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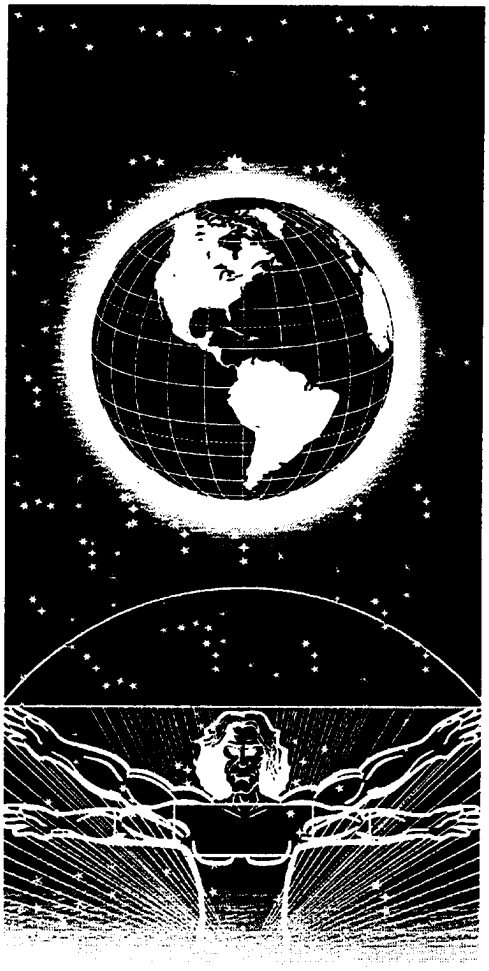

UNITED STATES AIR FORCE
ARMSTRONG LABORATORY

1995 TOXIC HAZARDS RESEARCH UNIT ANNUAL REPORT

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July 1996

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The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

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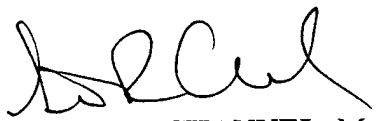

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13. ABSTRACT *(Maximum 200 words)*

This report presents a review of the activities of the ManTech Environmental Technology, Inc., Toxic Hazards Research Unit (THRU), for the period 01 October 1994 through 30 September 1995. ManTech Environmental's THRU is an on-site, contractor-operated, United States Air Force, Army, and Navy multidisciplinary research program. The THRU conducts descriptive, mechanistic, and predictive toxicology studies and toxicological risk assessments to provide data to predict health hazards and to assess the health risks associated with human exposure to chemicals and chemical materials of interest to the military. The major goal of the THRU's research efforts is to contribute to safe military operations, including safe occupational and environmental conditions. An additional goal of the THRU is to advance the state-of-the-art in toxicology research and risk assessment techniques.

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PREFACE

The 32nd Annual Report of the Toxic Hazards Research Unit (THRU) presents research and research support efforts conducted by ManTech Environmental Technology, Inc. on behalf of the U.S. Air Force, the U.S. Army, and the U.S. Navy under Department of the Air Force Contract No. F33615-90-C-0532. This document represents the fifth annual report for the current THRU contract and describes accomplishments from 01 October 1994 through 30 September 1995.

Operation of the THRU under this contract was initiated on 16 January 1991 under Project No. 6302, "Occupational and Environmental Toxic Hazards in Air Force Operations," Work Unit No. 63020002, "Toxic Hazards Research." This research effort is cosponsored by the Army Medical Research Detachment, Walter Reed Army Institute of Research (WRAIR), Work Unit Nos. 611102.S15L and 612787.878L, and by the Naval Medical Research Institute Detachment/Toxicology (NMRI/TD), Work Unit No. M0096.004.0006, "Criteria for Exposure Limits in Navy Operational Environments."

The Toxicology Division, Occupational and Environmental Health Directorate, Armstrong Laboratory (AL/OET), Wright-Patterson Air Force Base, Ohio, provided the technical direction for this contract. Lt Col Terry A. Childress, Director of the Toxicology Division, served as the Contract Technical Monitor. That portion of the work effort sponsored by the Army was under the direction of LTC Roland E. Langford, Detachment Commander, and LTC Daniel Caldwell, Senior Scientist of the Medical Research Detachment. That portion of the work effort sponsored by the Navy was under the direction of the NMRI/TD Officers-in-Charge, CAPT David A. Macys, MSC, USN and CAPT(S) Kenneth R. Still, MSC, USN. Darol E. Dodd, Ph.D. served as the ManTech Environmental THRU Program Manager.

The contents and the preparation of this report represent the combined effort of the ManTech Environmental staff of the THRU. Acknowledgment is made to Ms. Susie Godfrey and Ms. Shelia Brooks for their assistance in the preparation of this report.

TABLE OF CONTENTS

SECTION

SECTION 1

ABBREVIATIONS

Abbreviations

° C	Celsius
µg	Microgram
µM	Micromolar
ACCS	Accumulation Site
ADN	Ammonium Dinitramide
ADN	Ammonium Dinitramide
AL/OET	Armstrong Laboratory, Toxicology Division
AL/OEVM	Armstrong Laboratory, Comparative Medicine Branch
AP	Ammonium Perchlorate
aq	Aqueous
BES	Bioenvironmental Engineering
BF_3	Boron trifluoride
C	Control
CF_3I	Trifluoroiodomethane (Iodotrifluoromethane)
CH	Chloral hydrate
CO_2	Carbon dioxide
DCA	Dichloroacetic acid
DFA	Deferoxamine mesylate
DM-HMMS	Defense Mangement Hazardous Materials Management System
DOD	Department of Defense
DOT	Department of Transportation
DPA	2,2-Dichloropropionic acid
EM	Environmental Management
Fe(II)	Iron(II)
Fe(III)	Iron(III)
g	Grams
g/L	Gram/Liter
GD	Gestation Day
GLP	Good Laboratory Practice
h	Hours
H_2SO_4	Sulfuric acid
HM	Hazardous Material
HSC	Human Systems Center
HSR	Health and Safety Representative

HW	Hazardous Waste
IAP	Initial Accumulation Point
IP	Intraperitoneally
IU	International Units
m	Meter
M	Molar
mCi	Millicurie
metHb	Methemoglobin
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mM	milliMolar
mo	Month
mV	Millivolt
nmoles	Nanomoles
OI	Operating Instruction
OSHA	Occupational Health And Safety Act
pg	Picogram
pH	Negative $\log_{10}$ of the hydrogen ion concentration
pKa	Negative $\log_{10}$ of the acidity constant
ppm	Parts per million
QAA	Quality Assurance Associate
QAC	Quality Assurance Coordinator
QTIP	Quick Turn-in Process
RCRA	Resource Conservation and Recovery Act
SIDS	Screening Information Data Set
SOP	Standard Operating Procedure
T	Treatment
TCA	Trichloroacetic acid
TCE	Trichloroethylene
TCOG	Trichloroethanol glucuronide
TCOH	Trichloroethanol
THRU	Toxic Hazards Research Unit
TNB	1,3,5-Trinitrobenzene
TPH	Total Petroleum Hydrocarbons

SECTION 2

INTRODUCTION

INTRODUCTION

D.E. Dodd

This report presents a review of the activities of the ManTech Environmental Technology, Inc., Toxic Hazards Research Unit (THRU), for the period 01 October 1994 through 30 September 1995. ManTech Environmental's THRU is an on-site, contractor-operated, United States Air Force, Army, and Navy multidisciplinary research program. The THRU conducts descriptive, mechanistic, and predictive toxicology studies and toxicological risk assessments to provide data to predict health hazards and to assess the health risks associated with human exposure to chemicals and chemical materials of interest to the military. The major goal of the THRU's research efforts is to contribute to safe military operations, including safe occupational and environmental conditions. An additional goal of the THRU is to advance the state-of-the-art in toxicology research and risk assessment techniques.

The THRU conducts research on a variety of materials that may range from pure chemicals to poorly defined mixtures. They include, but are not limited to fuels, lubricants, solvents, additives, components of explosives, propellants, paints, solvents, structural materials, training agents, and combustion products. Descriptive toxicology is used to identify toxic effects, target organs, and dose-response effects associated with different exposure routes, concentrations, and durations. Mechanistic toxicology is performed to determine toxicokinetics, mechanisms of action, and dynamics of expression of the toxic effects of the material of interest. Predictive toxicology involves the development, validation, and application of computer simulation models to describe quantitative dose-response relationships based on quantified input parameters such as exposure concentration, partition coefficients, respiratory rate, blood flows, rate of metabolite formation, rate of chemical excretion, and metabolic enzyme constants. These models are used to define target organ toxicity based on the tissue-specific dose and are used in intra- and interspecies extrapolation. Data generated via descriptive, mechanistic, and predictive toxicology studies are used together with interpreted literature data to produce human health hazard risk assessments.

In accordance with the THRU contract's Statement of Work and specific technical directives (study requests) provided by the Contract Technical Monitor, the THRU also coordinates toxicology conferences, expert workshops, and program reviews. Research support benefitting both THRU and government research efforts is provided in the areas of special test equipment design, fabrication, validation, modification, and maintenance; mathematics and biometry; computer systems management and programming; necropsy and histology techniques; management of toxicology information databases; quality assurance; health and safety; and documentation and report preparation. The THRU's research support and administrative elements are integral to the quality, continuity, and productivity of its scientific research efforts.

The research and support efforts of the THRU represent a continuum of activities that may overlap two or more years depending upon the study scheduling and the extent of the research that is required. During this reporting period, studies performed in response to requirements of the Air Force included analyses of metabolites of trichloroethylene (TCE) in biological samples; development of methods for detecting and quantifying cell proliferation following administration of TCE; application of physiologically based pharmacokinetic (PBPK) models in predicting cardiac sensitization thresholds of

halon replacement chemicals; subchronic toxicity testing of CF_3I; gas uptake kinetics of HFC-236fa; and development of PBPK models for ethane exhalation or oxidative stress.

During this reporting period, the THRU continued to work on several study requests for toxicology research in support of the Army. The THRU conducted studies to evaluate the reproductive toxicity potential of 1,3,5-trinitrobenzene (TNB), ammonium dinitramide and modular artillery charge system (XM231/XM232). Mechanistic studies to determine the specific female reproductive effect of ammonium dinitramide or the cardiovascular response of XM231/XM232 were initiated. Additionally, the THRU provided specific analytical chemistry support for the Army's field and industrial hygiene program.

Toxic Hazards Research Unit technical directives that supported the Navy included investigation of trimethylolpropane phosphate-induced central nervous system sensitization; comparative toxicity of nitrophenols in human and rat hepatic tissue slices; and continuation of a statistical methods study to describe naval subpopulations for developing a basis for altering safety factors used in risk assessments applicable to military populations. Additionally, a number of technical support efforts in animal necropsy, histology, and equipment design and fabrication were conducted.

During this reporting period, the THRU provided work effort in support of several toxicology conferences and workshops, including the series of annual toxicology conferences that have been coordinated by the THRU since 1965. The proceedings of the 1994 toxicology conference, "Temporal Aspects in Risk Assessment for Noncancer Endpoints" were compiled by the THRU and distributed as a publication by the journal *Inhalation Toxicology* (Vol. 7, No. 6, 1995). The 1995 toxicology conference "Risk Assessment Issues for Sensitive Human Populations" was conducted in April at the Hope Hotel and Conference Center at Wright-Patterson Air Force Base (WPAFB). The proceedings of this conference have been compiled by the THRU for publication in the journal *Toxicology*. In August 1995, the THRU initiated its planning efforts for the 1996 toxicology conference, "Advances in Toxicology and Applications to Risk Assessment."

The execution of the THRU contract Statement of Work involves the integrated effort of a multidisciplinary staff of scientists, research technicians, research support, and administrative personnel. Sections 3 through 8 of this report emphasize the technical activities of the THRU. Sections 9 and 10 present highlights of the conferences and research support activities, including research engineering/fabrication, mathematics and statistics, pathology support, computer and electronic support, quality assurance, and health and safety. Section 11 of this report is a set of appendices that describe the THRU organization, its personnel, and its awards, publications and presentations.

Historically, the THRU has prepared annual reports on its research efforts since 1963. In general, these annual reports present summaries or highlights of the technical projects (study requests) that were directed by the Air Force, Army, and Navy. More descriptive reports on the THRU's research activities are prepared upon completion of study requests and are published as technical reports or peer-review publications (refer to "Products List for 1995" in Section 11). Technical reports also are prepared following the conferences and most workshops coordinated by the THRU. Copies of these technical reports are available from the National Technical Information Service or the Defense Technical Information Center.

SECTION 3

TRICHLOROETHYLENE CARCINOGENICITY PROJECT

3.1 CONVERSION OF TRICHLOROACETIC ACID TO DICHLOROACETIC ACID IN BIOLOGICAL SAMPLES

W.T. Brashear, M.M. Ketcha[1], D.K. Stevens[2], D.A. Warren[3], and C.T. Bishop[3]

ABSTRACT

Trichloroethylene (TCE) is an environmental contaminant in groundwater. Trichloroacetic acid (TCA), dichloroacetic acid (DCA), chloral hydrate (CH), trichloroethanol (TCOH), and trichloroethanol glucuronide (TCOG) have been identified as metabolites of TCE. Studies have shown that TCA and DCA are toxicologically significant metabolites which can induce liver tumors in B6C3F1 mice. Methods for the analysis of these metabolites are important for conducting pharmacokinetic studies. In this study, TCA and DCA were derivatized to their methyl esters by dimethyl sulfate under acidic conditions and analyzed by gas chromatography with electron capture detection. In developing a method for esterifying TCA and DCA, the conversion of TCA to DCA was observed in freshly drawn blood upon addition of acid. After blood was drawn from the animal, the amount of TCA converted to DCA by the addition of acid decreased with time. This conversion could be prevented by freezing blood samples overnight prior to the addition of acid. Further experiments demonstrated that this activity could be restored by the addition of dithionite to inactive blood samples, or by adding dithionite to methemoglobin prior to the addition of acid. The results reported here show that reduced hemoglobin may be involved in the acid catalyzed conversion of TCA to DCA.

INTRODUCTION

Trichloroethylene (TCE), a widely used degreasing and cleaning solvent, is an environmental contaminant commonly found in groundwater. Exposure to TCE is of concern because it has been found to be a rodent carcinogen (Bruckner et al., 1989). TCE is reported to be metabolized to chloral hydrate (CH), trichloroacetic acid (TCA), dichloroacetic acid (DCA), trichloroethanol (TCOH), and trichloroethanol glucuronide (TCOG) (Tanaka and Ikeda, 1968; Hathway, 1980). DCA and TCA, like TCE, cause mouse liver tumors (Bull et al., 1990; DeAngelo et al., 1991).

Analytical methods have been developed to analyze biological samples for TCE and its metabolites (Chen et al., 1993; Ikeda et al., 1972; Humbert and Fernandez, 1976; Breimer et al., 1974). Early colorimetric procedures used Fujiwara's method to analyze urine for TCA and TCOH (Ikeda et al., 1972). Later, more sensitive gas chromatographic methods analyzed urine for TCA, TCOH, and TCOG (Humbert and Fernandez, 1976). Urine was analyzed with and without sulfuric acid hydrolysis for TCOH and TCOG, and TCA was extracted into diethyl ether and derivatized to its methyl ester. Headspace gas chromatographic methods have been used for analyzing whole blood for TCA, TCOH and TCOG (Breimer et al., 1974). Blood was acidified with concentrated sulfuric acid to convert TCOG to free TCOH, and dimethyl sulfate esterified TCA to its volatile methyl ester. Here we report new findings regarding the conversion of TCA to DCA in freshly drawn whole blood samples. This conversion occurs in the presence of acid and reduced hemoglobin. Previous methods have recognized the need to

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prevent the conversion of TCE metabolites. Some investigators (Breimer et al., 1974) used lead acetate to prevent the enzymatic conversion of CH to TCOH in blood during incubation of samples for headspace analysis. They also reported that sulfuric acid will prevent the conversion of CH to TCOH by erythrocytes. Their recommendation is that blood samples be immediately mixed with lead acetate or sulfuric acid. Other methods have treated biological samples with sulfuric acid in order to extract DCA and TCA into an organic solvent (Hathway 1980; Gorecki et al., 1990). It is known that the addition of acid will affect TCOH levels since acid will hydrolyze TCOG to free TCOH (Breimer et al., 1974; Garrett and Lambert, 1966). Therefore, acid treated samples analyzed for TCOH will yield total TCOH since blood and urine have free and conjugated TCOH (Gorecki et al., 1990). Our experiments show that blood levels of TCA and DCA are also affected by the addition of acid. This metabolite interconversion occurs in freshly drawn blood which has been acidified with sulfuric acid or hydrochloric acid for the extraction and derivatization of TCA and DCA to their methyl esters.

Experimental

Laboratory Animals

Laboratory animals were obtained from Charles River Laboratories (Kingston, NY). Male B6C3F1 mice weighing 17-23 g (6-7 weeks) were used for this study. Animals were exposed to 600 ppm TCE via inhalation for 4 h, and sacrificed by CO_2 asphyxiation. Unexposed mice were also sacrificed by CO_2 asphyxiation for control tissue samples.

Chemicals & Equipment

TCE (99+%, 25,642-0), TCA (99+%, 25,139-9), DCA (99+%, D5,470-2), 2,2-dichloropropionic acid (94%, 29,115-3), methyl dichloroacetate (99+%, 10 840-5), methyl trichloroacetate (99%, 24, 249-7), and dimethyl sulfate (99+%, D18, 630-9) were obtained from Aldrich Chemical Company (Milwaukee, WI). Lead acetate was obtained from Mallinckrodt (Paris, KY). Sulfuric acid, hydrochloric acid, and hexane were obtained from Fisher Scientific (Fair Lawn, NJ). Deferoxamine mesylate (DFA), bovine albumin (fraction V), and hemoglobin (human, rat, and mouse) were obtained from Sigma Chemical Co. (St. Louis, MO). The hemoglobin was in the form of methemoglobin. Sodium trichloroacetate (97%), reagent grade sodium dithionite (sodium hydrosulfite), sodium nitrite, and ferrous sulfate were also obtained from Aldrich Chemical Co. A Haake-Buchler vortex evaporator (Saddlebrook, NJ) was used to vortex and heat samples. Samples were analyzed on a Hewlett Packard 5890 gas chromatograph with a Hewlett Packard 7673A liquid autosampler (Hewlett Packard, Avondale, PA). The chromatograph was equipped with an electron capture detector (63Ni, 15 mCi) and a 30m x 0.53mm Supelco Wax 10 column (Supelco, Belfonte, PA). Chromatographic conditions were: injector 175 °C; initial temperature 70 °C; initial time 15 min; heating rate 15 °C/min; final temperature 190 °C; final time 5 min; and detector temperature 300 °C. The carrier and make up gas was 5% methane in argon and the carrier flow rate was 6 mL/min.

Assay Procedures

Samples were analyzed for DCA and TCA by modification of a method by Maiorino et al. (Maiorino et al., 1980). To prevent the conversion of TCA to DCA in freshly collected blood, samples were frozen overnight at -20 °C prior to analysis. After thawing, a 0.1 mL sample of blood, was placed in a 2 mL vial with 0.1 mL of water, and 0.1 mL of 10 µg/mL 2,2-dichloropropionic acid (internal standard). The vials were placed on ice and allowed to cool for 30 min, 0.5 mL of concentrated sulfuric acid, and 0.1 mL aliquot of dimethyl sulfate were added. The vials were capped and vortexed on a

shaking incubator for 30 min at 60 °C. After vortexing, the vials were allowed to cool and 1 mL of hexane was added. The samples were extracted on the incubator shaker for 60 min at 55 °C and centrifuged at 2000xg for 10 min. The hexane layer was removed and analyzed by gas chromatography. Standards were made using blood from control animals which had been frozen overnight. A 0.1 mL aliquot was combined with 0.1 mL of 10 μg/mL 2,2-dichloropropionic acid (internal standard) and a 0.1 mL aliquot of an aqueous standard of DCA and TCA. The standards were derivatized and analyzed as previously described.

