	f	m
24-Nov-03	9	21a	10	0.00318	0.0318	f	*
24-Nov-03	9	21b	10	0.0083	0.083	*	m
2-Dec-03	10	22a	60	0.00058	0.0348	*	m
2-Dec-03	10	22b	60	0.00108	0.0648	f	*
8-Dec-03	11	23a	240	0.00037	0.0888	f	*
9-Dec-03	11	23b	240	0.00137	0.329	*	m
15-Dec-03	12	24a	10	0.0041	0.041	f	*
15-Dec-03	12	24b	10	0.0071	0.071	*	m
5-Jan-04	13	25a	60	0.00137	0.0822	f	*
5-Jan-04	13	25b	60	0.00364	0.218	*	m
12-Jan-04	14	26a	240	0.00287	0.689	*	m
13-Jan-04	14	27a	10	0.016	0.16	*	m
20-Jan-04	15	26b	240	0.00201	0.482	f	*
21-Jan-04	15	27b	10	0.0087	0.087	f	*

A2. STATISTICAL ANALYSIS OF PUPIL RESPONSE DATA

A2.1 Probit Analysis: Miosis@1hr vs Concentration, Sex&T.

Distribution: Lognormal base 10

Response Information

Variable	Value	Count	
Mio@1hr	1	90	(Event)
	0	210	
	Total	300	

Factor Information

Factor	Levels	Values
Sex&T	6	M10 M60 M240 F10 F60 F240

Estimation Method: Maximum Likelihood

300 cases were used
150 cases contained missing values ← **[RBC] these are the control rats**

Regression Table

Variable	Coef	Error	Standard Z	P
Constant	10.447	1.320	7.92	0.000
Conc	5.2435	0.6312	8.31	0.000
Sex&T				
M60	2.2307	0.4529	4.92	0.000
M240	3.8890	0.5503	7.07	0.000
F10	0.7650	0.3075	2.49	0.013
F60	3.9837	0.6135	6.49	0.000
F240	5.0348	0.6356	7.92	0.000
Natural Response	0.000			

Test for equal slopes: Chi-Square = 10.4321, DF = 5, P-Value = 0.064
Log-Likelihood = -117.951

Multiple degree of freedom test

Term	Chi-Square	DF	P
Sex&T	66.405	5	0.000

Goodness-of-Fit Tests

Method	Chi-Square	DF	P
Pearson	44.242	23	0.005
Deviance	42.147	23	0.009

Sex&T = M10

Tolerance Distribution

Parameter Estimates

Parameter	Estimate	Standard Error	95.0% Normal CI Lower	Upper
Location	-1.99247	0.04026	-2.07138	-1.91356
Scale	0.19071	0.02296	0.15063	0.24146

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
1	0.003663	0.000502	0.002630	0.004613
5	0.004941	0.000556	0.003803	0.006014
10	0.005796	0.000593	0.004601	0.006968
50	0.01017	0.000943	0.008521	0.01239
90	0.01786	0.002253	0.01441	0.02414

Sex&T = M60
Tolerance Distribution

Parameter Estimates

Parameter	Estimate	Standard Error	95.0% Normal CI Lower	Upper
Location	-2.41789	0.05478	-2.52526	-2.31052
Scale	0.19071	0.02296	0.15063	0.24146

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
1	0.001375	0.000211	0.000961	0.001798
5	0.001855	0.000250	0.001372	0.002372
10	0.002176	0.000279	0.001649	0.002768
50	0.003820	0.000482	0.003005	0.005003
90	0.006707	0.001052	0.005110	0.009688

Sex&T = M240

Tolerance Distribution

Parameter Estimates

Parameter	Estimate	Standard Error	95.0% Normal CI Lower	Upper
Location	-2.73416	0.04411	-2.82061	-2.64772
Scale	0.19071	0.02296	0.15063	0.24146

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
1	0.000664	0.000098	0.000465	0.000852
5	0.000896	0.000111	0.000672	0.001112
10	0.001051	0.000119	0.000813	0.001289
50	0.001844	0.000187	0.001513	0.002280
90	0.003238	0.000421	0.002586	0.004396