The recovery of DCA and TCA is a function of both the reaction efficiency of the dimethyl sulfate derivatization and the extraction efficiency of the methyl esters into hexane. The reaction efficiency was obtained by comparing the areas of DCA and TCA derivatized and extracted from spiked blood samples with the area of blood samples spiked with authentic methyl esters of DCA and TCA and extracted into hexane. The extraction efficiency was obtained by comparing the area of authentic methyl esters of DCA and TCA extracted from spiked blood samples into hexane with the areas of unextracted methyl esters in hexane.

To illustrate the dependency of the conversion of TCA to DCA on reduced hemoglobin, the following protocol was used. Into small glass centrifuge tubes were placed 100 μL each of neutral aqueous sodium trichloroacetate solution (20 μg/mL), the internal standard solution, and the sulfuric acid solution (10%), and either water, sodium dithionite (1 M), or sodium nitrite (1 M). One-hundred microliters of the test matrix was then added to the tubes. The contents were mixed by brief vortexing and allowed to stand at room temperature for approximately 30 min. The samples were then processed as above and analyzed for a change in the TCA to internal standard ratio as a result of TCA dechlorination to DCA. Specific quantitation of TCA and DCA was not performed. Aqueous bovine albumin (100 mg/mL) served as a proteinaceous negative control. The results of the other matrices were all referred to this matrix. The test matrices were: freshly drawn, heparinized mouse blood; fresh mouse blood + dithionite; fresh mouse blood + nitrite; 8 month-old human blood, drawn into a heparin tube and stored at 4 °C in a refrigerator (this sample was extremely hemolyzed); old human blood + dithionite; plasma drawn from centrifuged fresh mouse blood; plasma drawn from fresh mouse blood spiked with 20 μg/mL neutral sodium trichloroacetate, allowed to stand for 30 min prior to separation by centrifugation; an aqueous solution of methemoglobin (150 mg/mL); methemoglobin + dithionite. Additionally, the effects of deferoxamine (DFA), a known Fe(II) chelator, also was examined. Two different addition sequences were used. In the first sequence, DFA was added to blood prior to the addition of the blood to the tubes containing TCA in sulfuric acid. This was designated as "blood + DFA a". Secondly, neutralized TCA was mixed with fresh blood, prior to the addition of DFA to blood, which was added to sulfuric acid. This was designated "blood + DFA b", and more closely resembled the situation of an actual sampling protocol. The activity of an aqueous solution of iron(II) sulfate was also examined. Using the above protocol, concentrations of 0, 2.5, 5, 10, 25, and 50 mM iron(II) sulfate were tested.

RESULTS

DCA, TCA Assay

The assay developed for DCA and TCA had a limit of detection of 1.0 μg/mL for the analysis of 0.1 mL blood samples. This was based on a signal-to-noise level greater than three. The on-column limit of detection for the methyl esters of DCA and TCA was estimated at 50 pg, which was also based on a signal-to-noise ratio greater than three. The method produced quadratic standard curves for DCA and TCA as shown in Figure 3.1-1.

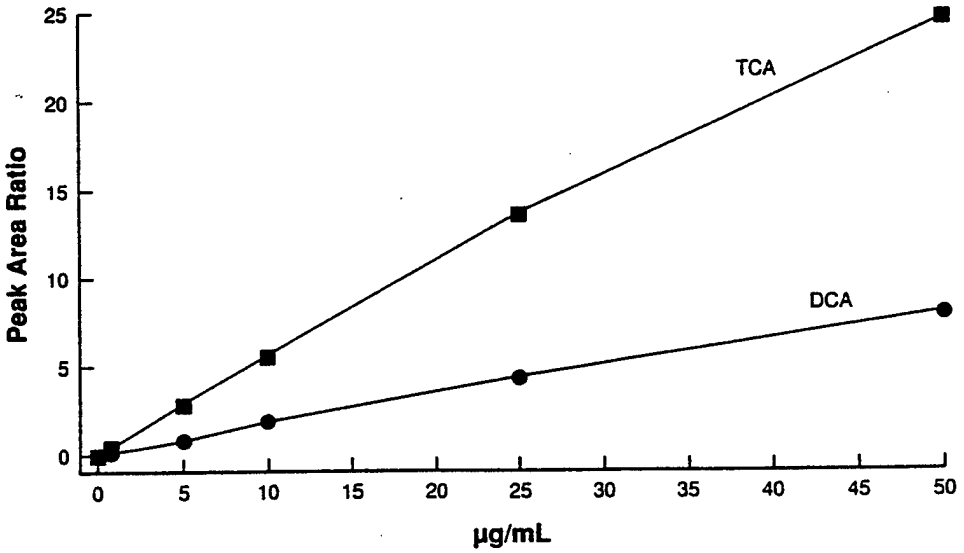

Figure 3.1-1 **Standard curves for DCA and TCA extracted and derivatized from whole blood at 1, 5, 10, 25, and 50 μg/mL each. The peak area ratio relative to the internal standard (DPA,10 μg/mL) is plotted against the amounts of DCA and TCA (μg/mL).**

Typical curves for DCA and TCA were: $y=-0.0510x^2+1.836x+0.0484$, $r^2>0.999$, and $y=-0.0203x^2+5.93x+0.0808$, $r^2>0.999$, respectively. A chromatogram of DCA and TCA extracted and derivatized from blood spiked at 10 μg/mL each is

shown in Figure 3.1-2. The retention times of TCA, DCA, and DPA (internal standard) were 8.0, 9.1, and 5.1 min, respectively. The reaction efficiency of the dimethyl sulfate derivatization averaged 75% (n=12), and the extraction efficiency averaged 67% (n=12). The extraction and reaction efficiencies were measured in duplicate at 5, 25, and 50 μg/mL for both DCA and TCA. The precision of this method was evaluated in whole blood. The intra-day variability for DCA and TCA had an average coefficient of variation of 7%, 4%, and 4% at 5, 25, and 50 μg/mL, respectively (n=7). The inter-day variability for DCA and TCA had an average coefficient of variation of 8%, 8%, and 7% at 5, 25, and 50 μg/mL, respectively (n=6).

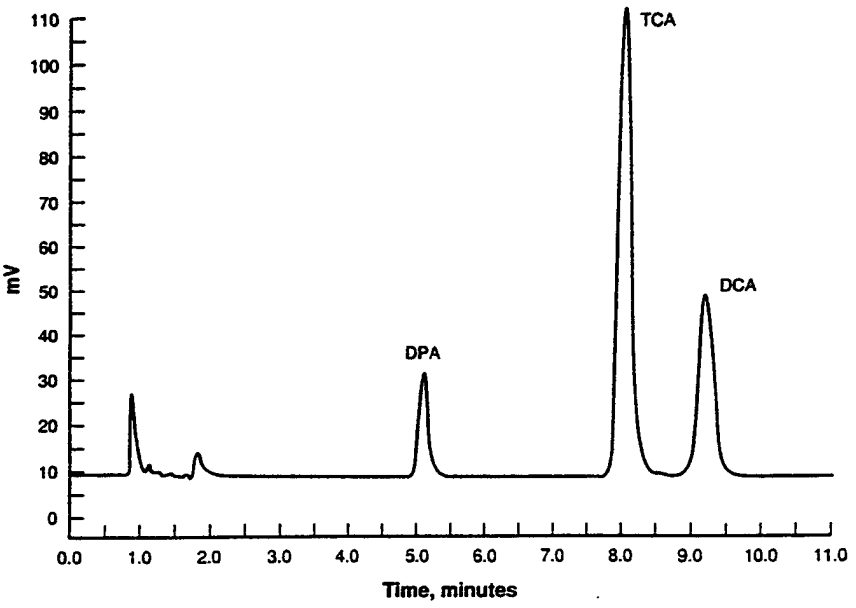

Figure 3.1-2 **Chromatogram of 10 μg/mL TCA and DCA in whole blood with internal standard DPA (10 μg/mL). Retention times of DPA, TCA, and DCA were 5.1, 8.0, and 9.1 min, respectively. Units of the y axis is detector response in millivolts.**

Conversion of TCA to DCA

Fresh control blood was collected from mice and 0.1 mL aliquots were placed into tubes kept at room temperature which contained 0.1 mL of 33 μg/mL neutral TCA (20 nmoles). At time intervals of 0, 1, 5, 10, 15, 30, and 60 min, 0.1 mL of 10% sulfuric acid was added. Three hours after the blood was taken, the samples were derivatized with dimethyl sulfate and analyzed. The results, shown in Figure 3.1-3, suggest that TCA is converted to DCA by the addition of 10% sulfuric acid to freshly drawn blood. The conversion of TCA to DCA also occurred when 15% hydrochloric acid, and other concentrations

of sulfuric acid (5%, 25%, 50%, and 100%) were added to fresh blood (data not shown). The data shown in Figure 3.1-3 represents an average of four experiments in which TCA in fresh mouse blood was converted to DCA under acidic conditions with 10% H_2SO_4. The amount of conversion of TCA to DCA varied from 20% to 70%. However, the sum of DCA and TCA remained constant over the course of the experiment (20 ± 0.9 nmoles).

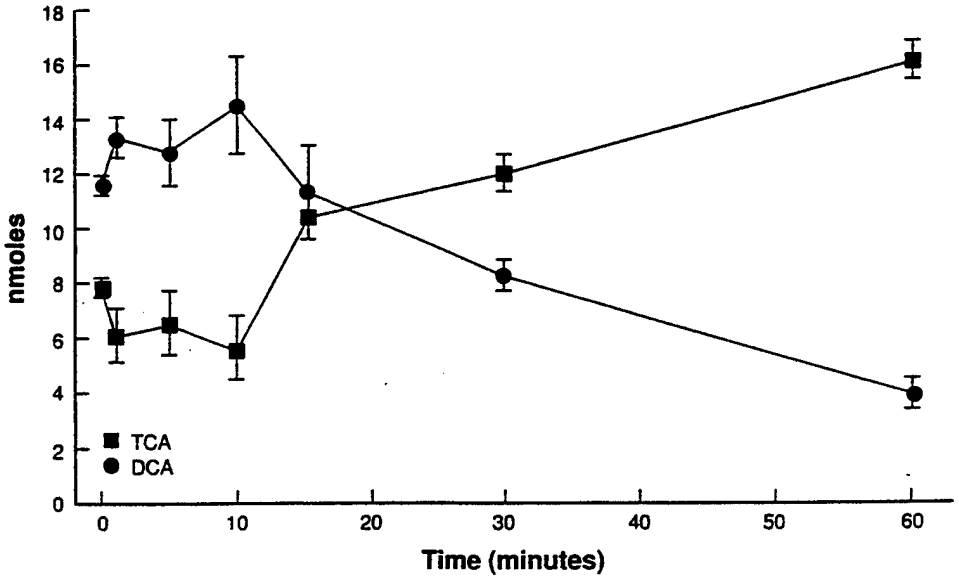

Figure 3.1-3 Conversion of TCA to DCA in fresh blood samples from B6C3F1 mice spiked with 0.1 mL of 33 μg/mL TCA (20 nmoles). Concentrated sulfuric acid was added at t=0, 1, 5, 10, 15, 30, and 60 min.

A 0.1 mL aliquot of fresh blood from a control animal was added to an ice-cold vial which contained both TCA and 20% lead acetate. Addition of 0.1 mL 10% sulfuric acid converted 80% of the TCA to DCA. However, if a TCA-spiked blood sample was first frozen overnight at -20 °C prior to analysis, TCA was not converted to DCA with the addition of 10% sulfuric acid. In this sample, the TCA level was consistent with the expected value. The addition of sulfuric acid to fresh mouse plasma spiked with TCA did not convert any TCA to DCA.

To demonstrate the effect of treating blood samples from exposed mice with acid, mice were exposed to 600 ppm TCE for 7 h. Following exposure, individual mice were sacrificed and blood samples were obtained at: t= 0.08, 0.25, 0.5, 0.75, 1.5, 3, 5, 7, 9, and 18 h. Blood samples were collected at each time point and immediately split into two portions. One portion was put into an equal volume of 10% sulfuric acid and kept on ice, the second was combined with an equal volume of distilled water and kept on ice. After collection, samples were stored at -20 °C for 24 h and analyzed for DCA and TCA. As shown in Figure 3.1-4, the samples immediately combined with acid contained DCA and TCA. Samples which were not combined with 10% sulfuric acid had higher concentrations of TCA, but no DCA.

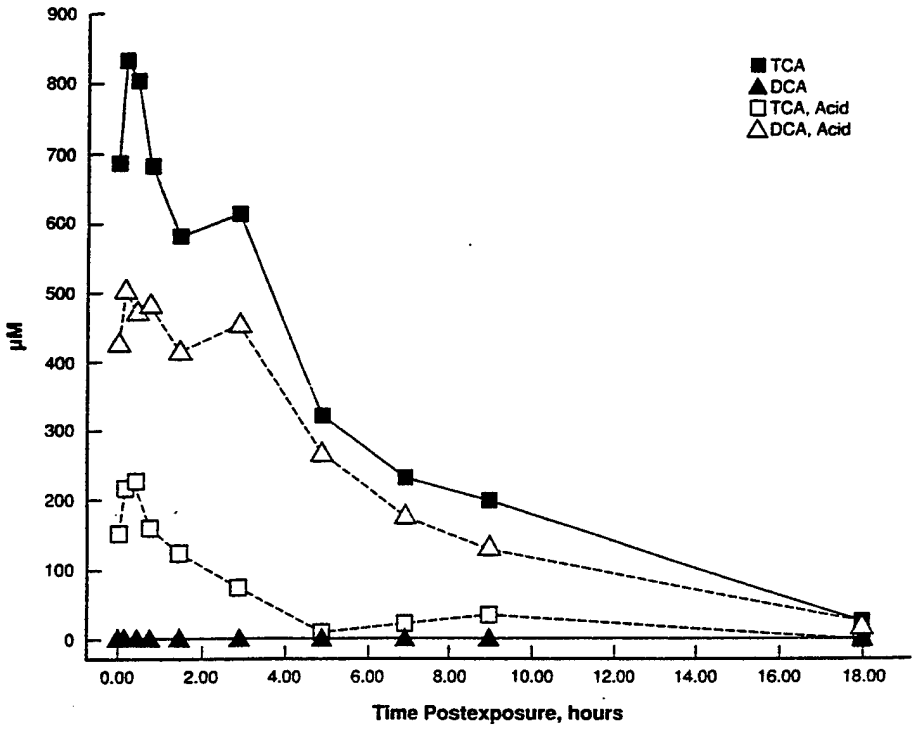

Figure 3.1-4 **Conversion of TCA to DCA in fresh blood samples from mice exposed to TCE. Samples at each time point were split into 0.1 mL portions. One portion was combined with 0.1 mL 10% sulfuric acid (DCA, Acid and TCA, Acid), and the second with 0.1 mL water (DCA, TCA).**

Table 3.1-1 summarizes the ability of various matrices to catalyze the conversion of TCA to DCA. The loss of TCA is expressed relative to the bovine albumin control in which no conversion of TCA was expected and none was observed. Reduced blood samples, i.e., those to which dithionite had been added were all active in catalyzing this reaction, even 8-month-old (hemolyzed) blood, as well as reduced, purified hemoglobin. Fresh blood was partially active while fully oxidized bloods, either the 8-month-old blood sample or the fresh blood to which nitrite had been added, as well as the plasma

samples, had no activity towards dechlorination of TCA. There was no difference in TCA content of freshly separated plasma spiked with TCA with that of whole blood which had been spiked and allowed to stand for 30 min prior to plasma separation. Methemoglobin had no activity towards the dechlorination of TCA, while methemoglobin with dithionite (reduced hemoglobin) extensively dechlorinated TCA. There was a difference in the effect of DFA depending on whether it was added to blood containing TCA (blood + DFA b), or whether it was added to the blood prior to addition to the TCA in sulfuric acid (blood + DFA a). The latter inhibited the conversion of TCA to DCA, while the former did not. Finally, none of the aqueous solutions of Fe(II) were capable of catalyzing the dechlorination of TCA.

TABLE 3.1-1 EFFECT OF DIFFERENT MATRICES ON THE CONVERSION OF TCA TO DCA

Matrix	Ave % Loss*	SEM (n=3)	DCA Formed
Fresh Blood (mouse)	34	9	Yes
Fresh Blood (mouse) + Nitrite	<5	ND	No
Fresh Blood (mouse) + Dithionite	83	3	Yes
Stored Human Blood	<5	ND	No
Stored Human Blood + Dithionite	90	2	Yes
Plasma (mouse)	<5	ND	No
Plasma (mouse) from TCA Spike Blood	<5	ND	No
metHb	<5	ND	No
metHb + Dithionite	93	1	Yes
Blood (mouse) + DFA a**	<5	ND	No
Blood (mouse) + DFA b***	40	10	Yes
Fe(II) aq	<5	ND	No

* **Expressed as percent of bovine albumin control.**
** **DFA added prior to the addition of TCA.**
*** **DFA added after the addition of TCA.**

DISCUSSION

This assay was developed for the determination of DCA and TCA in biological samples. However, the analysis of DCA and TCA in blood is complicated by an artifactual conversion of TCA to DCA. The conversion of TCA to DCA in fresh blood is an important concern for the analysis of these metabolites of TCE. Published methods for the analysis of TCA (Ikeda et al., 1972; Breimer et al., 1974; Gorecki et al., 1990) in biological fluids routinely acidify samples with strong acid. This step is necessary in order to extract acids such as DCA (pKa 1.3) and TCA (pKa 0.7) into an organic solvent for methylation. Similar derivatization reactions developed for trifluoroacetic acid, such as the formation of methyl esters with

N,N-dimethylformamide dimethylacetal (Karashima et al., 1977a), and derivatization with ethereal phenyldiazomethane (Karashima et al., 1977b) adjust biological samples to low pH values for the same reason. An alternative method for the derivatization of TCA is needed, and there are several possibilities. One method would be to extract and derivatize plasma. This would eliminate any conversion catalyzed by reduced hemoglobin. Conversion of TCA to DCA can be prevented by freezing blood samples at -20 °C overnight before adding acid for derivatization. Freezing hemolyzes red blood cells and facilitates the oxidation of Fe(II) to Fe(III). Blood samples may be treated with sodium nitrite which will oxidize Fe(II) to Fe(III). It may also be possible to derivatize TCA in a biological sample by adding base, lyophilizing to remove water, and dissolving the residue in an organic solvent such as methanolic BF_3 for derivatization.

Our work has shown that the acidification of fresh whole blood samples can lead to erroneous results. This has been demonstrated to occur *in vitro* with fresh blood spiked with TCA, and also with fresh blood obtained from mice exposed to TCE. Plasma proteins do not appear to be responsible for the conversion of TCA to DCA since the reaction does not occur in fresh plasma from mice. The finding that pretreating blood with lead acetate does not prevent the TCA to DCA conversion indicates that the conversion may not be due to enzymatic activity. The necessary dependence on reduced hemoglobin is clearly evident in the data presented in Table 3.1-1. Only fresh blood samples, and samples which had dithionite (an Fe(III) reducing agent) added, dechlorinated TCA. The data shown in Figure 3.1-3 demonstrate that the ability of fresh blood to convert TCA to DCA decreases with time. This may be attributed to the loss of reduced hemoglobin from blood while standing at room temperature. The purified reduced hemoglobin data, shown in Table 3.1-1, indicate that the hemoglobin component of the blood is the active constituent. The fact that no difference was observed between the spiked plasma sample and plasma separated from spiked blood indicates that this conversion only takes place upon acidification in the presence of the hemoglobin, and that TCA is stable in blood at physiologic pH, an important consideration in sample handling.