Sex&T = F10

Tolerance Distribution

Parameter Estimates

Parameter	Estimate	Standard Error	95.0% Normal CI Lower	Upper
Location	-2.13837	0.04135	-2.21943	-2.05732
Scale	0.19071	0.02296	0.15063	0.24146

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
1	0.002618	0.000377	0.001847	0.003335
5	0.003531	0.000421	0.002672	0.004344
10	0.004142	0.000449	0.003237	0.005029
50	0.007272	0.000692	0.006041	0.008873
90	0.01277	0.001592	0.01030	0.01714

Sex&T = F60

Tolerance Distribution

Parameter Estimates

Parameter	Estimate	Standard Error	95.0% Normal CI Lower	Upper
Location	-2.75222	0.04812	-2.84654	-2.65790
Scale	0.19071	0.02296	0.15063	0.24146

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
1	0.000637	0.000083	0.000469	0.000800
5	0.000859	0.000096	0.000669	0.001055
10	0.001008	0.000107	0.000803	0.001233
50	0.001769	0.000196	0.001443	0.002260
90	0.003106	0.000466	0.002410	0.004455

Sex&T = F240

Tolerance Distribution

Parameter Estimates

Parameter	Estimate	Standard Error	95.0% Normal CI Lower	Upper
Location	-2.95266	0.04290	-3.03675	-2.86858
Scale	0.19071	0.02296	0.15063	0.24146

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
1	0.000401	0.000064	0.000273	0.000523
5	0.000542	0.000072	0.000396	0.000680
10	0.000635	0.000076	0.000481	0.000785
50	0.001115	0.000110	0.000913	0.001360
90	0.001958	0.000233	0.001587	0.002578

Table of Relative Potency

Factor: Sex&T

Comparison	Relative Potency	95.0% Fiducial CI Lower	Upper
M10 VS M60	0.3755	0.2773	0.5133
M10 VS M240	0.1813	0.1376	0.2375
M10 VS F10	**0.7147**	**0.5473**	**0.9276**
M10 VS F60	0.1739	0.1329	0.2324
M10 VS F240	0.1096	0.08249	0.1426
M60 VS M240	0.4828	0.3475	0.6606
M60 VS F10	1.9034	1.3802	2.5835
M60 VS F60	**0.4631**	**0.3371**	**0.6434**
M60 VS F240	0.2919	0.2079	0.3974
M240 VS F10	3.9427	2.9852	5.2029
M240 VS F60	0.9593	0.7236	1.3055
M240 VS F240	**0.6046**	**0.4507**	**0.7986**
F10 VS F60	0.2433	0.1851	0.3285
F10 VS F240	0.1534	0.1154	0.2008
F60 VS F240	0.6303	0.4569	0.8339

[DRS]—for all three exposure durations (above bolded lines), there is a statistically significant difference between male and female rats, with the female rats being more sensitive with respect to pupil response.