DFA inhibited the dechlorination of TCA only when DFA was added to blood prior to the addition to the TCA in sulfuric acid (blood + DFA a). When DFA was added to blood already containing TCA (blood + DFA b), DFA failed to inhibit the dechlorination of TCA. The latter more closely resembles actual sampling situations, and hence it can be concluded that the addition of DFA would not be a solution preventing the TCA to DCA conversion. However, in the absence of TCA, DFA is able to sequester the iron from the later, subsequent addition of TCA. Finally, the activity of aqueous solutions of Fe(II) were assessed. Concentrations ranging from 0 to 50 mM were inactive towards dechlorination of TCA. This indicates that for the electron transfer reaction to occur, the iron must be complexed, either with sulfur, as in ferredoxin, or extensively delocalized nitrogens, as in the case of the pyrrolic porphyrin ring structure of hemoglobin.

Similar reactions have been previously reported. Cammack et al. (1992) reported that ferredoxin, an iron-sulfur containing protein involved in photosynthesis, was capable of photochemically reducing TCA to DCA using visible light. It was found that the ferredoxin iron had to be maintained in the reduced state with dithionite for the reaction to occur. They speculate that the iron-sulfur clusters were the origin of the reducing electrons. Rusling et al. (1990) found that TCA could be reductively dehalogenated by Vitamin B_{12}, a transition metal complex of cobalt (16). This reaction occurred at pH 3 and was found to be spontaneous when Vitamin B_{12} was in the reduced form. Additionally, Manno et al. (1989) and Ferrara et al.

(1993) have reported that human hemoglobin was capable of reductive activation of carbon tetrachloride. Their system was totally anaerobic and, again, the reduced form of the iron was maintained with dithionite.

Conclusion

The results reported here show that it is critically important to evaluate the ability of metabolites to interconvert during analytical procedures, particularly when collecting data for pharmacokinetic analysis and assessing the metabolite(s) of TCE responsible for its carcinogenicity. Our data has shown that the addition of acid to fresh blood samples can convert TCA to DCA. Blood samples need to be frozen overnight at -20 °C prior to the addition of acid for DCA/TCA analysis.

Acknowledgment

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3.2 IMMUNOHISTOCHEMICAL DETECTION AND QUANTIFICATION OF PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA)-POSITIVE HEPATOCYTES AFTER ADMINISTRATION OF TRICHLOROETHYLENE TO MALE MICE

J.R. Latendresse, J.H. Grabau[1], K.T. Geiss,[1] and S.R. Channel[2]

ABSTRACT

Trichloroethylene (TCE) is a widely used industrial solvent and degreasing agent. Because it is commonly detected as a ground water contaminant, its potential to adversely affect human health has been studied and debated for years. One potential mechanism of TCE-induced mouse hepatocellular tumors is the formation of excessive active oxygen species (AOS), possibly during lipid peroxidation in hepatocytes. Target (proteins, lipids, DNA) interaction with free radicals can result in activation of signaling molecules, transcription factors, and/or the induction of oxidative stress-responsive genes which are potential pathways for triggering cell proliferation, paramount to tumorigenesis. To test this hypothesis using our experimental approach, the non-invasive proliferating cell nuclear antigen (PCNA) method was selected over the invasive bromodeoxyuridine and H3-thymidine incorporation mehtods, both which require invasive pre-administration that could potentially generate AOS. The purposes of this study were to utilize immunohistochemistry to determine the efficacy of PCNA antibody to detect TCE-induced hepatocellular proliferation and to measure the magnitude and time course of the proliferative response. Groups of mice were orally administered water, corn oil, or TCE (400, 800, or 1200 mg/kg) in corn oil in equal volumes (once/day, 5 days/week) for 2, 3, 6, 10 14, 21, 28, 35, 42, 49, and 56 days.

Histopathology, immunohistochemistry, and cell counts by image analysis revealed that PCNA antibody is an effective method for detection of TCE-induced proliferation of hepatocytes in tissue sections, and the results suggest that TCE is best classified as a mitogen rather than a cytotoxicant.

INTRODUCTION

Trichloroethylene (TCE) is a widely used industrial solvent and degreasing agent. Because it is commonly detected as a ground water contaminant, its potential to adversely affect human health has been studied and debated for many years. The primary concern about TCE environmental exposures arises from National Toxicology Program (NTP) studies that have shown TCE to be a mouse hepatocarcinogen when given in high doses by corn oil gavage (NCI, 1976; NTP, 1990).

One potential mechanism for hepatocellular carcinomas caused by exposure to TCE is the formation of excessive active oxygen species (AOS), possibly during lipid peroxidation in hepatocytes. Target (proteins, lipids, DNA) interaction with free radicals can result in activation of signaling molecules, transcription factors, and/or the induction of oxidative stress-responsive genes, all of which are potential pathways for triggering cell proliferation, paramount to tumorigenesis. To test our hypothesis of biological effects following exposure to TCE, we proposed to measure AOS by electron paramagnetic

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resonance (Steel-Goodwin et al., 1995), lipid peroxidation by measurement of thiobarbituric acid reactive species (TBARS) (Channel et al., 1995), oxidative DNA damage by measurement of 8-hydroxy-deoxyguanosine using HPLC (Kidney and Channel, 1995), and cell proliferation.

Cell proliferation can be detected in tissue sections utilizing [3H]-thymidine (Elcombe et al., 1985) or bromodeoxyuridine (BrdU) incorporation (Wilson et al., 1987) and colorimetric labeling of cell cycle-specific proteins expressed during DNA replication (PCNA for example, Foley et al., 1991). Both [3H] - thymidine and BrdU require invasive pre-administration by injection or mini-pump implant that could potentially generate AOS. Tritiated thymidine has the added disadvantage of a lengthy detection interval. Because we intended to measure potential AOS caused by TCE exposure, we preferred the non-invasive PCNA method. Levels of PCNA, a 33 kD nuclear auxiliary protein of DNA polymerase delta, increase rapidly as the cells go into mid-G1 phase, peak during the DNA-proliferative S phase, and decline in early G2 phase (Celis and Celis, 1985; Kurki et al., 1986). The purposes of this study were to utilize immunohistochemistry to determine the efficacy of PCNA antibody to detect TCE-induced hepatocellular proliferation and to measure the magnitude and time course of the proliferative response.

METHODS

Male B6C3F1 mice were obtained at 12 weeks of age (25-30 g) from Charles River Laboratories (Kingston, NY). They were housed 5 mice per plastic cage provided with hardwood chip bedding and maintained on a 12-h light/dark cycle at a constant temperature of 22±1 °C and humidity 35-50%. The mice were provided with water and Purina Forumlab #5008 *ad libitum*. One week prior to the study start, mice were implanted with subcutaneous magnetic identification micro-transponders (BMDS, Biomedic Data Systems, Maywood, NJ) as per manufacturer's instructions. Mice were then randomly assigned to treatment groups and returned to their appropriate cages. All animals were housed in the same room under the conditions stated previously. Animal weights were recorded at the study start and weekly thereafter.

Trichloroethylene, 99.5+% without antioxidant additives, was ordered from Aldrich Chemical Company (Milwaukee, WI, Lot No. MF 01428EF). Gavage solutions were prepared in corn oil (Mazola™ , Best Foods, Somerset, NJ) in concentrations, based on body weight, to deliver 0, 400, 800, and 1200 mg/kg in a volume of 0.5 mL. In addition to the vehicle control, a water-only group was included. Fresh solutions were prepared each week, and the concentration adjusted on the mean body weight of each treatment group. Dosing solutions were analyzed by gas chromatography immediately following preparation, and again at weeks' end to insure potency. All gavage solutions were stored at 4 °C.

Gavage was performed each morning at 0800 h, five days a week for 8 weeks using 20 ga. feeding needles fitted to glass syringes. Animals were closely observed after each dosing.

Liver samples were obtained on study days 2, 3, 6, 10 14, 21, 28, 35, 42, 49, and 56, starting at 1300 h exactly on each day to control for time between dosing and harvest. Mice from each treatment group were randomly selected and weighed. Euthanasia was accomplished by rapid CO_2 asphyxiation. The liver was quickly removed, and a 2mm thick cross-section of the entire median lobe was fixed in 10% neutral buffered formalin for 15 h, dehydrated in ethanol and Hemo-De (Fisherbrand, Fisher Scientific, Pittsburgh, PA), and paraffin-embedded at less than 60 °C using a Histomatic MVP (Fisher

Scientific, Pittsburgh, PA). Four μm-thick sections were cut, mounted on ChemMate Plus slides (BioTek Solutions, Santa Barbara, CA), and air dried. Sections were deparaffinized in xylene and then rehydrated through a graded ethanol series to PBS buffer (pH 7.4).

Immunohistochemical processing of the sections was performed on a TechMate 1000 (BioTek Solutions, Santa Barbara, CA) automated immunostaining system using a modification of the method of Foley et al.,1991. This allowed us to perform consistent and reproducible immunoanalysis of the samples using an optimized programmed method, process a number of slides (20-60) at a time, and remove a degree of human variability while performing the analysis. This instrument utilizes capillary gap flow technology in performing the pre-programmed immunohistochemical protocols. Briefly, for antigen retrieval, sections were microwaved in citrate buffer (BioTek Solutions, Santa Barbara, CA), then reacted with hydrogen peroxide (3%) (20 min), and blocked with normal horse serum (BioTek Solutions, Santa Barbara, CA) (20 min). Slides were then incubated overnight at room temperature with mouse monoclonal antibody generated by immunizing BALB/c mice with recombinant PCNA and fusing with myeloma (SP2/0-Ag 14) (PCNAZ [Ab-1]; Oncogene Science, Cambridge, MA). Detection of PCNA-positive nuclei was performed using biotinylated horse anti-mouse secondary antibody (BioTek Solutions, Santa Barbara, CA) and avidin-biotin complex (ABC kit, BioTek Solutions, Santa Barbara, CA) (20 min incubation for each, at room temperature), followed by incubation in peroxidase/diaminobenzidine (BioTek Solutions, Santa Barbara, CA) for 21 min. Tissues were counterstained with hematoxylin (BioTek Solutions, Santa Barbara, CA).

Quantitative image analysis was performed using a Quantimet 570c Image Analysis System (Leica, Inc., Deerfield, IL). Digital color images of liver sections immunohistologically labeled for PCNA were analyzed to detect positive and negative hepatocyte nuclei. Detection criteria were feature color identification between brown (positive) and blue (negative) nuclei, as well as nuclear morphological characteristics (area, fullness, aspect ratio and roundness). Serial images were analyzed from each liver section by digitizing adjacent microscopic fields along a linear path that transversed the section along its greatest width. Data were collected in this manner for a minimum of 18 fields per liver section.

Treatment differences were determined by ANOVA and plotted using SigmaStat™ and SigmaPlot™ software, respectively (Jandel Scientific, San Rafael, CA). If found to be significant, appropriate post-hoc comparisons such as Dunnett's method were applied.

RESULTS

Oral gavage of TCE in corn oil caused a significant burst of cell proliferation centered around Day 10 (Figure 3.2-1). Although the elevation was only statistically significant in the 1200 mg/kg treatment group, a dose response trend was evident. PCNA-positive cells, as well as mitotic figures, were present in centrilobular, midzonal, and periportal regions with no apparent predilection for a particular lobular distribution (Figure 3.2-2). Cytotoxicity manifested as hepatocellular necrosis was not observed in any dose groups.

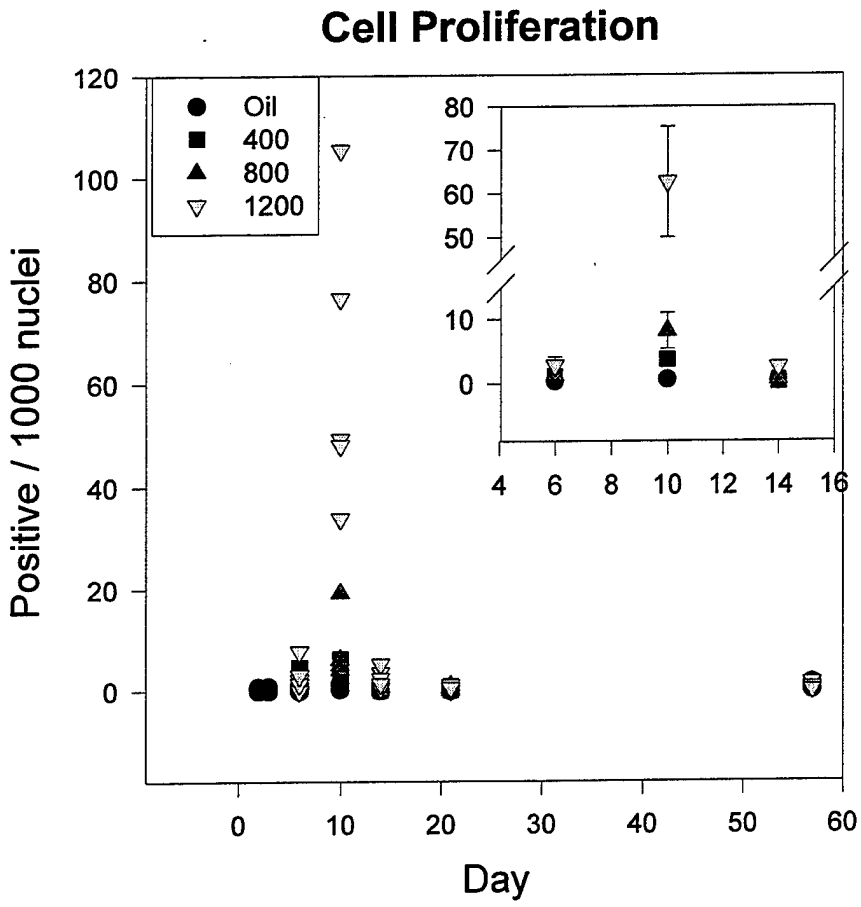

Figure 3.2-1. **Hepatocyte Proliferation Indices Detected by Immunoreactivity to PCNA in Liver Sections. Cell proliferation was significantly ($p<0.05$) increased on treatment day 10 in the 1200 mg/kg TCE-exposure group compared to corn oil controls. Note: N=5 for all groups**

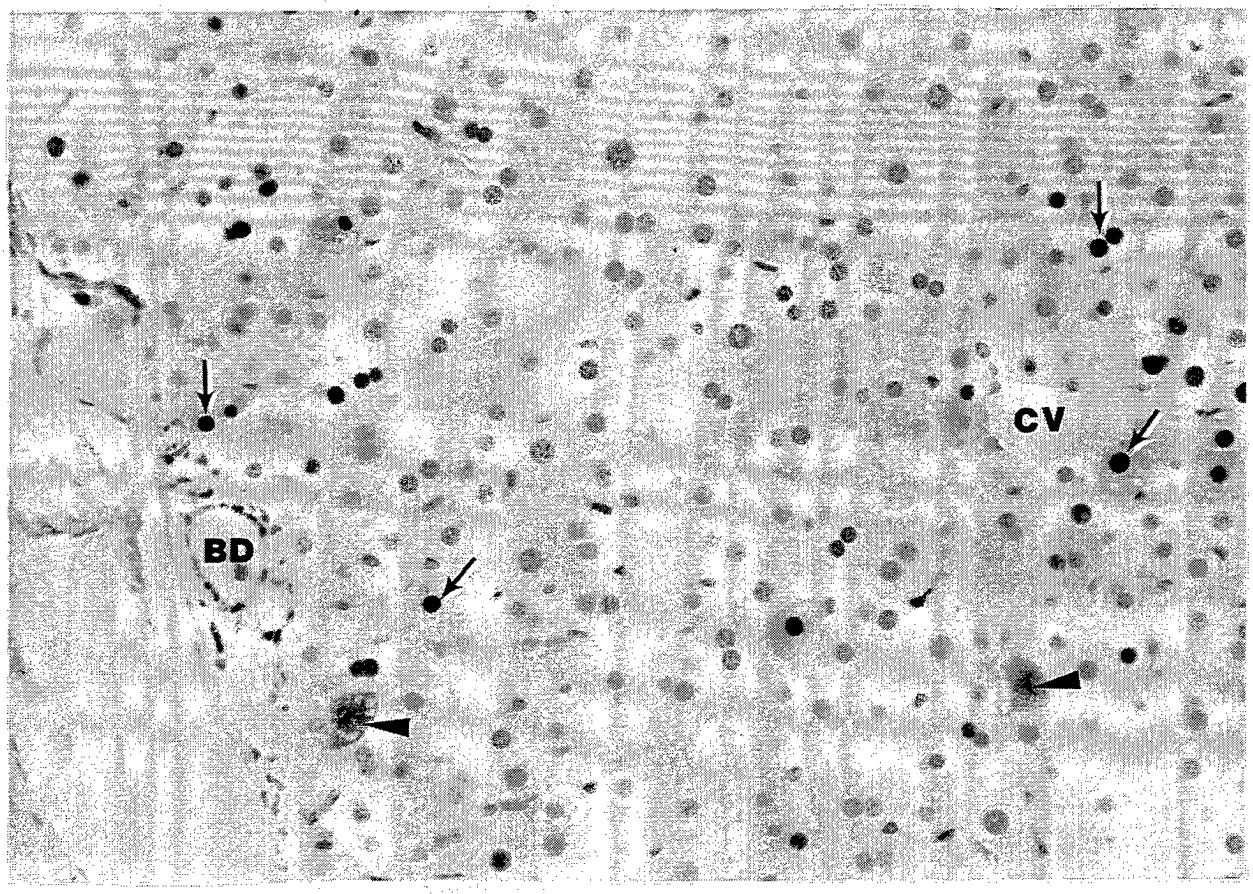

Figure 3.2-2. Day 10. Photomicrograph of immunolabeled PCNA-positive hepatocellular nuclei (arrows) from a 1200 mg/kg TCE-exposed mouse. Immunolabeled nuclei (arrows) are present in both periportal (near bile duct, BD) and centrilobular (near central vein, CV) regions of the hepatic lobule. Note cells in mitosis (arrow heads). Hematoxylin counter-stained. x333.

DISCUSSION

Our data shows that detection of PCNA utilizing immunohistochemistry is an effective means to detect and quantify cell proliferation over time in liver resulting in exposure to TCE. Moreover, this *in situ* method allows for assessment of anatomical distribution of the proliferative response within the hepatic lobule.

Cell proliferation resulting from chemical exposures in the liver is often classified as either cytotoxic or mitogenic (Goldsworthy et al., 1991; Butterworth et al., 1992). Cytotoxicants produce necrosis and regenerative growth. Sustained cell

proliferation resulting from regenerative growth may increase the frequency of spontaneous mutations or fix mutations prior to DNA repair. In contrast, hepatic mitogens produce a transient increase in hepatocyte proliferation in the absence of hepatotoxicity. Because no liver cytotoxicity was apparent in this study and the proliferative response was transient, the data supports the conclusion that TCE is a hepatocellular mitogen. Mitogenic agents may provide a selective growth advantage to initiated cells by inhibition of normal hepatocyte proliferation or by increasing cell proliferation or decreasing apoptosis (or both) in preneoplastic cell populations. The mechanism(s) by which TCE provides a selective growth advantage to mouse hepatocytes is unknown.

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SECTION 4

HALON 1301 REPLACEMENT TOXICITY PROJECT

4.1 CARDIAC SENSITIZATION THRESHOLDS OF HALON REPLACEMENT CHEMICALS PREDICTED IN HUMANS BY PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELING

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ABSTRACT

Human exposure to halons and halon replacement chemicals is often regulated on the basis of cardiac sensitization potential. The dose-response data obtained from animal testing are used to determine the no observable adverse effect level (NOAEL) and lowest observable adverse effect level (LOAEL) values. This approach alone does not provide the information necessary to evaluate the cardiac sensitization potential for the chemical of interest under a variety of exposure concentrations and durations. In order to provide a tool for decision makers and regulators tasked with setting exposure guidelines for halon replacement chemicals, a quantitative approach was established which allowed exposures to be assessed in terms of the chemical concentrations in blood during the exposure. A physiologically based pharmacokinetic (PBPK) model was used to simulate blood concentrations of Halon 1301 (bromotrifluoromethane, CF3Br), HFC-125 (pentafluoroethane, CHF2CF3), HFC-227ea (heptafluoropropane, CF3CHFCF3), HCFC-123 (dichlorotrifluoroethane, CHCl2CF3), and CF_3I (trifluoroiodomethane) during inhalation exposures. This work demonstrates a quantitative approach for use in linking chemical inhalation exposures to the levels of chemical in blood achieved during the exposure.