A2.2 Ordinal Logistic Regression: SizeScore vs LogC, LogT, Sex.

```
Link Function:  Normit
Response Information

Variable  Value       Count   Description
SizeScore 0              90         diameter < 50% = small pupils = miosis
          1             135   50% < diameter < 84%
          2              75   84% < diameter
          Total         300

  300 cases were used
  150 cases contained missing values ← [RBC] these are the control rats

Logistic Regression Table

Predictor       Coef    SE Coef        Z      P
Const(1)      5.1107     0.5508     9.28  0.000
Const(2)      6.8141     0.6009    11.34  0.000
LogC          3.5946     0.3208    11.21  0.000
LogT          2.1772     0.2258     9.64  0.000
Sex          -0.29844    0.07546   -3.96  0.000

Log-likelihood = -242.313
Test that all slopes are zero: G = 155.630, DF = 3, P-Value = 0.000

[RBC] Additional Calculations:
6.8141 - 5.1107 = 1.7034 normits of % rats = 1 normit of % pupil size

toxic load exponent = 3.5946/2.1772 = 1.6510 with SE = .0921

Normit(fraction of rats) = 5.1107 + 3.5946*LogC + 2.1772*LogT - 0.29844 Sex

Z(pupil diameter fraction) = -3.0003 - 2.1103*LogC - 1.2781*LogT + 0.1752*Sex
Z(shrinkage fraction) =       3.0003 + 2.1103*LogC + 1.2781*LogT - 0.1752*Sex
standard error(coefficient):   .3234    .1883         .1326         .0443

Goodness-of-Fit Tests

Method      Chi-Square    DF      P
Pearson        102.302    55  0.000
Deviance       110.102    55  0.000

Measures of Association:
(Between the Response Variable and Predicted Probabilities)

Pairs           Number  Percent     Summary Measures
Concordant       23699    81.7%     Somers' D                0.66
Discordant        4657    16.0%     Goodman-Kruskal Gamma    0.67
Ties               669     2.3%     Kendall's Tau-a          0.42
Total            29025   100.0%

Data Display: Variance-Covariance Matrix of Estimated Parameters

  const(1)  const(2)     LogC      LogT       Sex
 0.303396  0.324332  0.161494  0.070841 -0.014769
 0.324332  0.361077  0.178622  0.081021 -0.016027
 0.161494  0.178622  0.102906  0.061079 -0.009433
 0.070841  0.081021  0.061079  0.050997 -0.005696
-0.014769 -0.016027 -0.009433 -0.005696  0.005694

[RBC]
Standard error of toxic load exponent = (3.5946/2.1772)*sqrt(.102906/3.5946^2
+ 0.050997/2.1772^2 - 2*0.061079/(3.5946*2.1772)) = 0.0921268
```

A2.3 Analysis of Recovery Time of Pupil Diameters.

[RBC] Note: Group accounts for sex, exposure concentration, and exposure duration; Control is coded 0 for exposed rats and 1 for control rats.

1 Hr – the ratio of pupil diameter at 1 hr post-exposure to pre-exposure pupil diameter, adjusted for the same ratio of same-sex control rats in the exposure group

General Linear Model: 1 Hr vs Group, Control

```
Factor      Type Levels Values
Group       fixed     30 13F 13M 14F 14M 15F 15M 16F 16M 17F 17M 18F 18M 19F 19M
                         20F 20M 21F 21M 22F 22M 23F 23M 24F 24M 25F 25M 26F 26M
                         27F 27M
Control     fixed      2 0 1

Analysis of Variance for 1 Hr, using Adjusted SS for Tests

Source           DF     Seq SS     Adj SS     Adj MS       F      P
Group            29   10.63276    5.33174    0.18385    7.13  0.000
Control           1   15.39934   15.36414   15.36414  595.78  0.000
Group*Control    29    5.30252    5.30252    0.18285    7.09  0.000
Error           389   10.03158   10.03158    0.02579
Total           448   41.36619
```

2 Hr – the ratio of pupil diameter at 2 hr post-exposure to pre-exposure pupil diameter, adjusted for the same ratio of same-sex control rats in the exposure group

General Linear Model: 2 Hr vs Group, Control

```
Factor      Type Levels Values
Group       fixed     30 13F 13M 14F 14M 15F 15M 16F 16M 17F 17M 18F 18M 19F 19M
                         20F 20M 21F 21M 22F 22M 23F 23M 24F 24M 25F 25M 26F 26M
                         27F 27M
Control     fixed      2 0 1

Analysis of Variance for 2 Hr, using Adjusted SS for Tests

Source           DF     Seq SS     Adj SS     Adj MS       F      P
Group            29   10.58128    5.28573    0.18227    7.59  0.000
Control           1   11.79687   11.83819   11.83819  493.26  0.000
Group*Control    29    5.20962    5.20962    0.17964    7.49  0.000
Error           387    9.28804    9.28804    0.02400
Total           446   36.87581
```

1 Day – the ratio of pupil diameter at 1 day post-exposure to pre-exposure pupil diameter, adjusted for the same ratio of same-sex control rats in the exposure group

General Linear Model: 1 Day vs Group, Control

```
Factor      Type Levels Values
Group       fixed     30 13F 13M 14F 14M 15F 15M 16F 16M 17F 17M 18F 18M 19F 19M
                         20F 20M 21F 21M 22F 22M 23F 23M 24F 24M 25F 25M 26F 26M
                         27F 27M
Control     fixed      2 0 1
```

Analysis of Variance for 1 Day, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Group	29	2.02672	0.96610	0.03331	1.97	0.002
Control	1	1.47514	1.51632	1.51632	89.47	0.000
Group*Control	29	0.97702	0.97702	0.03369	1.99	0.002
Error	386	6.54159	6.54159	0.01695		
Total	445	11.02048				

2 Days – the ratio of pupil diameter at 2 days post-exposure to pre-exposure pupil diameter, adjusted for the same ratio of same-sex control rats in the exposure group

General Linear Model: 2 Days vs Group, Control

Factor	Type	Levels	Values
Group	fixed	30	13F 13M 14F 14M 15F 15M 16F 16M 17F 17M 18F 18M 19F 19M 20F 20M 21F 21M 22F 22M 23F 23M 24F 24M 25F 25M 26F 26M 27F 27M
Control	fixed	2	0 1

Analysis of Variance for 2 Days, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Group	29	0.98749	0.48543	0.01674	0.95	0.541
Control	1	0.02817	0.03018	0.03018	1.72	0.191
Group*Control	29	0.52155	0.52155	0.01798	1.02	0.437
Error	386	6.79004	6.79004	0.01759		
Total	445	8.32725				

7 Days – the ratio of pupil diameter at 7 days post-exposure to pre-exposure pupil diameter, adjusted for the same ratio of same-sex control rats in the exposure group

General Linear Model: 7 Days vs Group, Control

Factor	Type	Levels	Values
Group	fixed	30	13F 13M 14F 14M 15F 15M 16F 16M 17F 17M 18F 18M 19F 19M 20F 20M 21F 21M 22F 22M 23F 23M 24F 24M 25F 25M 26F 26M 27F 27M
Control	fixed	2	0 1

Analysis of Variance for 7 Days, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Group	29	0.66792	0.33006	0.01138	0.93	0.576
Control	1	0.00189	0.00230	0.00230	0.19	0.665
Group*Control	29	0.33700	0.33700	0.01162	0.95	0.546
Error	387	4.74382	4.74382	0.01226		
Total	446	5.75063				

[DRS] p-values for Control and Group*Control can be used to indicate when exposed pupils recover from VX vapor exposure. The two parameters are statistically significant (with greater than 99% confidence) for 1 hr, 2 hr and 1 day post exposure. However, for 2 days or greater, the p-values are greater than 0.19 (i.e, not significance). So, recovery occurred between 1 day and 2 days post exposure.

A3. STATISTICAL ANALYSIS OF BLOOD CHE DEPRESSION DATA

A3.1 General Linear Model: Aratio vs Group, Control.

```
Factor      Type Levels Values
Group      fixed     30 13F 13M 14F 14M 15F 15M 16F 16M 17F 17M 18F 18M 19F 19M
                        20F 20M 21F 21M 22F 22M 23F 23M 24F 24M 25F 25M 26F 26M
                        27F 27M
Control    fixed      2 0 1

Analysis of Variance for Aratio, using Adjusted SS for Tests

Source          DF     Seq SS     Adj SS     Adj MS       F      P
Group           29    5.79568    3.01728    0.10404    2.40  0.000
Control          1    3.76974    3.79051    3.79051   87.59  0.000
Group*Control   29    2.47764    2.47764    0.08544    1.97  0.002
Error          388   16.79004   16.79004    0.04327
Total          447   28.83310
```

[DRS] All factors and interactions are significant (with greater than 99.8% confidence). Thus, there was significant AChE depression for one-hr post-exposure.

A3.2 General Linear Model: Bratio vs Group, Control.

```
Factor      Type Levels Values
Group      fixed     30 13F 13M 14F 14M 15F 15M 16F 16M 17F 17M 18F 18M 19F 19M
                        20F 20M 21F 21M 22F 22M 23F 23M 24F 24M 25F 25M 26F 26M
                        27F 27M
Control    fixed      2 0 1

Analysis of Variance for Bratio, using Adjusted SS for Tests

Source          DF     Seq SS     Adj SS     Adj MS       F      P
Group           29    2.47421    1.30724    0.04508    0.83  0.722
Control          1    0.01376    0.00655    0.00655    0.12  0.729
Group*Control   29    1.21319    1.21319    0.04183    0.77  0.801
Error          379   20.59889   20.59889    0.05435
Total          438   24.30005
```

[RBC] Nothing significant: no need to analyze BChE ratio data.