INTRODUCTION

Cardiac sensitization potential is a marker often used by federal regulators to determine the human health risk associated with acute exposure to halogenated hydrocarbons. Of particular interest are the halogenated hydrocarbons proposed as replacements for Halon 1301 (bromotrifluoromethane, CF3Br) in occupied space fire-fighting applications. Most of the currently proposed Halon 1301 replacements are at least partially halogenated and likely have cardiac sensitization potential at some exposure concentration.

Cardiac sensitization tests currently accepted by federal regulators are conducted using dogs which are exposed to the chemical of interest in combination with an epinephrine challenge (U.S. EPA, 1994). In such tests, the animals are exposed first to an epinephrine challenge, followed 5 minutes later by an exposure via inhalation to the chemical for 5 minutes. Then the animals are challenged with epinephrine and monitored while the exposure continues for an additional 5 minutes. During the exposure period the cardiac electrical activity in each animal is monitored for cardiac arrhythmias. After a range of chemical concentrations has been evaluated using the cardiac sensitization protocol, the dose-response data can be used to establish the no observable adverse effect level (NOAEL) and lowest observable adverse effect level (LOAEL).

Utilization of the animal testing data for assessing potential risks to humans requires development of appropriate risk assessment methods. These methods must address the unusual exposure circumstances involved in the use of chemicals as

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fire suppressants. In particular, potential exposures would be for relatively brief but varying periods of time at concentrations high enough to effectively extinguish fires.

Under its Significant New Alternative Policy (SNAP) program, the U.S. EPA is required to evaluate whether chemicals are acceptable for use in occupied or unoccupied spaces. In addition, egress times need to be established for people occupying a facility at the time of chemical agent discharge. This is the major focus of the work described herein. Currently, the LOAEL determined in dogs has been applied directly by the U.S. EPA in evaluating acceptable use and allowable exposure limits for humans such as Emergency Guidance Levels (EGLs) (Jarabek et al., 1994). This is in contrast to the common use of the NOAEL, which is typically divided by uncertainty factors for risk assessment of noncancer effects.

Use of the LOAEL is considered to be protective of human health in emergency situations for several reasons. The cardiac sensitization test is considered to be extremely sensitive due to the use of the epinephrine challenge and scoring the response as positive if a single dog out of the test group responds at a given concentration. The doses tested are generally closely spaced so there is little error in estimating the effect threshold due to the arbitrary spacing of doses in the study. In addition, the risks from an uncontrolled fire are potentially great and are implicitly considered in establishing allowable exposure limits using the LOAEL.

Establishing egress times from the 5-minute exposure LOAEL requires careful consideration of the relationship between exposure concentration, duration, and the temporal aspects of the biological response. Under steady-state conditions, the concentration in blood and tissues would be constant regardless of the exposure duration. With pre-steady-state conditions, concentrations vary with time. Using the quantitative relationship between inhalation exposure at the LOAEL and the resulting concentration of chemical in blood is a way to make scientifically based decisions about the egress times. Cardiac sensitization is considered to be a function of the concentration of parent chemical in heart tissue which under perfusion limited conditions is proportional to parent chemical in blood. Therefore, the approach developed herein involves estimating a target level defined as the chemical concentration in human blood that is achieved after 5 minutes of inhalation exposure at the chemical LOAEL as determined in dogs. This approach more closely aligns the currently accepted cardiac sensitization testing procedure with the human emergency exposure scenarios subject to regulatory activity.

A physiologically based pharmacokinetic (PBPK) model provides the quantitative link between exposure concentration and blood levels of chemical achieved following inhalation. The PBPK model is a mathematical description of the uptake, distribution, metabolism, and elimination of a chemical in the species of interest. The physiological compartments that compose the model are based on appropriate physiological and anatomical properties for the species of interest as well as the chemical specific properties of the test compound. The use of PBPK models for kinetic description of chemical interaction with biological systems has been well represented in the scientific literature over the last decade (Andersen, 1981; Conolly and Andersen, 1991; Leung, 1991; Ramsey and Andersen, 1984). Additionally, PBPK modeling approaches have been instrumental in activities requiring the extrapolation of kinetic or biochemical data between species (Clewell and Andersen, 1985). The focus of this work was to apply a PBPK modeling approach that, given appropriate input data, could better define the duration of chemical exposure and physiological conditions required to reach target levels of chemical in blood for Halon

1301 and proposed replacement chemicals: HFC-125 (pentafluoroethane, CHF_2CF_3); HFC-227ea (heptafluoropropane, CF_3CHFCF_3); HCFC-123 (dichlorotrifluoroethane, $CHCl_2CF_3$); and CF_3I (trifluoroiodomethane).

METHODS

Target Levels of Chemical in Blood

A human PBPK model was used to estimate the target levels of chemical in blood by simulating a 30-minute inhalation exposure to a LOAEL concentration of the test chemical. The blood level achieved 5 minutes into the LOAEL inhalation exposure was determined to be the target chemical concentration in blood and was subsequently used as the blood level at which cardiac sensitization was likely to occur. This simulation was conducted for humans under both rest and moderate (35-50 Watt) activity levels.

Physiologically Based Pharmacokinetic (PBPK) Model

The human PBPK model used in this work was described by Williams et al. (in press). Physiological compartments described in the model were liver, fat, lung, gut, slowly perfused and rapidly perfused tissues. Tissue blood flows and ventilation rates for humans under rest and exercise conditions were obtained from work published by Dankovic and Bailer 1993, and are shown in Table 4.1-1.

TABLE 4.1-1. ANATOMIC AND PHYSIOLOGIC PARAMETERS FOR HUMANS
(Dankovic and Bailer [1993])

Parameter	Resting	Moderate Activity
Gut Blood Flow *	0.2192	0.2093
Liver Blood Flow*	0.0885	0.0602
Fat Blood Flow*	0.0288	0.0400
Rapid Perfused Blood Flow*	0.4616	0.3188
Slowly Perfused Blood Flow*	0.2019	0.4319
Volume Fat **	0.215	0.215
Volume Gut**	0.022	0.022
Volume Liver**	0.027	0.027
Volume Rapid Perfused**	0.041	0.041
Volume Slowly Perfused**	0.575	0.575
Ventilation ***	17.4	43.1
Cardiac Output***	12.9	20.7
Body Weight (Kg)	70.0	70.0

*** The values are given as the proportion of cardiac output.**
**** The values are given as the proportion of body weight.**
***** The values are given as Liters/Hour/Kg body weight.**

Chemical-Specific Model Parameters and Values

In some cases human tissue partition coefficient data were available and in others only rodent data were available. Rodent metabolic rate constants were available for these chemicals, but none of them had human metabolism data. The assumption made for this work was that rat metabolism data can be extrapolated to humans using allometric scaling ([body weight]$^{0.75}$). Variance from this assumption should not dramatically impact the work here because this group of chemicals shows as a general characteristic extremely low metabolic activity. This is particularly true for the highly fluorinated chemicals in the group. Chemical-specific data used in the model are shown in Table 4.1-2. Partition coefficients were determined using a vial equilibration method described by Gearhart et al. (1993). Gas uptake for determining metabolic constants was conducted using the methods described by Gargas et al. (1986).

TABLE 4.1-2 CHEMICAL SPECIFIC MODEL PARAMETERS AND VALUES

	Chemical				
Parameter	Halon 1301	CF_3I	HFC-227ea	HFC-125	HCFC-123[a]
Molecular Weight (g/mole)	148.9	195.9	170.0	120.0	152.9
Blood:air Partition Coefficient (Human)	0.34	0.97	0.23[b]	0.12[b]	1.90
Blood:air Partition Coefficient (Rat)	0.72	1.75	0.45	0.23	3.20
Vmaxc, Max. Metabolic Rate (mg/h/kg)	0.00	0.38	0.00	0.00	8.80
Km, Affinity Constant (mg/L)	N/A	0.10	N/A	N/A	0.70
Liver:air Partition Coefficient	0.85[c]	1.22[c]	0.42[c]	0.26[c]	3.00
Gut:air Partition Coefficient	0.69[c]	1.57[c]	0.45[c]	0.37[c]	1.60
Fat:air Partition Coefficient	3.95[c]	11.24[c]	1.58[c]	0.45[c]	49.00
Rapidly Perfused:air Partition Coefficient	0.85	1.22	0.42	0.26	3.00
Slowly Perfused:air Partition Coefficient	0.59	1.27	0.36	0.34	3.00

[a] Partition coefficients of human blood and tissue (Williams et al., [in press]) and the partition coefficient of rat blood and metabolic constants (Vinegar et al., 1994) were determined previously.
[b] The human blood:air partition coefficient used was one-half of that determined in rats. This extrapolation was based on experience with other halogenated hydrocarbons.
[c] Tissue:air partition coefficients were measured in the current study using rat tissues.

Arrows point to the 5-minute and 30-minute blood concentrations which are given in Table 4.1-3. Rest and work blood concentrations are shown together for the LOAEL exposure in Panel C of Figures 4.1-2 through 4.1-6. Time to reach steady-state blood concentrations required that simulations be run for longer time periods under rest and work conditions at the LOAEL exposure concentration (Panel D of Figures 4.1-2 through 4.1-6). The time course of blood concentration during 30 minutes of exposure to HFC-227ea at the LOAEL and for 30 minutes postexposure is shown in Figure 4.1-7.

RESULTS

A physiologically based pharmacokinetic model was used to estimate the blood levels that would be achieved in humans under resting and moderate activity level conditions when challenged with inhalation of Halon 1301 replacement chemicals. A simulated time course of HFC-125 in the blood during inhalation is shown in Figure 4.1-1 Five-minute exposure to the LOAEL (10.0%) of the chemical gave the target chemical concentration in blood of approximately 30 mg/L. The time required to reach the target chemical concentration in blood for exposure concentrations less than and greater than the

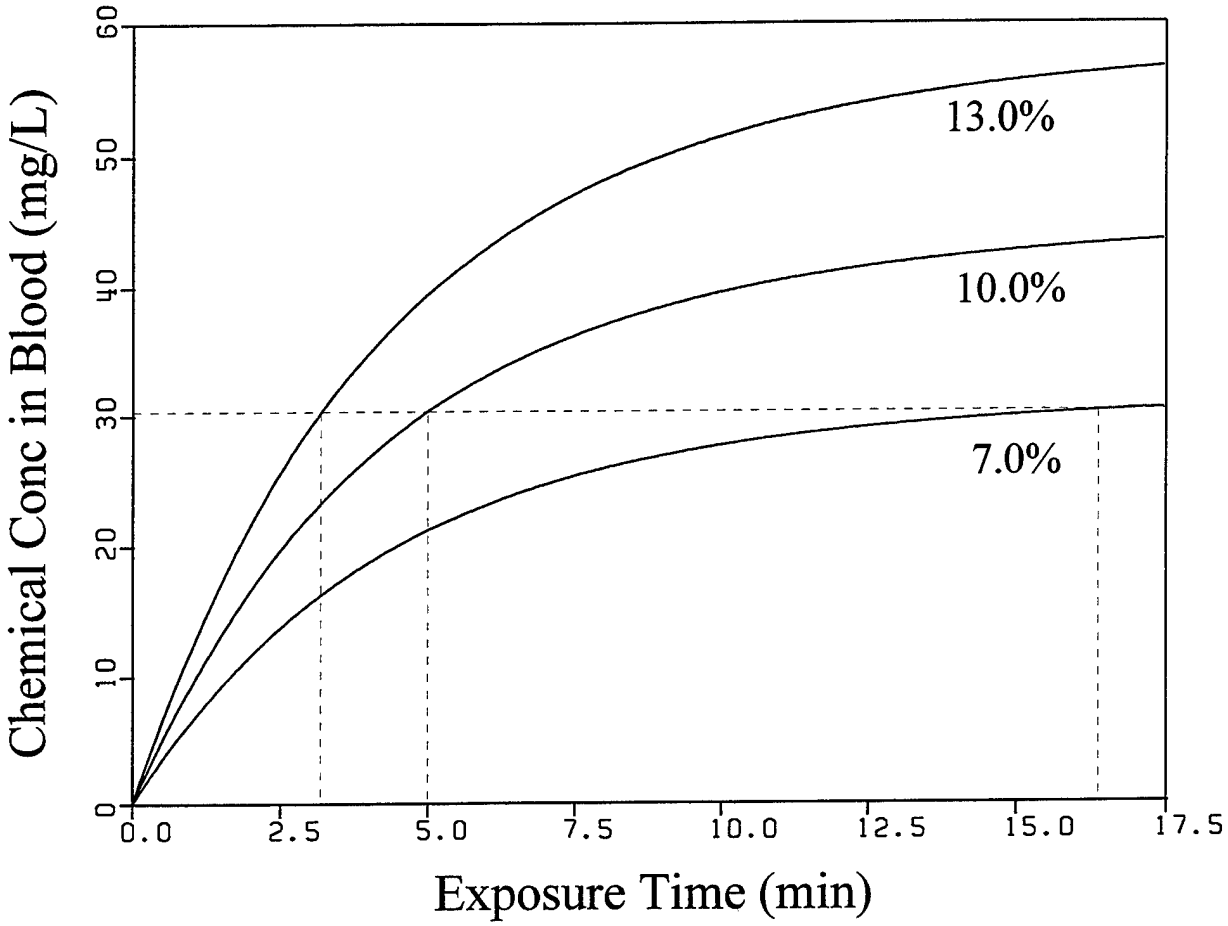

Figure 4.1-1. Chemical Concentration in Blood During Inhalation of HFC-125. The percentages shown are the exposure concentrations. The LOAEL for this chemical is 10%. The 5 min. vertical dotted line intersects the 10.0% (LOAEL) exposure concentration curve at the blood concentration of about 30 mg/L. The other 2 vertical dotted lines indicate the times, approximately 3.0 and 16.5 minutes, to reach the LOAEL blood concentration with exposure concentrations of 13.0% and 7.0%, respectively.

LOAEL are shown as intersection points of the dashed lines and the x-axis. For none of the chemicals reported herein does the concentration in blood reach its steady-state value after 5 minutes of exposure. However, since a 5-minute period of chemical inhalation has usually been used as the exposure duration in cardiac sensitization tests conducted using a widely

accepted protocol, and a 30-minute period of chemical inhalation is being used by at least one laboratory, the blood levels at the 5-minute and 30-minute points were recorded during the simulations.

Simulations were performed for 30-minute exposures of a 70 kg human to Halon 1301, HFC-125, HCFC-123, CF_3I, and HFC-227ea. The time courses of blood concentrations are shown in panels A-C of Figures 4.1-2 through 4.1-6. Panels A and B, respectively, of each figure show simulations while resting or working during exposure to the LOAEL and NOAEL concentrations. If the NOAEL was greater than 50% of the LOAEL, then a third simulation was performed at 50% of the LOAEL.

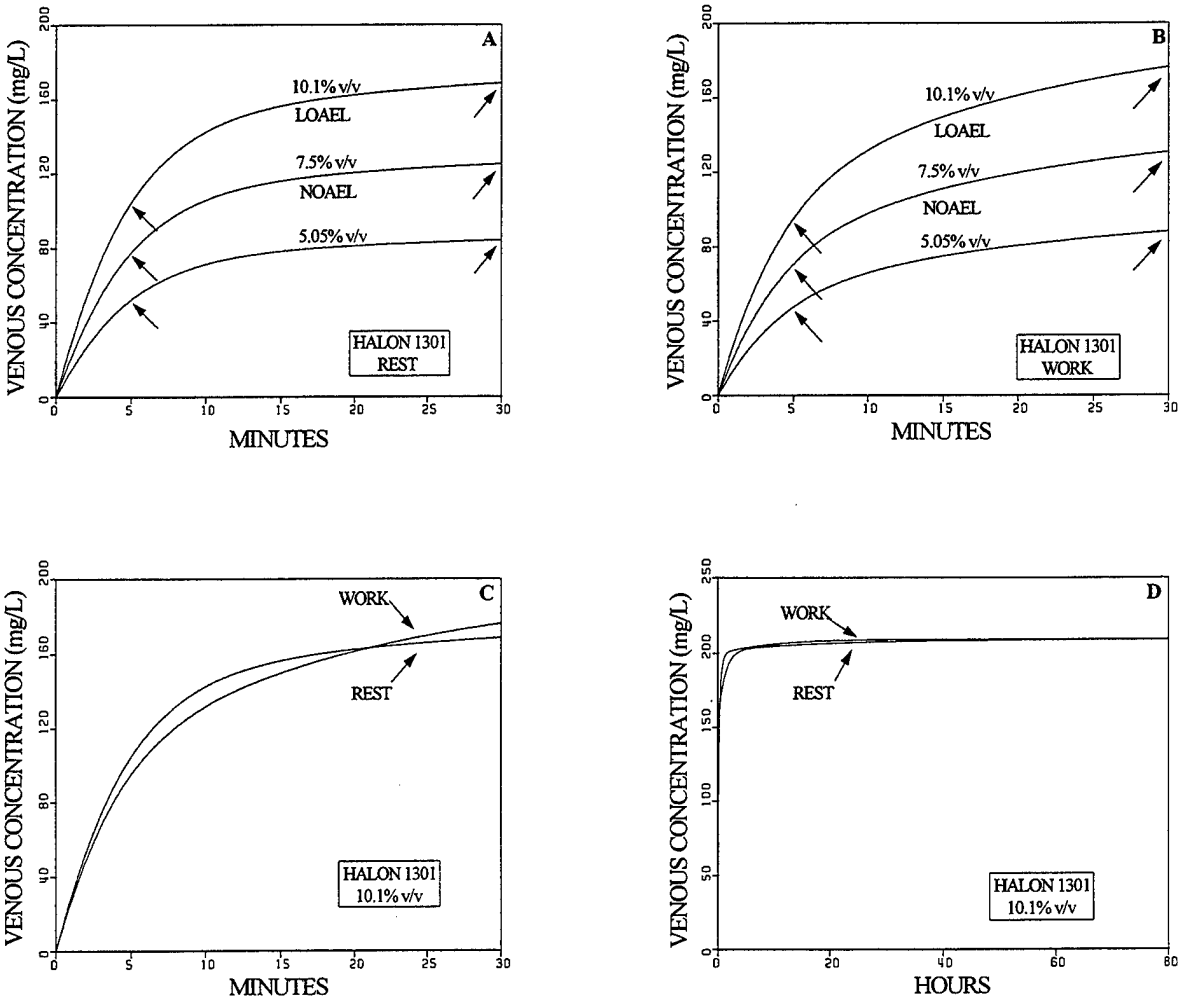

Figure 4.1-2. Chemical Concentration in Blood During Inhalation of Halon 1301. Blood concentrations during exposure to the LOAEL, NOAEL, and one-half the LOAEL concentrations under conditions of rest (A) and work (B). Arrows point to the blood concentrations at 5 and 30 minutes of exposure. Blood concentrations during exposure to the LOAEL under conditions of rest and work for 30 minutes (C) and 80 hours (D).

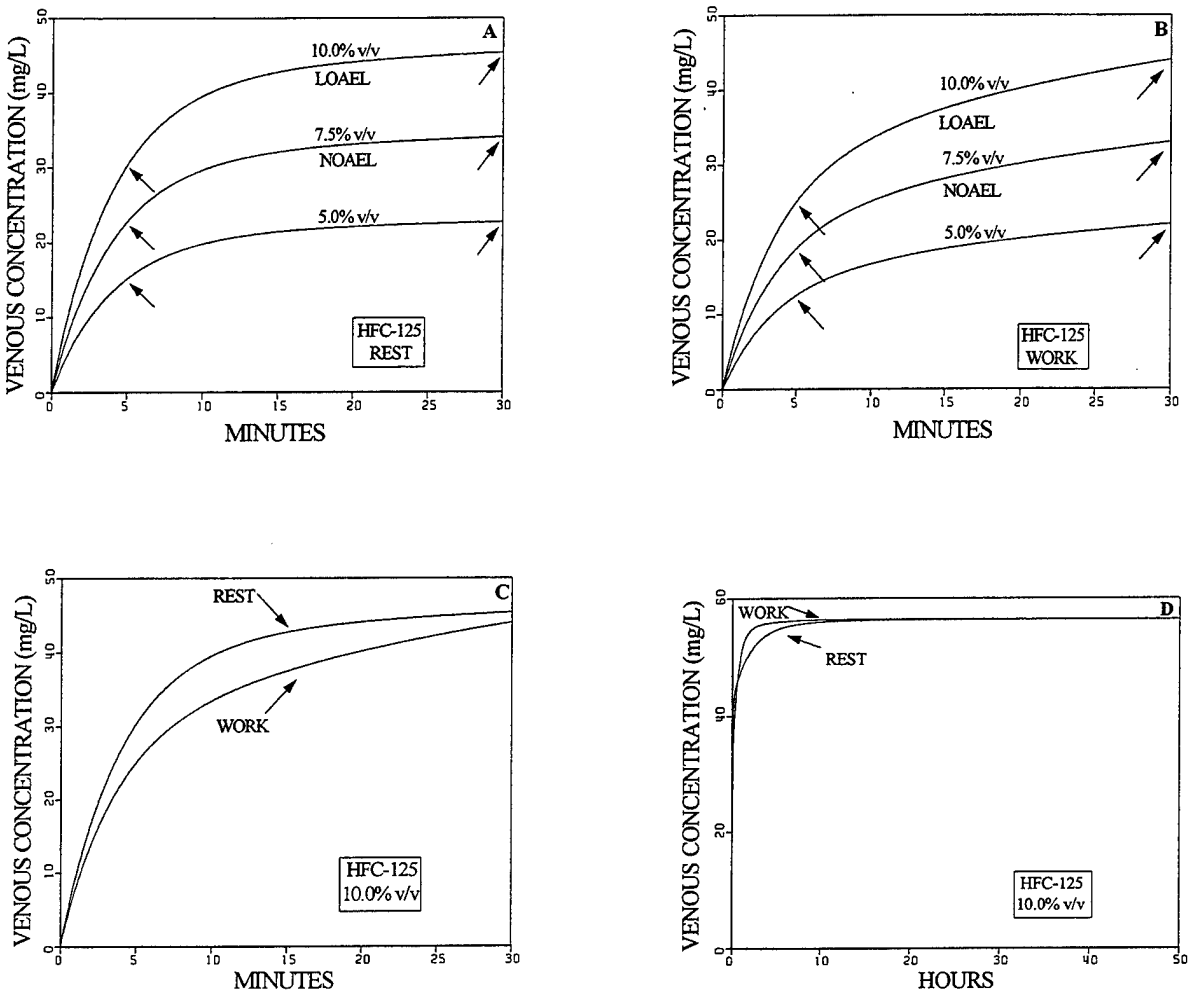

Figure 4.1-3. **Chemical Concentration in Blood During Inhalation of HFC-125. Blood concentrations during exposure to the LOAEL, NOAEL, and one-half the LOAEL concentrations under conditions of rest (A) and work (B). Arrows point to the blood concentrations at 5 and 30 minutes of exposure. Blood concentrations during exposure to the LOAEL under conditions of rest and work for 30 minutes (C) and 80 hours (D).**

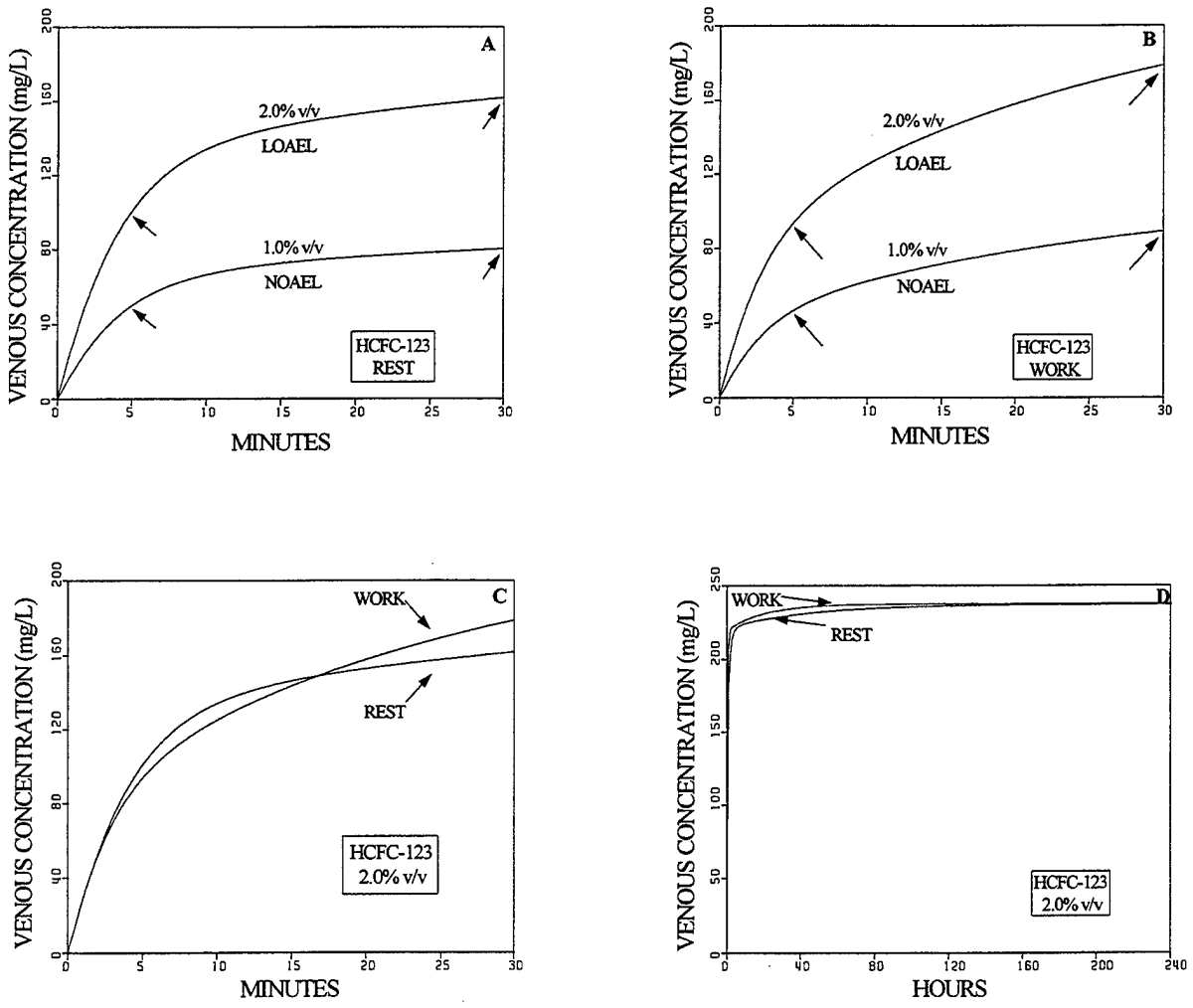

Figure 4.1-4 **Chemical Concentration in Blood During Inhalation of HCFC-123. Blood concentrations during exposure to the LOAEL and NOAEL concentrations under conditions of rest (A) and work (B). Arrows point to the blood concentrations at 5 and 30 minutes of exposure. Blood concentrations during exposure to the LOAEL under conditions of rest and work for 30 minutes (C) and 80 hours (D).**

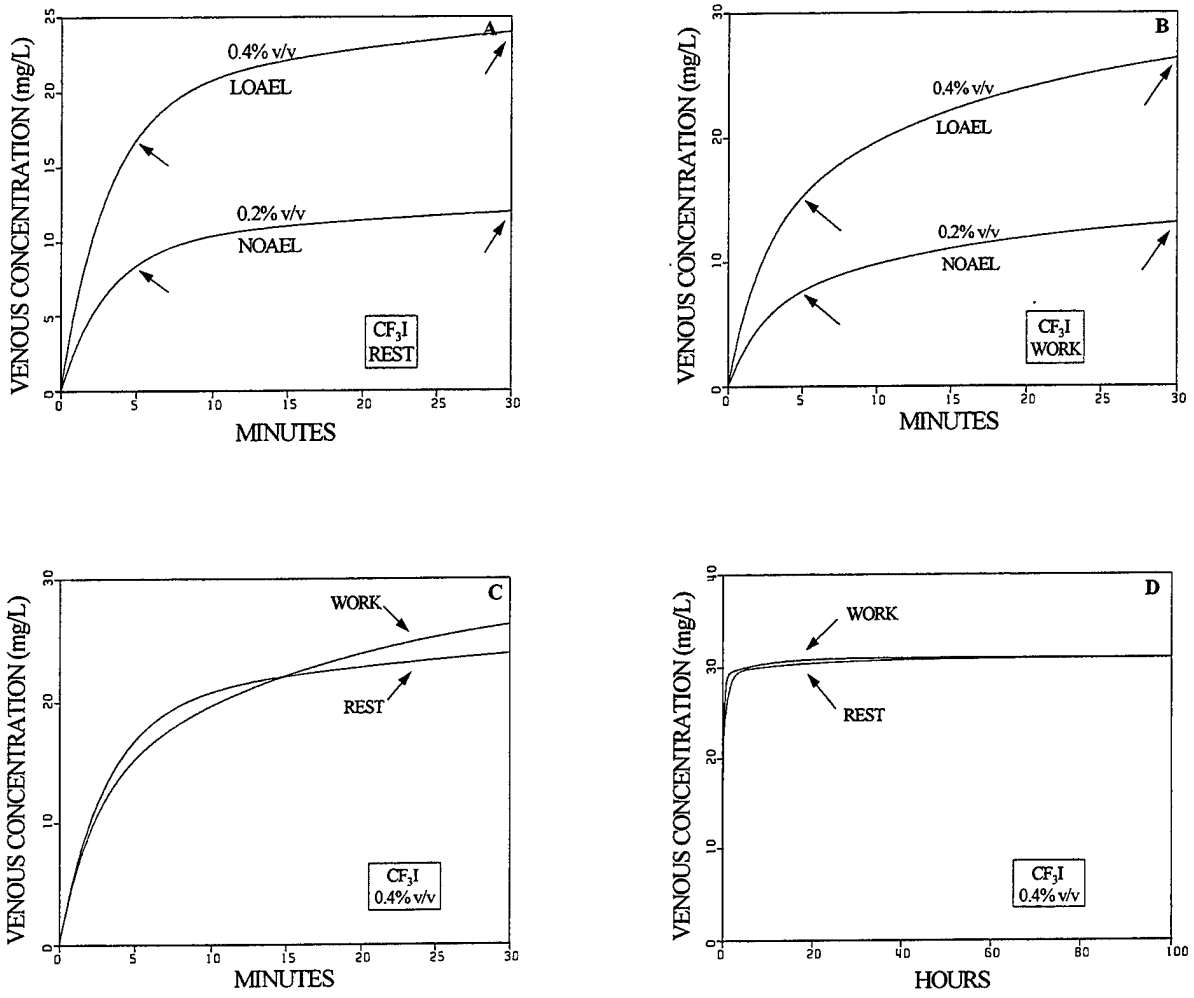

Figure 4.1-5. **Chemical Concentration in Blood During Inhalation of CF_3I. Blood concentrations during exposure to the LOAEL and NOAEL concentrations under conditions of rest (A) and work (B). Arrows point to the blood concentrations at 5 and 30 minutes of exposure. Blood concentrations during exposure to the LOAEL under conditions of rest and work for 30 minutes (C) and 80 hours (D).**

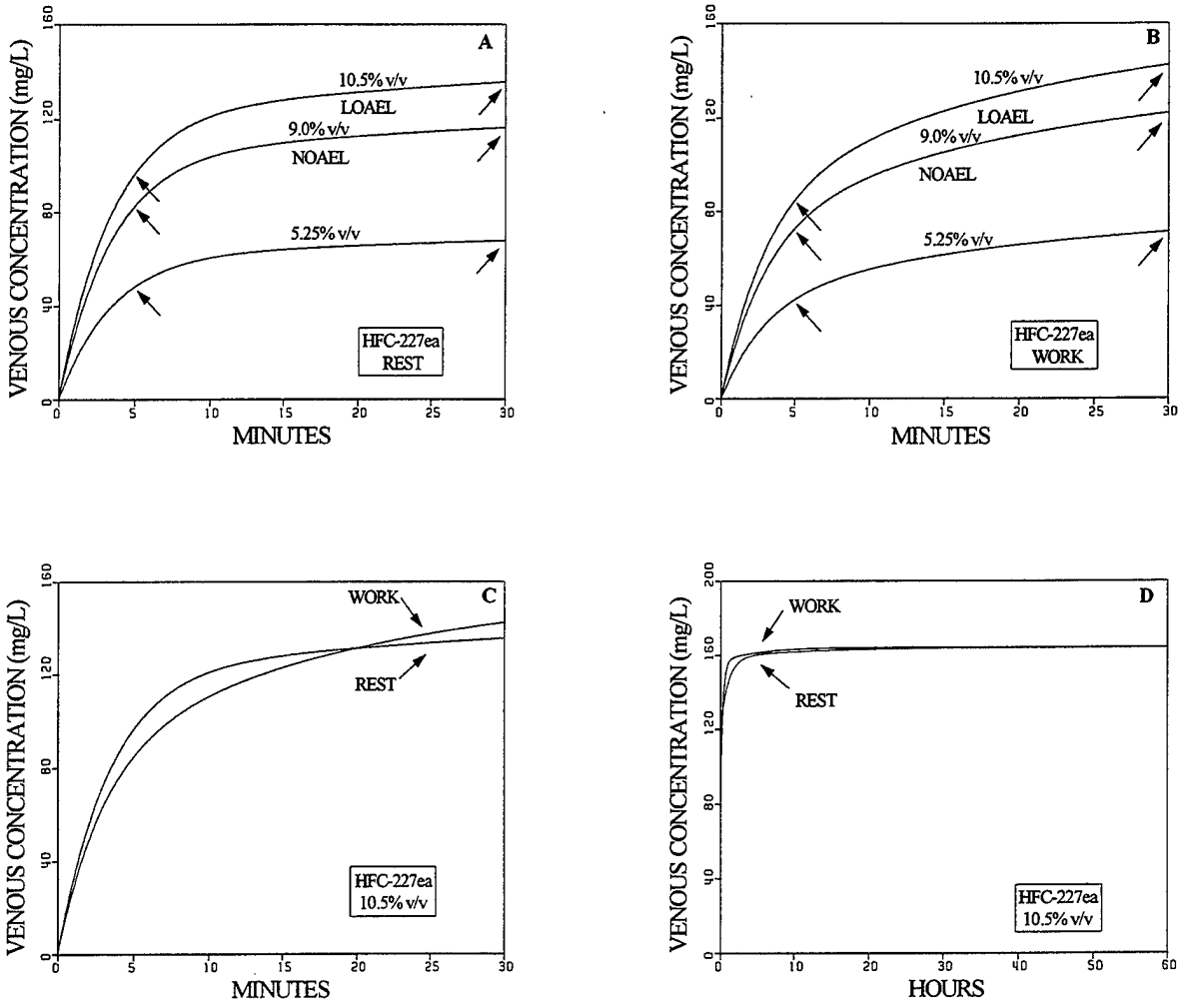

Figure 4.1-6. **Chemical Concentration in Blood During Inhalation of HFC-227ea. Blood concentrations during exposure to the LOAEL, NOAEL, and one-half the LOAEL concentrations under conditions of rest (A) and work (B). Arrows point to the blood concentrations at 5 and 30 minutes of exposure. Blood concentrations during exposure to the LOAEL under conditions of rest and work for 30 minutes (C) and 80 hours (D).**

TABLE 4.1-3. SIMULATED HUMAN BLOOD CONCENTRATIONS AT 5 AND 30 MINUTES OF INHALATION EXPOSURE

Chemical	Exposure Concentration % v/v	Simulated Blood Concentration (mg/L)			
		Rest		Work	
		5 Minutes	30 Minutes	5 Minutes	30 Minutes
Halon 1301	10.1†(1)	104.21	168.75	94.76	176.40
	7.5‡(1)	77.38	125.31	70.37	130.99
	5.05	52.10	84.38	47.38	88.20
HFC-125	10.0†(2)	30.33	45.39	25.07	44.04
	7.5‡(2)	22.75	34.04	18.81	33.03
	5.0	15.16	22.70	12.54	22.02
HCFC-123	2.0†(3)	100.53	161.81	93.50	178.75
	1.0‡(3)	49.91	80.53	46.56	89.18
CF_3I	0.4†(4)	16.85	23.98	15.29	26.33
	0.2‡(4)	8.40	11.97	7.63	13.15
HFC-227ea	10.5†(5)	96.45	135.59	84.76	142.39
	9.0‡(5)	82.67	116.22	72.65	122.05
	5.25	48.22	67.79	42.38	71.19

†**LOAEL**
‡**NOAEL**

([1] Mullin et al., 1979; [2]Hardy et al., 1994; [3]Trochimowicz and Mullin, 1973; [4]Kenny et al., 1995; [5]Chengelis, 1993)

DISCUSSION

The simulations of chemical concentrations in blood during an inhalation exposure were conducted using a physiologically based pharmacokinetic model. PBPK models have been used extensively to describe chemical fate in biological systems, and as such, have become substantial tools in health risk assessment applications. However, PBPK models have physiological and chemical data requirements, which play an important role in the utility of the model. For the most part, the anatomical and physiological parameters required for a human or rat PBPK model are defined well enough to construct compartments for blood and the major organs. Examples of the physiological parameters are the blood flows to the tissues, tissue volumes, ventilation rate, and cardiac output. (Table 4.1-1). These physiological parameters define a particular biological system and are independent of the chemical of interest as long as the chemical does not produce a kinetically relevant change in the physiological processes.

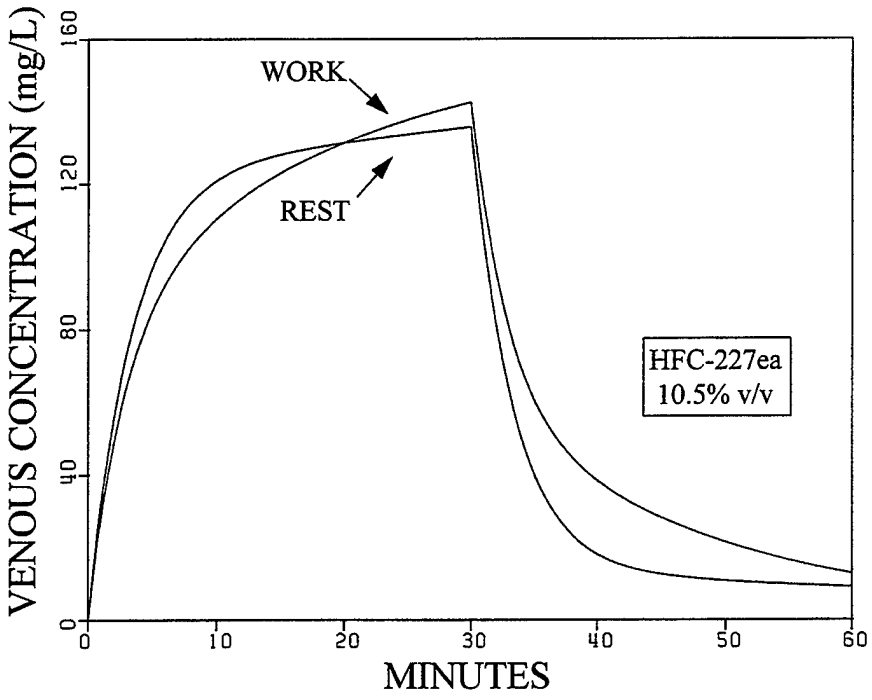

Figure 4.1-7. Chemical Concentration in Blood During and After Inhalation of HFC-227ea. Blood concentrations during and after exposure to the LOAEL under conditions of rest and work.