A3.3 Probit Analysis: AChE Depression vs C, Sex&T.

```
Distribution:  Lognormal base 10

Response Information

Variable  Value       Count
AChE      1              35  (Event)
          0             265
          Total         300

Factor Information

Factor   Levels Values
Sex&T         6 F10  F240 F60  M10  M240 M60

Estimation Method:  Maximum Likelihood

  300 cases were used
  150 cases contained missing values ← [RBC] these are the control rats
```

Regression Table

Variable	Coef	Standard Error	Z	P
Constant	6.755	1.555	4.34	0.000
C	3.7199	0.7107	5.23	0.000
Sex&T				
F240	3.1554	0.5972	5.28	0.000
F60	2.4270	0.6837	3.55	0.000
M10	-0.4332	0.3958	-1.09	0.274
M240	2.7985	0.5613	4.99	0.000
M60	1.4203	0.5092	2.79	0.005
Natural Response	0.000			

Test for equal slopes: Chi-Square = 4.1342, DF = 5, P-Value = 0.530
Log-Likelihood = -81.382

Multiple degree of freedom test

Term	Chi-Square	DF	P
Sex&T	35.066	5	0.000

Goodness-of-Fit Tests

Method	Chi-Square	DF	P
Pearson	35.324	23	0.048
Deviance	33.610	23	0.071

Sex&T = F10
Tolerance Distribution

Parameter Estimates

Parameter	Estimate	Standard Error	95.0% Normal CI Lower	Upper
Location	-1.81594	0.09839	-2.00878	-1.62311
Scale	0.26882	0.05136	0.18486	0.39093

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
1	0.003620	0.000773	0.002042	0.005165
5	0.005519	0.000981	0.003636	0.007738
10	0.006911	0.001185	0.004795	0.009901
50	0.01528	0.003461	0.01056	0.02845
90	0.03377	0.01166	0.02013	0.09450

Sex&T = F240

Tolerance Distribution

Parameter Estimates

Parameter	Estimate	Standard Error	95.0% Normal CI Lower	Upper
Location	-2.66418	0.06986	-2.80110	-2.52726
Scale	0.26882	0.05136	0.18486	0.39093

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
1	0.000513	0.000130	0.000245	0.000756
5	0.000783	0.000151	0.000461	0.001071
10	0.000980	0.000165	0.000636	0.001311
50	0.002167	0.000349	0.001627	0.003243
90	0.004790	0.001263	0.003209	0.01040

Sex&T = F60

Tolerance Distribution

Parameter Estimates

Parameter	Estimate	Standard Error	95.0% Normal CI Lower	Upper
Location	-2.4684	0.1215	-2.7065	-2.2302
Scale	0.26882	0.05136	0.18486	0.39093

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
1	0.000806	0.000182	0.000462	0.001211
5	0.001229	0.000255	0.000787	0.001895
10	0.001539	0.000324	0.001015	0.002479
50	0.003401	0.000951	0.002163	0.007365
90	0.007519	0.002989	0.004129	0.02443

Sex&T = M10

Tolerance Distribution

Parameter Estimates

Parameter	Estimate	Standard Error	95.0% Normal CI Lower	Upper
Location	-1.69949	0.08798	-1.87192	-1.52706
Scale	0.26882	0.05136	0.18486	0.39093

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
1	0.004733	0.001032	0.002583	0.006720
5	0.007217	0.001249	0.004699	0.009856
10	0.009036	0.001454	0.006287	0.01243
50	0.01998	0.004047	0.01430	0.03458
90	0.04416	0.01411	0.02735	0.1145

Sex&T = M240

Tolerance Distribution

Parameter Estimates

Parameter	Estimate	Standard Error	95.0% Normal CI Lower	Upper
Location	-2.56825	0.07148	-2.70834	-2.42816
Scale	0.26882	0.05136	0.18486	0.39093