Chemical-specific parameter values are required for each chemical that is to be evaluated using the PBPK model. Examples of chemical-specific parameters are tissue:blood partition coefficients and metabolic rate constants. These chemical-specific parameter values are often lacking since they are not collected as part of the currently used toxicity test protocols commonly employed for hazard identification purposes. The chemicals used in this effort are examples of just such a situation. Cardiac sensitization levels were determined based on cardiac electrical activity, but the partitioning and kinetic data required to construct a quantitative tool for extrapolating between species and exposure scenarios were lacking. No usable human partitioning (except for HCFC-123) or kinetic data were available for the chemicals of interest. In order to accomplish the simulation of chemical concentration in human blood during an inhalation exposure, kinetic and tissue partitioning data were collected from rats. The rat data were then mathematically scaled to humans and used in the simulations. One exception to the data deficit was human blood:air partition coefficients. The availability of human blood made these partition coefficient determinations practical. This is particularly important since the blood:air partition coefficient is the major determinant of the amount of these volatile chemicals that gains access to the biological system during the inhalation exposure.

As a consequence of the lack of human data, obviously the model could not be validated against actual human exposures to the halon replacement chemicals of concern. However, the model has been used to extrapolate the kinetics of HCFC-123 from rat to human by showing the model's ability to describe halothane kinetics from both rat and human *in vivo* data (Williams et al., [in press]). Halothane differs from HCFC-123 only by a bromine for chlorine substitution. The ability of the model to simulate human exhaled breath data during 5 minutes of halothane exposure is demonstrated in Figure 4.1-8.

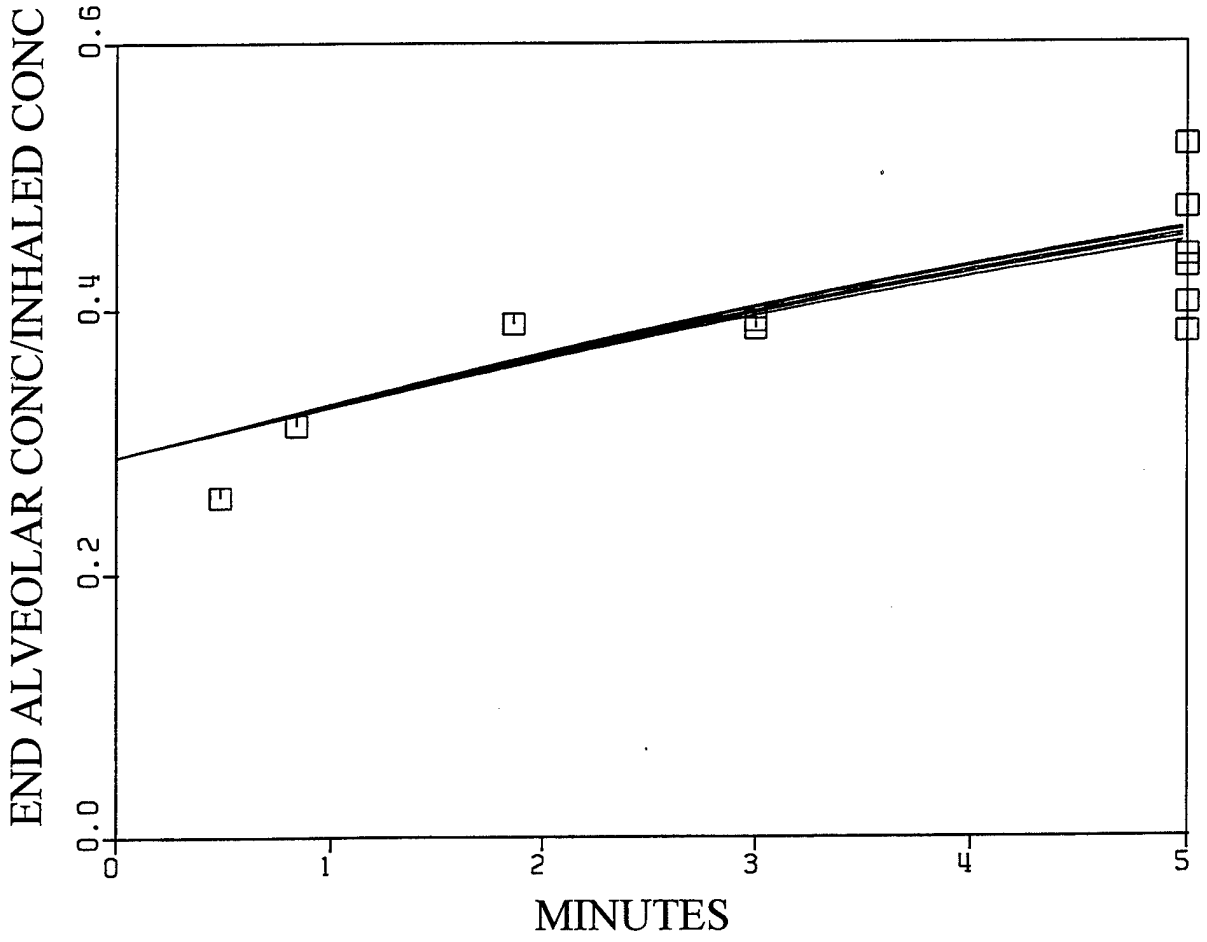

Figure 4.1-8. **End Alveolar Concentration/Inhaled Concentration During Inhalation of Halothane. The continuous lines represent simulations of 6 different inhalation exposures (1100 ppm x 2, 2000 ppm, 2260 ppm, 3000 ppm, and 4300 ppm. The individual data points are taken from Cahalan et al. (1981), Carpenter et al. (1986), Torri et al. (1972), and Yasuda et al. (1991).**

Several facts can be gleaned from examination of the blood time course curves (Figures 4.1-2 through 4.1-6). It is important when specifying LOAEL and NOAEL exposure concentrations for cardiac sensitization that the duration of chemical exposure before epinephrine challenge be defined. The LOAEL and NOAEL exposures shown assume that a 5-minute exposure protocol was used. Thirty-minute exposures to Halon 1301, HFC-125, and HFC-227ea at their respective NOAELs would result in blood concentrations that exceed those obtained by 5 minutes of LOAEL exposure. In contrast, exposures to HCFC-123 and CF_3I for 30 minutes at their NOAELs would result in blood concentrations not exceeding those obtained by 5 minutes of LOAEL exposure. The NOAELs of the latter two chemicals are one-half of the LOAELs. Exposure of the former three chemicals at one-half of the LOAEL likewise would result in blood concentrations not exceeding those obtained by the 5-minute LOAEL exposures.

The LOAEL and NOAEL of 2.0% and 1.0%, respectively, for HCFC-123 represent a difference in concentration of 1.0%, with a NOAEL that is 50% of the LOAEL. The LOAEL and NOAEL of 10.0% and 7.5%, respectively, for HFC-125 represent a larger difference in concentration of 2.5%, with a NOAEL that is 75% of the LOAEL. Thus, the relationship between LOAEL and NOAEL is a function of the pharmacokinetics of the chemical and the selection of the concentrations for performing the cardiac sensitization test. A small difference in percent concentration for a potent cardiac sensitizer (1% difference with a 50% change for HCFC-123) represents a larger proportionate change in concentration than for a weaker cardiac sensitizer (2.5% difference with a 25% change for HFC-125).

At 30 minutes, chemical concentrations in blood are at only 68% to 83% of steady-state under resting, and 75% to 87% under working conditions (C & D in Figures 4.1-2 through 4.1-6). The percent of steady-state that is reached by 30 minutes depends on chemical-specific properties (partitioning and metabolism) and physiological properties (blood flows and tissue volumes). Physiological properties influence the shorter term kinetics, as can be seen by the differences in the time course of venous concentration between resting and light work blood flows (Figures 4.1-2 through 4.1-6). Blood concentrations drop rapidly when the chemical exposure is stopped (Figure 4.1-7).

Cardiac sensitization data collected for exposure to HFC-227ea were actually collected in response to a 30-minute rather than a 5-minute exposure as in the case of the other chemicals. The LOAEL and NOAEL exposure concentrations were based on 30 minutes of exposure. The blood concentrations at 30 minutes are greater than for 5 minutes of exposure (Figure 4.1-6 and Table 4.1-3). Thus, LOAEL and NOAEL concentrations based on a 5-minute exposure would actually be higher than those based on a 30-minute exposure. Under conditions of rest the LOAEL and NOAEL concentrations would be 14.76% and 12.65%, respectively, and under conditions of light work the LOAEL and NOAEL concentrations would be 17.64% and 15.12%, respectively.

Figure 4.1-1 illustrates several points. Exposure concentrations above the LOAEL take less than 5 minutes to reach the target chemical concentration in blood. Some exposure concentrations below the LOAEL can reach target chemical concentrations in blood if the exposure continues long enough. However, other exposures will not produce the target chemical concentrations no matter how long the exposure continues. These points may be useful in deciding on appropriate egress times based on the expected exposure concentrations.

The blood time course shown in Figure 4.1-1 demonstrates the importance of exposure time in the cardiac sensitization test. Since there are currently protocols that expose animals to the chemical via inhalation for 5, 10, and 30 minutes, the animals could be at very different levels relative to their steady-state chemical concentration in blood. Exposures of 30 minutes bring blood concentrations close to steady-state, whereas at 5 minutes of exposure, concentrations are considerably less than steady-state. The time it takes to reach steady-state is governed by physiological variables, while the steady-state concentrations are governed by partition coefficients. If the animals are not at or near steady-state when the cardiac sensitization test is conducted, there is potential for greater error in assignment of exposure concentrations that reach cardiac sensitization thresholds because physiological conditions tend to vary more than steady-state partitioning. Since the cardiac sensitization studies are conducted in dogs, and the testing stops when a response is obtained in one dog, the LOAEL obtained is dependent on the physiological state of the one animal if a 5-minute test were done, whereas it would be dependent on the partition coefficients of the chemical being tested if a 30-minute test were performed. Ideally, 30-minute tests done with multiple animals at each concentration would give the most reliable data.

The quantitative relationship between chemical time courses in blood and cardiac sensitization potential demonstrates an approach that can be used to assess appropriate egress times. The PBPK model simulations of chemical concentrations in blood during inhalation exposures showed that it is possible to be exposed to concentrations above the LOAEL for a short period of time without reaching the target blood concentration of chemical. Additionally, the importance of cardiac sensitization test exposure times for appropriate determination of LOAEL and NOAEL values was demonstrated. The use of a PBPK model to link exposure scenarios to biological effects represents an approach which, when properly validated, could benefit regulators and decision makers working on halon replacement activities. The benefit of this approach could be greatly enhanced if kinetic and partitioning data were routinely collected as part of the toxicity tests relevant to halon replacement issues. Even with the current data limitations, this approach adds a tool for making scientifically based decisions to ensure an appropriate balance between operational effectiveness and human safety.

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4.2 EVALUATION OF IN VITRO ALTERNATIVES TO THE DOG CARDIAC SENSITIZATION ASSAY

J. M. Frazier

ABSTRACT

Many halogenated hydrocarbons have been shown to be cardiac sensitizers, i.e., chemicals that alter the sensitivity of the heart to endogenous chemical signals such that under conditions of stress cardiac failure may occur. The best known examples of these agents are solvents and propellants used in aerosol dispensers that have lead to the death of "glue sniffers" as a result of cardiac arrest. The standard toxicological test for cardiac sensitization involves the exposure of dogs to the test agent and the recording of the EKG to determine the cardiac response to a systemic injection of epinephrine. In some cases the experimental procedure is lethal. It would be useful, both from a practical as well as a humane point of view, to have non-whole animal test procedures, possibly *in vitro* tests, that would provide quantitative evaluation of the risk for cardiac sensitization of candidate chemicals for DOD applications. This report reviews various *in vitro* models that potentially could be developed for such purposes, including primary cell cultures, cell lines and isolated organ cultures. At this time, none of the models have been developed for this application. It is apparent that a better understanding of the mechanism of cardiac sensitization would allow for the more rapid development and validation of non-whole animal test systems.

Model System

Cardiac Myocyte Cultures In Vitro

Beginning in the 1960s myocardial cell cultures were developed for mechanistic studies of cardiac metabolism and function. The following is a summary of some of the mammalian cardiac cell culture systems that have been described in the literature. A significant research effort has been invested in chick embryo myoblast cell culture. However, for the purposes of this project these activities have been excluded because of the problems of extrapolation from avian to mammalian cardiac toxicity that would require extensive validation.

Neonatal Rat Heart Cell Cultures

Wenzel et al. (1970) investigated the effects of nicotine on cultured neonatal rat heart cells. Using a procedure described by Mark and Strasser (1966) to dissociate heart cells from two- to four-day old Sprague-Dawley rats, they evaluated techniques to separate myocardial cells from endothelial cells which tended to overgrow the muscle cell cultures. Having improved the purity of the myocardial cell cultures, they demonstrated that nicotine increased spontaneous cell beating rates. At higher concentrations of nicotine, there was evidence of cytotoxicity (vacuole formation and nuclear swelling).

Schanne et al. (1977) described the electrogenic properties of a preparation of rat (Sprague-Dawley) neonatal ventricular myocytes derived from 24- to 48-h old rats (the original description of this preparation is in German). Cardiac myocytes derived from the lower third of the neonatal hearts are cultured in streptomycin-free media, since streptomycin was found to interfere with spontaneous electrical activity. Various studies were conducted to characterize the nature of the spontaneous beating activity. The authors concluded that the electrogenic pacemaker activity is a result of a slow, tetrodotoxin insensitive - manganese sensitive, inward current. Some of the properties of this culture system differ from those observed in other

model systems.

McCall (1976) describes studies of myocardial cells in culture using the method of Harary and Farley (1963) as modified by Blondel et al. (1971) to improve the purity of the myocardial cell preparations. The purpose of the studies was to investigate the effect of quinidine, a drug used to control tachycardia, on cardiac cell function. Using Na flux measurements and cell contraction rates as experimental parameters, McCall was able to demonstrate that quinidine reduced the spontaneous beating rate of the myocardial cells in culture and this effect was correlated with a reduction in Na influx. Na influx and spontaneous beating rate was insensitive to tetrodotoxin (Na channel blocker) and highly sensitive to verapamil (Ca channel - slow inward current blocker). Furthermore, quinidine only affected the verapamil sensitive Na influx.

Welder et al. (1991a) used primary cultures of neonatal myocardial cells to investigate the effects of dietary manipulations during gestation. The investigators used the method of Wenzel et al. (1970) to isolate myocardiac cells. Several observations of this study are important. First, there were significant differences between the cells isolated from neonates of dams given a full-calorie diet during gestation versus those given a 50% calorie-restricted diet. This observation indicates that cells retain treatment-specific characteristics even after isolation. Second, the investigators determined the ß-adrenergic receptor density (receptors per cell) and affinity (K_D) using a radio-ligand binding assay. Since epinephrine acts on cardiac myocytes through the ß-adrenergic receptor, these studies indicate the presence of the appropriate receptor in the cultured cells.

Details of the preparation of neonatal rat myocardial cells is given by Welder and Acosta (1993).

Adult Rat Heart Cell Cultures

Early investigations of adult rat myocardial cell cultures (Vahouny et al., 1970) indicated that spontaneously beating cells could be obtained and cultured. Heart tissue was minced and treated with digestive enzymes to isolate myocytes. Primary cultures exhibited spontaneous beating rates ranging from 20 to 120 beats per minute at room temperature. The beating rate was highly dependent on medium conditions.

Farmer et al. (1977) described a different procedure to produce myocyte cultures from adult rats involving the perfusion of multiple rat hearts to increase yield. The cell preparations obtained exhibited a 70% viability. In many respects these cell cultures exhibit normal metabolic regulation but under low Ca culture conditions ([Ca] < 10^{-5} M) the myocytes do not beat spontaneously - random twitching is reported. However, if the calcium concentration is increased, rapid twitching of most cells is observed. This is referred to as the "calcium paradox" and is related to a loss of control of Ca permeability of the cell membrane. The investigators demonstrated that isoproterenol (a $ß_1$-/$ß_2$-adrenergic receptor agonist) increased intracellular cAMP and this effect was blocked by propranolol (a $ß_1$-adrenergic receptor antagonist) indicating that the $ß_1$-adrenergic receptors, through which epinephrine acts, was present and functional.

Welder et al. (1991b) recently described an improved perfusion technique to obtain adult rat myocytes. Their procedure is based on previous work by Farmer et al. (1983) and Khetarpal et al. (1988). This system was used to investigate the cardiac toxicity of amitriptyline, a tricyclic antidepressant.

Rabbit Heart Cell Cultures

Dani et al. (1979) developed a perfusion technique to isolate myocardiac cells from the rabbit heart. The cell membrane of the cultured myocytes was electrochemically shunted and freely permeable to Ca, therefore the cells did not exhibit spontaneous beating. The objective of these studies was to investigate calcium transport in relation to contractile activity and this preparation proved useful for this purpose.

Canine Heart Cell Cultures

Vahouny et al. (1979) described a procedure to isolate canine ventricular myocytes, which is based on their procedure to isolate adult rat heart cells (Vahouny et al., 1970). Cells isolated by this technique lost their spontaneous beating activity rapidly, within the first hour, but could be stimulated to contract with high Ca concentrations (calcium paradox), ouabain and epinephrine.

Recent Applications

In vitro cellular model systems have been used extensively for basic cardiac research activities. A review of the recent literature (1992-1994) is summarized in Table 4.2-1.

TABLE 4.2-1

MODEL	LIFE STAGE	SPECIES	REFERENCE
Myocardial cells	Fetal	Human	Goldman and Wurzel, 1992
Cardiomyocytes	Neonatal	Rat	Flanders et al., 1993, Yamazaki et al., 1993 Ito et al. 1993, Suzuki et al., 1993, Hilenski et al., 1992
Ventricular cardiomyocytes	Neonatal	Rat	Orita et al., 1993, Atkins et al., 1992, Dostal et al., 1992; Ogawa et al., 1992
Ventricular cardiomyocytes	Neonatal	Canine	Liu et al., 1992
Cardiomyocytes	Adult	Rat	Clark et al., 1993, Venema and Kuo, 1993 Hartmann and Schrader, 1992 Sadoshima et al., 1992a and Sadoshima et al., 1992b Blank et al., 1992, Springhorn et al., 1992 Schulz et al., 1992
Cardiomyocytes	Adult	Rabbit	Hung and Lew, 1993, Liu et al. 1993
Cardiomyocytes	Adult	Canine	Youker et al., 1992
Cardiomyocytes	Adult	Feline	Woosley et al., 1993
Ventricular cardiomyocytes	Adult	Guinea pig	Failli et al., 1992, Walsh and Long, 1992 Adamantidis et al., 1992
Ventricular cardiomyocytes	Adult	Rat	Eid et al., 1992

CONCLUSIONS

There is a significant history of the isolation and culture of myocardial cells from various mammalian species. Over the years the techniques have improved significantly such that current cell culture systems retain the ability to spontaneously beat in the absence of artificial manipulations of culture conditions. It appears that cultures of either neonatal rat heart myoblasts or adult rat myocytes could form the basis of an experimental *in vitro* test system to investigate the cardiac sensitization potential of chemicals. Any model system selected for further investigation should be characterized with respect to expression and response of β_1-adrenergic receptors (the target of epinephrine) and Na and Ca fluxes before cardiac sensitization validation studies can be initiated.