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
1	0.000640	0.000152	0.000321	0.000923
5	0.000976	0.000175	0.000601	0.001313
10	0.001222	0.000192	0.000824	0.001615
50	0.002702	0.000445	0.002035	0.004142
90	0.005974	0.001642	0.003950	0.01351

Sex&T = M60

Tolerance Distribution

Parameter Estimates

Parameter	Estimate	Standard Error	95.0% Normal CI Lower	Upper
Location	-2.1978	0.1099	-2.4131	-1.9824
Scale	0.26882	0.05136	0.18486	0.39093

Table of Percentiles

Percent	Percentile	Standard Error	95.0% Fiducial CI Lower	Upper
1	0.001503	0.000377	0.000778	0.002303
5	0.002291	0.000500	0.001375	0.003475
10	0.002869	0.000607	0.001816	0.004440
50	0.006342	0.001604	0.004131	0.01235
90	0.01402	0.005040	0.008080	0.03998

Table of Relative Potency

Factor: Sex&T

Comparison	Relative Potency	95.0% Fiducial CI Lower	Upper
F10 VS F240	0.1418	0.07961	0.2205
F10 VS F60	0.2226	0.1294	0.4097
F10 VS M10	1.3075	0.7808	2.1080
F10 VS M240	0.1769	0.1024	0.2739
F10 VS M60	0.4151	0.2318	0.7325
F240 VS F60	1.5697	0.9527	3.1705
F240 VS M10	9.2192	6.0280	15.5573
F240 VS M240	**1.2472**	**0.8217**	**1.9450**
F240 VS M60	2.9269	1.7662	5.4778
F60 VS M10	5.8733	3.1507	9.8541
F60 VS M240	0.7946	0.4068	1.3006
F60 VS M60	1.8647	0.9481	3.3785
M10 VS M240	0.1353	0.08245	0.2067
M10 VS M60	0.3175	0.1836	0.5619
M240 VS M60	2.3468	1.4170	4.2720

[DRS]—for all three exposure durations (above bolded lines), there is no statistically significant difference between male and female rats, with the female rats being more sensitive with respect to pupil response.

A3.4 Binary Logistic Regression: AChE Depression vs logC, logT, Sex.

Link Function: Normit

Response Information

Variable	Value	Count	
AChE	1	35	(Event)
	0	265	
	Total	300	

300 cases were used
150 cases contained missing values ← these are the control rats

```
Logistic Regression Table

Predictor       Coef     SE Coef        Z      P
Constant      3.5240      0.9854     3.58  0.000
logC          3.2388      0.5848     5.54  0.000
logT          2.0605      0.3529     5.84  0.000
sex          -0.2140      0.1162    -1.84  0.066

Log-Likelihood = -82.335
Test that all slopes are zero: G = 51.468, DF = 3, P-Value = 0.000

Data Display

Estimated Parameters (Coefficients):

 Intercept     logC      logT       sex
   3.52401   3.23881   2.06051  -0.21395

Note: sex coded -1 for female and 1 for male.

 Variance-Covariance Matrix of Estimated Parameters:

 0.971028  0.543010  0.230580 -0.038771
 0.543010  0.341939  0.179392 -0.023391
 0.230580  0.179392  0.124566 -0.011837
-0.038771 -0.023391 -0.011837  0.013496
```

Toxic load exponent = 3.23881/2.06051 = 1.57 with standard error = 0.14 from the propagation of error formula, (3.23881/2.06051)*Sqrt[0.341939/3.23881^2 + 0.124566/2.06051^2 – 2*0.179392/(3.23881*2.06051)].

DEPARTMENT OF THE ARMY
U.S. Army Edgewood Chemical Biological Center
Aberdeen Proving Ground, Maryland 21010-5424

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AUTHORS	Bernard J. Benton, et al.
DATE	March 2005
CLASSIFICATION	UNCLASSIFIED

A copy of ECBC-TR-428 was mailed to you in April 2005. Please replace the front cover currently on the document with the attached, revised cover.

Sandra J. Johnson

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Chief, Technical Releases Office