Cardiac SubCellular Organelles

Several studies have utilized subcellular organelles derived from cardiac tissues to investigate mechanisms of cardiac function. Studies on cardiac mitochondrial function are probably not relevant to the issue of cardiac sensitization. However, models for calcium transport processes may be useful if the membrane effects of sensitizing agents alter calcium pump activity and/or regulation. Recent studies have utilized canine sarcolemma vesicles (Qu et al., 1992) and rat ventricular sarcoplasmic reticulum (Pessah et al. 1992). These systems may be useful for mechanistic studies.

Isolated Heart Tissues

Heart tissues have been explored as research models for cardiac studies. Minces of heart have been employed in receptor binding studies (Hull et al., 1993). Many studies, summarized in Table 4.2-2, utilize intact tissue, mostly atrial tissue, from adult animals as the research model. These preparations have advantages over isolated cell cultures for electrophysiological studies of signal conduction *in situ*.

TABLE 4.2-2

MODEL	SPECIES	REFERENCES
Isolated atrial strips	Rat	Salvatici et al., 1992a
Spontaneously beating atria	Rat	Schaefer et al., 1992 Agnoletti et al., 1992 Ward et al., 1992 Salvatici et al., 1992b
Isolated atria	Rabbit	Cusack et al., 1993
Spontaneously beating atria	Guinea pig	Lechner, 1993 Wilhelm et al., 1992 Eglen et al., 1992

Isolated Heart Preparation

The isolated heart preparation has been used for many years to study regulation of cardiac function. The system has the advantage of maintaining the integrated architecture of the entire heart. Electrophysiological studies can be conducted to measure action potential conduction velocities that could be relevant to the cardiac sensitization question. Some recent studies described in the literature (1992-1994) are listed in Table 4.2-3

TABLE 4.2-3

MODEL	SPECIES	REFERENCES
Isolated perfused heart	Rat	Klabunde et al., 1992 Uusimaa et al., 1992 Van Wylen et al., 1992
Isolated perfused heart	Mouse	Ganim et al., 1992
Isolated perfused heart	Rabbit	Moller and Covino, 1993; Ballagi et al., 1992
Isolated perfused heart	Guinea pig	Gallenberg et al., 1993

Special Preparations

Several experimental models have been developed for special research projects to investigate mechanistic questions concerning action potential initiation and conduction. These models are listed in Table 4.2-4.

TABLE 4.2-4

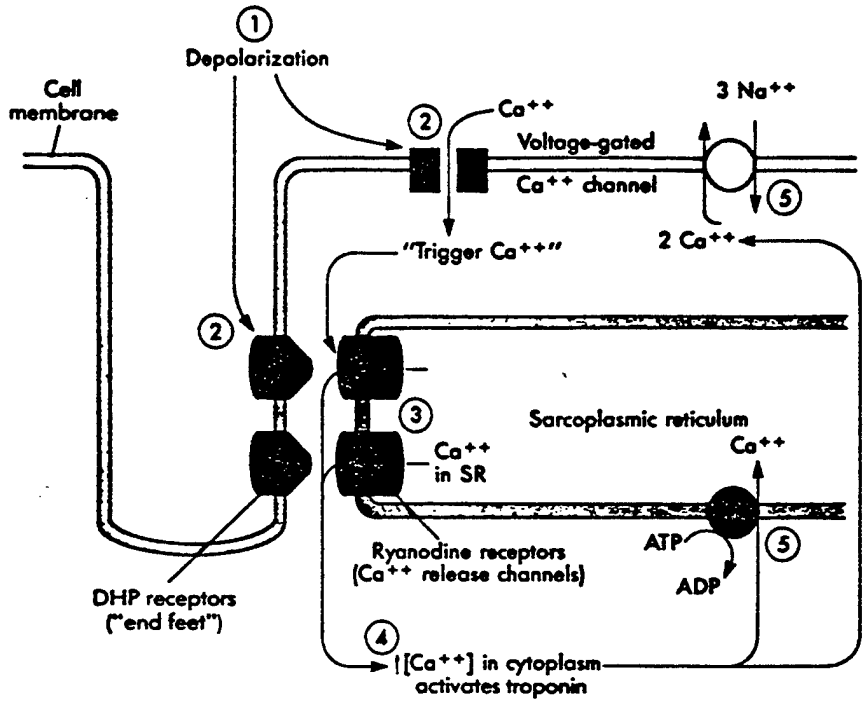
MODEL	SPECIES	REFERENCES
Isolated cardiac ganglia	Rat	Selyanko, 1992
Cardiac Purkinje fibers	Canine	Hanck and Sheets, 1992; Phillips et al., 1992
Cardiac Purkinje fibers	Sheep	Pacini et al., 1992 Rowan et al., 1992 Abete and Vassalle, 1992
Isolated perfused SA node	Guinea pig	Gonzalez and Vassalle, 1993

In Vitro Assays as Alternatives

Cardiac sensitization by halogenated hydrocarbons appears to be a result of a physical-chemical interaction of the toxic agent with the heart tissues. The ultimate effect appears to be an increase in the sensitivity of the cardiac myocytes to the effect of epinephrine resulting in arrhythmia and potentially ventricular fibrillation. The observation of a critical concentration of the chemical in the plasma below which the effect does not occur and the immediate reversibility of the effects when the sensi-

tizing agent is removed argue strongly in favor of a mechanistic hypothesis that the effect is a direct action of the chemical on membrane structure of cardiac cells, although other systemic effects cannot be ruled out completely. The development of an *in vitro* assay as an alternative to the *in vivo* dog assay for the detection of cardiac sensitization requires development and validation of a system that retains the essential cellular components of the process.

A basic schematic of the processes involved in cardiac muscle contraction are illustrated in Figure 4.2-1. The main components of this process are depolarization, regulation of calcium release, activation of the molecular elements of the contraction system (troponin - actin - myosin), and repolarization/restoration of calcium reservoirs. The key characteristic of the cardiac sensitization effect is the increased sensitivity of the heart to epinephrine. The cardiac effects of epinephrine are twofold - an effect on the rate of contraction, and an effect on the strength of contraction. Epinephrine acts on heart tissue



Pathways of excitation-contraction coupling in myocardial cells. Depolarization (1) opens (2) both membrane voltage-gated Ca^{++} channels and SR Ca^{++} channels controlled by T tubule DHP receptors. Entry of trigger Ca^{++} potentiates release of (3) Ca^{++} from the SR (Ca^{++}-induced Ca^{++} release). The number of crossbridges that become active is determined by the amount of Ca^{++} released into the cytoplasm (4). Relaxation occurs as Ca^{++} is removed from the cytoplasm (5) by the Ca^{++} pump of the SR and by Na^{+}/Ca^{++} exchange across the cell membrane.

Figure 4.2-1: **General schematic of processes involved in muscle contraction. Taken from *Human Physiology*, Second Edition, David F. Moffett, Stacie B. Moffitt and Charles L. Schauf, Mosby, St. Louis, 1993.**

through the β_1 adrenergic receptor (Figure 4.2-2). The response of cardiac tissues to epinephrine at the cellular level is mediated through cyclic AMP. Thus, the cardiac sensitizing chemical could affect the highly regulated response of cardiac myocytes to epinephrine at any stage of the signal transduction-myocyte contraction continuum. Mechanistic studies should focus on the effects of known cardiac sensitizers on signal transduction through the β_1-adrenergic receptor and the regulation of ion fluxes during spontaneous contraction.

Requirements for an *in vitro* model for the detection of a cardiac sensitizing agent include a cardiac culture system that:

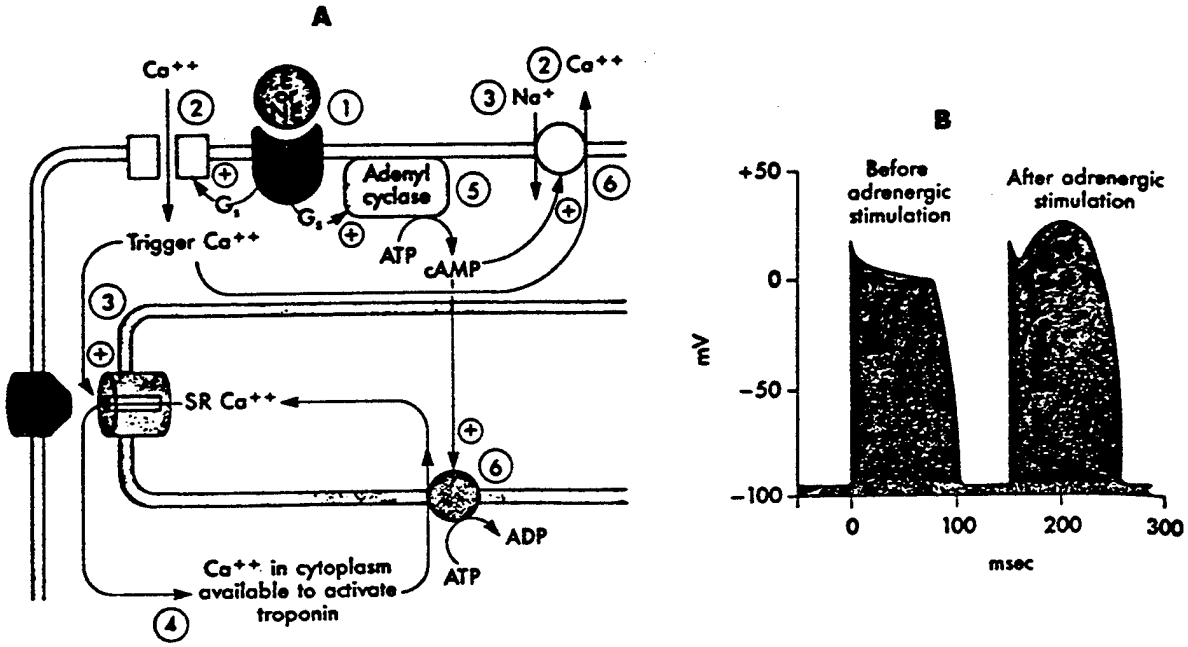
(1) exhibits synchronized spontaneous beating,

(2) is responsive to epinephrine,

(3) exhibits sensitivity to known cardiac sensitizers, and

(4) can be cultured in a controlled atmosphere where the system can be exposed to the agents of concern, mostly volatile organics.

The establishment of such a system is technically feasible considering the current state of knowledge in the field.



A Mechanisms of sympathetic inotropic effect. Binding of epinephrine or norepinephrine (1) activates the beta$_1$ receptor, sending a G_s signal to (2) Ca^{++} channels, resulting in an increase in the amount of trigger Ca^{++} that enters during the plateau phase. This induces a greater release of Ca^{++} from the SR when (3) Ca^{++} release channels are activated by depolarization. The ultimate result is that (4) cytoplasmic Ca^{++} rises higher and more cross-bridges are activated, making systole more forceful. The cAMP second message (5) set in motion by the receptor stimulates Ca^{++} pumping (6), making systole briefer.
B Two action potentials from the same myocardial cell before (left) and after (right) adrenergic stimulation. The effect of activating beta$_1$ receptors increases the Ca^{++} current that flows during the plateau, heightening it.

Figure 4.2-2: **The influence of the ß1-adrenergic receptor on muscle contraction. Taken from *Human Physiology*, Second Edition, David F. Moffett, Stacie B. Moffitt and Charles L. Schauf, Mosby, St. Louis, 1993.**

In the context of the long-term goal to develop new extinguishing agents, the development of a validated *in vitro* screening test system to identify and rank the cardiac sensitizing potential of candidate agents is a high priority. The time and cost ultimately saved by such a test system will far outweigh the initial investment in development and validation. The value of mechanistic studies should not be underestimated. As a result of our lack of understanding of the mechanistic basis of the cardiac sensitization process, the development of an *in vitro* screen is based on intuition and can only be validated empirically by mathematical correlations. This limits the reliability of model predictions and introduces uncertainties that may not be acceptable to regulatory agencies. In this case the currently accepted animal test, the canine cardiac sensitization test, will still be required for meeting regulatory standards. This does not mean that the alternative test cannot be used effectively for screening purposes with the prospect that chemicals with low toxicity *in vitro* will easily pass the *in vivo* test. With a better understanding of the mechanisms involved in the cardiac sensitization process, better *in vitro* tests can be developed that ultimately will gain regulatory acceptance in addition to their value as screening tools.

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4.3 90-DAY NOSE-ONLY INHALATION TOXICITY STUDY OF TRIFLUOROIODOMETHANE (CF_3I) TO MALE AND FEMALE FISCHER 344 RATS

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ABSTRACT

Trifluoroiodomethane (CF_3I) is being considered as a replacement for Halon fire suppressants. Its structure is similar to that of Halon 1301 (CF_3Br), but it has very low ozone depletion potential. Male and female Fischer 344 rats were exposed to 0 (air only), 2, 4, or 8% CF_3I vapor (via nose-only), 2 h/day, 5 days/wk for 13 weeks. Rats exposed to 8 or 4% CF_3I had lower mean body weights than the controls. Deaths observed in the 2 and 8% groups were attributed to accidents involving the restraint system employed. Mild, but consistent hematologic alterations in the 8% group included a reduction in RBC count and total lymphocytes. Increases in the frequency of micronucleated bone marrow polychromatic erythrocytes were observed in rats of all three CF_3I groups. Serum chemistry alterations observed in rats of the 8% group included decreases in calcium, ALT, triglycerides and T_3; thyroglobulin, rT_3, T_4, and TSH were increased. Alterations in thyroglobulin, T_3, rT_3, T_4, and TSH were observed also in rats of the 4 and 2% CF_3I groups. Relative organ weight increases (8% CF_3I group) occurred in the brain, liver, and thyroid; decreases were observed in the thymus and testes. Histopathological findings included a mild inflammation in the nasal turbinates of rats exposed to 4 or 8% CF_3I, mild atrophy and degeneration of the testes (4 and 8% CF_3I groups), and a mild increase in thyroid follicular colloid content in rats of all CF_3I exposure groups. Though NOAELs were observed for select target organs (e.g., nasal turbinates, testes), NOAELs were not apparent in all target organs examined (e.g., thyroid, bone marrow.)

INTRODUCTION

Environmental concern over the depletion of stratospheric ozone and global warming has led to an international treaty called the The Montreal Protocol (1984) which calls for the phaseout of halons by the year 2000. Presently, the Air Force is using Halon 1301 (CF_3Br) as a flooding agent for extinguishing in-flight aircraft and electronic equipment fires and for fire extinguishment in confined spaces. Because it is has less ozone depleting activity and excellent fire suppression properties, trifluoroiodomethane (CF_3I) is being considered as a possible replacement for Halon 1301 in unoccupied spaces.

Little information is available in the literature concerning CF_3I toxicity. A modified acute inhalation toxicity test was performed in which rats were exposed in a nose-only chamber to 12% CF_3I for 15 min (Skaggs et al.,1993). Excess salivation was observed in the rats upon removal from the chamber; however, all rats appeared to be fully recovered by 2 h postexposure. A 15-min, nose-only inhalation study in rats determined the LC_{50} value to be 27% CF_3I (Ledbetter, 1994). No deaths occurred following an acute 4-h nose-only inhalation exposure to either 0.5 or 1.0% CF_3I (Kinkead et al., 1994). Additionally, no treatment-related signs of toxic stress were noted immediately following exposure. Histopathologic

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examination of select tissues from animals examined immediately following exposure, three days postexposure, or after a 14-day postexposure observation period showed no lesions of pathologic significance. No repeated exposure toxicity studies with CF_3I were found in the literature.

In other toxicity testing protocols, CF_3I was positive in the *Salmonella typhimurium* histidine reversion mutagenesis assay (Mitchell, 1995a). The L5178Y/*tk* mouse lymphoma cell mutagenesis assay showed that CF_3I did not induce gene or chromosomal mutations in mammalian cells *in vitro* (Mitchell, 1995c). However, a positive evaluation in the mouse bone marrow erythrocyte micronucleus test indicated that CF_3I was clastogenic *in vivo* (Mitchell, 1995b). Cardiac sensitization testing of CF_3I vapor using beagle dogs showed a no observable effect level at 0.2% and a lowest observable adverse effect level at 0.4% (Kenny et al., 1995). This toxicity precludes the use of this compound as a flooding agent in occupied spaces, but it may still be used in unoccupied spaces (Vinegar et al., 1995).

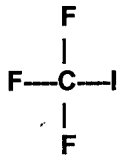
CF_3I has a high vapor pressure under ambient conditions (541 mmHg at 25 °C), thus inhalation is a major route of exposure for persons in the workplace. The objectives of this study were to 1) obtain repeated (subchronic) inhalation exposure data in rats to assess potential target organ effects and 2) to obtain a no-observable-adverse-effect-level (NOAEL). Since CF_3I contains an iodine atom, it is possible that CF_3I exposure might interfere with thyroid function (Capen, 1995). In this investigation, emphasis was placed on monitoring several serum thyroid hormone levels and on histopathological examination of the thyroid, including image analysis of thyroid follicular colloid.

MATERIALS AND METHODS

Test Material

Pertinent physical and chemical properties of trifluoroiodomethane follow.

Structure



CAS No.	2314-97-8
Systematic Name	Iodotrifluoromethane
Molecular Weight	195.91
Empirical Formula	CF_3I
Physical State	Colorless gas
Specific Gravity	2.3608 g/mL (-42 °C)
Melting Point	-110 °C
Boiling Point	-22.5 °C
Flash Point	None
Flammability Limits	Nonflammable
Vapor Pressure	541 mm Hg @ 25 °C
Solubility in H_2O	0.862%

CF_3I, a liquid under pressure at room temperature, was obtained from Deepwater Iodide (Carson, CA), through Combat Systems Activity, Aberdeen Proving Ground, MD. Eleven propane tanks were received, each containing between 36 and 69 pounds of CF_3I. The purity of the CF_3I test material was determined to be 99.6+% by GCMS (Tekmar 7000 Headspace Analyzer, Forster City, CA; Hewlett-Packard 5890A GC, HP 5970B Mass Selective Detector, and HP Vectra 386/25 Data System, Palo Alto, CA).

Exposure Chamber Design and Operation

Nose-only rather than whole-body exposures were performed, because of limited supply and high cost of the CF_3I test material. This decision reduced considerably the amount of test material required to carry out the 90-day study. The chamber selected for nose-only exposure was a stainless-steel flow past system designed by Cannon et al., 1983. Each chamber had 52 ports for exposure of animals. In this study, 30 ports were selected randomly for rat exposure. Rat restraining tubes made of Lucite® were plugged into the animal ports resulting in the outward extension of the tubes radially from the main body of the chamber.

Generation of Test Material

The CF_3I and dilution air were delivered from pressurized systems and were controlled through flow meters. Fine control of chamber concentration was made by minor adjustment of the CF_3I flow in response to chemical analysis of the chamber atmosphere. Total chamber air flow resulted in the delivery of more than 300 mL/min of mixed CF_3I/air at each animal port.

A portion of the diluent air passed through a gas washing bottle (Ace Glass, Vinland, NJ, Model 7166-26) containing water to provide adequate relative humidity to the chamber input air stream. Relative humidity and temperature of the exposure atmosphere were constantly monitored and recorded using HY-CAL dual probes (Models CT830, HY-CAL, Atlanta, GA) and a data acquisition system.

GC/MS analysis of mixed CF_3I/air collected from the nose-only ports did not differ from the CF_3I vapor collected from the cylinders demonstrating no breakdown products of the test material during the generation of test atmospheres.

Chamber Analysis

Continuous analysis of the chamber air for CF_3I was performed using infrared absorption spectrometers (Miran 1A, Foxboro Analytical, South Norwalk, CT). A short path (10-cm) cell in combination with a low intensity absorption band at 9.6 to 9.7 microns facilitated the analysis of the chamber CF_3I vapor concentrations involved in this study. Instrumental calibration was performed using known concentrations of freshly prepared CF_3I in air contained in tedlar sample bags (231 series, SKC, Eighty Four, PA). Calibration checks were performed at two-week intervals during the study.

Exposure Concentration Selection

In addition to the acute inhalation toxicity studies with CF_3I that were described in the Introduction, a two-week inhalation exposure range-finding study was performed (Kinkead et al., 1995). Four groups, each containing five male Fischer 344 rats, were exposed via nose only at target concentrations of 12, 6, 3, or 0 (air-only control)% (vol/vol) CF_3I. Exposures were 2 h/day, 5 days/wk, for a total of ten exposures. No deaths were observed, though lethargy and slight incoordination were

noted in the high- and mid-concentration groups at the conclusion of each daily 2-h exposure. Mean body weight gains were depressed in the high-level group ($p<0.01$) at 7 days and 14 days, and in the mid-level group ($p<0.05$) at 7 days only. Serum thyroglobulin (HTG) and reverse T_3 (rT_3) values were significantly increased at all exposure levels. At necropsy, no gross lesions or differences in absolute or relative organ weights were noted. Histopathologic examination of the thyroid and parathyroids indicated no morphological abnormalities in CF_3I-exposed rats.

Failure of the high-concentration rats to gain weight during the two-week period, clinical signs of inactivity, and increases in serum HTG and rT_3 indicated that 12% CF_3I might be too stressful as a target concentration for the 90-day study. The mid-concentration group (6%) showed some minimal clinical signs of stress, such as transient weight-gain suppression and mild increases in serum HTG and rT_3 values. Increases in serum HTG and rT_3 were the only clinical findings noted in the low-concentration (3%) group. Given that the increases in serum HTG and rT_3 were not supported with microscopic findings in the thyroid and parathyroids, target concentrations chosen for the 90-day study were 8, 4, 2, and 0% CF_3I. The 8% concentration level was expected to produce clinical signs and, possibly, microscopic effects in the thyroid gland of CF_3I-exposed rats. The mid-concentration level (4%) was expected to produce minimal to no adverse effects in rats and the low-concentration level (2%) was expected to produce no adverse effects (i.e., a NOAEL).

Test Animals

Sixty male and 60 female Fischer 344 (F-344) rats were purchased from Charles River Breeding Laboratories, Raleigh, NC. The rats were six weeks of age upon arrival and nine weeks of age at the initiation of the 90-day exposure regimen. Complete details on quality control and animal husbandry conditions are available in a technical report (Kinkead et al., 1996).

Exposure Regimen and Response Assessment

Fifteen male and 15 female F-344 rats were exposed 2 h/day, 5 days/week, for up to 13 weeks (65 exposures over 90 days) to 0 (air-only), 2, 4, or 8% CF_3I. Prior to initiation of the CF_3I exposures, rats were acclimated to the nose-only chamber restraint system, breathing air only, 2 h/day for one week. Five male and five female rats per group were necropsied after 30 days on study (20 exposures). Records were maintained for body weights (weekly), and signs of toxicity including mortality (twice daily). Euthanasia was via CO_2 inhalation overdose. At the 30- or 90-day sacrifice, gross pathology was performed and tissues were harvested for histopathologic examination. Wet tissue weights were determined on adrenals, brain, heart, kidneys, liver, lungs, ovaries, spleen, testes, thymus, and thyroids (including parathyroids). Complete details of the methods used for general and specific target organ toxicity assessments are available in a technical report (Kinkead et al., 1996).

Statistical Analysis

Comparisons of mean body weights were performed using the multivariate analysis of covariance for repeated measures test (Rosner, 1990). A two-factorial (treatment and sex) analysis of variance with multivariate comparisons was used to analyze the hematology, clinical chemistry and organ weight data. The histopathology data were analyzed using Yates' corrected Chi-square (Zar, 1974). Chi-square analysis and one-way analysis (ANOVA) were used to evaluate data for the micronuclei induction and the PCE/NCE ratio changes.

RESULTS

Exposure System Analysis

The specified target concentrations of 8, 4, and 2% CF_3I were maintained during the daily 2-h exposures. The exposure mean concentrations were maintained within ± 2% of the desired concentrations. Mean concentrations for each exposure, along with the lowest and highest daily mean concentrations are provided in Table 4.3-1. The daily mean relative humidity ranged between 47 and 52%, while the daily mean temperature ranged from 67 to 73 °F.

TABLE 4.3-1. CF_3I CONCENTRATIONS INHALED BY MALE AND FEMALE F-344 RATS

Target Concentration (%)	8.0	4.0	2.0
Mean Concentration (%)	8.05	4.03	2.02
Standard Error	0.01	>0.01	>0.01
Lowest Daily Mean (%)	7.94	3.95	1.99
Highest Daily Mean (%)	8.18	4.10	2.05
Mean Temperature (°F)	70.2	70.7	70.5
Mean Relative Humidity (%)	49.6	49.4	49.4

In-Life Observations

During the daily 2-h exposures, rats of the 8% CF_3I group were highly active compared to air-only exposed rats; the 4% exposure group were moderately active and the 2% exposure group were mildly active. Since the rats were restrained in horizontal exposure tubes, the activities observed consisted of twisting or turning of the whole body, pushing backward from the front (exposure) end of the tube, and constant moving and/or pushing of the limbs against the tubular wall. (Note: An animal with increased activity continues to inhale the test material, due to the flow-past design of the nose-only exposure system.) For comparison, the rats of the control group were generally motionless and appeared to sleep throughout the exposure period. Following exposure, rats of the 8% group appeared lethargic.

Six male rats from the 2% CF_3I group died during the ninth day of exposure. A male rat from the 8% exposure group was found dead in its cage on the morning following 10 days of exposure. Another male rat of the 2% group died during the thirteenth exposure. Following the loss of 6 male rats on Exposure Day 9, the remaining male rats (all study groups) were placed into larger nose-only exposure tubes when their body weight reached 225 g. No additional changes in tube size were made once an animal was moved to a larger exposure tube. Because of the unexpected deaths, male rats from the 2% CF_3I group were not available for necropsy at the scheduled 30-day animal sacrifice.

A female rat assigned to the control group appeared moribund and was removed from the study during the pre-exposure acclimation period. This event resulted in a control group size of 14 female rats for the 90-day exposure regimen.

Mean body weights of the male and female rats of the 8% CF_3I group showed a slight decrease compared to control values through the first three weeks of the study. Mean body weights of the male group did not return to their pretreatment weight

until 28 days into the study (Figure 4.3-1). Similarly, mean body weights of the 8% CF_3I female rats did not return to their pretreatment weight until 42 days into the study (data not shown). The mean body weights of both the male and female high-concentration rats were statistically significantly ($p<0.01$) less than control values for the duration of the study. Mean

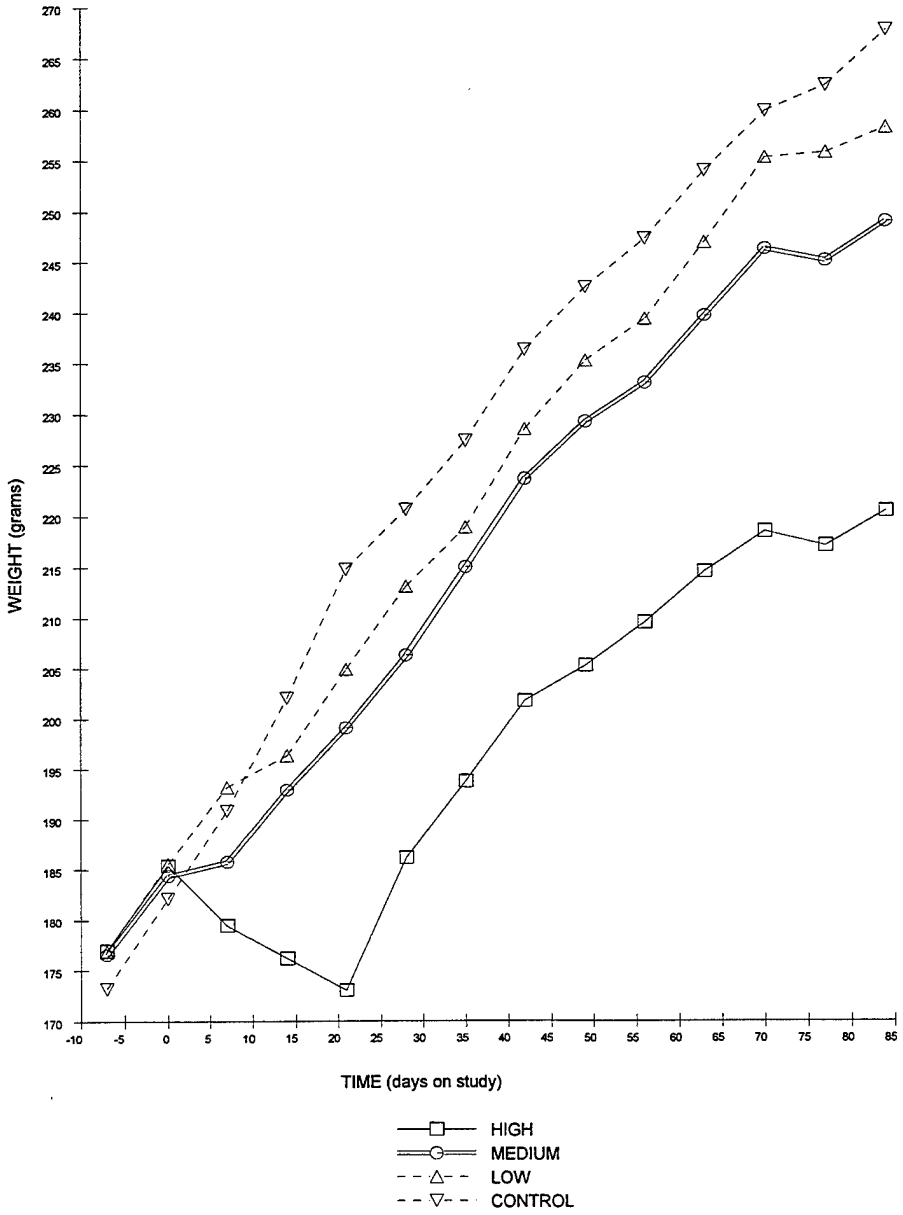


Figure 4.3-1. **Mean Body Weights of Male F-344 Rats Exposed to CF_3I Vapor for 90 Days in Nose-Only Chambers.**

body weights of the 4% CF_3I male rats were also less than control means ($p<0.01$) beginning on Study Day 14 and continuing through the remainder of the study. Mean body weights of the 4% CF_3I female rats differed ($p<0.05$) from the control means on Study Day 7 only. Mean body weight values for the 2% CF_3I rats did not differ from the body weight means of control rats.

Hematology

30 Days. The mean values of hemoglobin concentration, red blood cell count, and lymphocyte percentage were statistically significantly decreased in the 8% CF_3I male rats compared to control male rat values; neutrophil percentage was increased. Mean lymphocyte percentages were also less than the control means in the 4% CF_3I male rat group and the 8% female rat group. Details of the 30-day hematologic results are available in a technical report (Kinkead et al., 1996).

90 Days. Mean red blood cell count and lymphocyte percentage in the 8% CF_3I-exposed male rats remained statistically significantly less than control values. Mean lymphocyte percentages were also decreased in the 8% female rat group. The observed decreases in mean hematologic indices due to CF_3I exposure were mild (< 10%) in magnitude. Details of the 90-day hematologic results are available in a technical report (Kinkead et al., 1996).

Serum Chemistry

30 Days. Mean calcium and glucose levels were decreased, and ALT and globulin levels were statistically significantly increased compared to control values in the 8% CF_3I male and female rat groups. The mean triglycerides value was decreased in the 8% male rats only. A number of statistically significant treatment- and/or concentration-related effects were observed in several thyroid hormone values. Mean thyroglobulin (HTG) levels were increased at all CF_3I exposure levels in female rats and at the 8% CF_3I level in male rats when compared to respective female and male rat control values. Mean thyroxine (T_4) levels were increased in all CF_3I exposure groups. Mean triiodothyronine (T_3) levels were decreased in male rats of the 8 and 4% CF_3I groups; neither the 2% male rats nor the female rats were assayed. Mean reverse T_3 (rT_3) levels were increased in the 8% male rats and the 4 and 8% female rats. Mean thyroid stimulating hormone (TSH) concentrations were elevated in all CF_3I-exposed female rat groups, as well as in the 8% male rats. Details of the 30-day serum chemistry results are available in a technical report (Kinkead et al., 1996).

90 Days. Compared to control means, statistically significant decreases in calcium and ALT values were observed in both male and female rats exposed to 8% CF_3I (Tables 4.3-2 and 4.3-3).

Mean glucose was decreased in the 8% female rats, and mean trigylcerides were decreased in the 8% male rats. As observed in the 30-day animal sacrifice, a number of statistically significant treatment- and/or concentration-related effects were observed in several thyroid hormone values (Tables 4.3-2 and 4.3- 3). Increases in mean HTG, T4 and TSH, and decreases in mean T_3 were observed in all CF_3I-exposed male and female rat groups, except for HTG concentration in the 2% female rats. Mean rT_3 values were increased in both male and female rats of the 4 and 8% CF_3I groups.

TABLE 4.3-2. MEAN VALUES[a] OF SERUM CHEMISTRY PARAMETERS FOR MALE F-344 RATS FOLLOWING 90 DAYS OF TREATMENT WITH CF_3I

	CONTROL	LOW	MEDIUM	HIGH
UN (mg/dL)	20.7 ± 1.2	21.0 ± 1.7	19.7 ± 0.72	20.4 ± 0.60
Creatinine (mg/dL)	0.43 ± 0.02	0.44 ± 0.03	0.42 ± 0.01	0.41 ± 0.01
Calcium (mg/dL)	11.1 ± 0.15	10.9 ± 0.12	11.0 ± 0.24	10.9 ± 0.13[c]
Phosphorus (mg/dL)	9.22 ± 0.28	9.06 ± 0.36	9.39 ± 0.22	9.52 ± 0.32
Total Protein (g/dL)	6.79 ± 0.06	6.66 ± 0.11	6.52 ± 0.07	6.78 ± 0.09
AST (IU/L)	114 ± 7.6	106 ± 5.2	107 ± 6.1	105 ± 8.5
ALT (IU/L)	66.4 ± 4.8	65.8 ± 2.3	68.2 ± 2.9	62.7 ± 2.3[c]
Alkaline phosphatase	268 ± 11	285 ± 6.6	272 ± 9.8	257 ± 7.7
Albumin (g/dL)	3.83 ± 0.06	3.73 ± 0.10	3.70 ± 0.06	3.91 ± 0.08
Globulin	2.93 ± 0.04	2.84 ± 0.06	2.82 ± 0.04	2.84 ± 0.02
A/G Ratio	1.31 ± 0.03	1.32 ± 0.06	1.31 ± 0.02	1.36 ± 0.03
Glucose (mg/dL)	148 ± 3.9	150 ± 6.0	162 ± 6.5	137 ± 3.0
Sodium (mmol/L)	151 ± 1.5	155 ± 2.2	150 ± 1.2	154 ± 2.0
Triglycerides (mg/dL)	117 ± 19	133 ± 17	95.5 ± 7.5	74.8 ± 7.8[c]
Magnesium (mg/dL)	2.88 ± 0.10	2.76 ± 0.10	2.85 ± 0.10	2.90 ± 0.09
Potassium (mmol/L)	5.20 ± 0.09	5.29 ± 0.14	5.32 ± 0.09	5.49 ± 0.15
Cholesterol (mg/dL)	55.2 ± 1.9	53.3 ± 1.5	47.5 ± 7.0	56.8 ± 1.5
Thyroglobulin (ng/mL)	0.27 ±<0.01	0.46 ± 0.01[b]	0.60 ± 0.02[b]	1.11 ± 0.02[b]
T_3 (ng/mL)	271 ± 7.6	192 ± 5.5[b]	176 ± 7.2[b]	134 ± 4.8[b]
T_4 (mg/dL)	2.84 ± 0.2	3.53 ± 0.1[c]	3.10 ± 0.2[c]	3.09 ± 0.2[c]
rT_3 (ng/dL)	6.66 ± 0.1	6.96 ± 0.2	8.91 ± 0.2[b]	9.66 ± 0.3[b]
TSH (ng/mL)	3.21 ± 0.1	4.68 ± 0.1[b]	6.14 ± 0.2[b]	8.14 ± 0.2[b]

[a]Mean ± SEM.
[b]Significantly different from control, $p<0.01$.
[c]Significantly different from control, $p<0.05$.

TABLE 4.3-3. MEAN VALUES[a] OF SERUM CHEMISTRY PARAMETERS FOR FEMALE F-344 RATS FOLLOWING 90 DAYS OF TREATMENT WITH CF_3I

	CONTROL	LOW	MEDIUM	HIGH
UN (mg/dL)	21.0 ± 1.0	18.9 ± 2.3	21.2 ± 1.2	23.9 ± 1.2
Creatinine (mg/dL)	0.44 ± 0.04	0.46 ± 0.02	0.44 ± 0.02	0.42 ± 0.02
Calcium (mg/dL)	10.8 ± 0.23	10.6 ± 0.15	10.6 ± 0.19	10.4 ± 0.14[c]
Phosphorus (mg/dL)	8.74 ± 0.21	8.41 ± 0.21	8.61 ± 0.17	9.03 ± 0.34
Total Protein (g/dL)	6.47 ± 0.11	6.13 ± 0.06[c]	6.31 ± 0.09	6.43 ± 0.10
AST (IU/L)	154 ± 45	140 ± 31	149 ± 20	141 ± 15
ALT (IU/L)	60.7 ± 4.8	57.3 ± 2.1	67.0 ± 8.8	52.5 ± 1.8[c]
Alkaline phosphatase	265 ± 16	269 ± 11	278 ± 6.3	275 ± 7.2
Albumin (g/dL)	3.62 ± 0.10	3.42 ± 0.05	3.48 ± 0.09	3.58 ± 0.10
Globulin	2.81 ± 0.04	2.73 ± 0.03	2.83 ± 0.03	2.85 ± 0.03
A/G Ratio	1.28 ± 0.04	1.26 ± 0.02	1.24 ± 0.04	1.27 ± 0.03
Glucose (mg/dL)	136 ± 7.5	135 ± 5.5	131 ± 3.5	128 ± 4.1[b]
Sodium (mmol/L)	155 ± 3.4	150 ± 1.9	152 ± 2.2	151 ± 2.5
Triglycerides (mg/dL)	56.4 ± 5.9	54.7 ± 6.1	50.8 ± 4.4	46.6 ± 3.4
Magnesium (mg/dL)	2.68 ± 0.11	2.69 ± 0.07	2.73 ± 0.08	2.74 ± 0.09
Potassium (mmol/L)	5.82 ± 0.14	5.53 ± 0.12	5.78 ± 0.19	5.90 ± 0.16
Cholesterol (mg/dL)	67.2 ± 2.6	57.2 ± 1.8	57.6 ± 1.2	56.7 ± 2.4
Thyroglobulin (ng/mL)	0.44 ± 0.01	0.51 ± 0.01	0.76 ± 0.02[b]	1.10 ± 0.04[b]
T_3 (ng/mL)	274 ± 8.5	190 ± 6.3[b]	167 ± 3.8[b]	123 ± 3.0[b]
T_4 (mg/dL)	2.82 ± 0.1	4.20 ± 0.2[c]	4.09 ± 0.2[c]	3.92 ± 0.2[c]
rT_3 (ng/dL)	6.72 ± 0.2	7.12 ± 0.01	8.64 ± 0.2[b]	10.17 ± 0.4[b]
TSH (ng/mL)	4.36 ± 0.1	5.73 ± 0.2[b]	7.77 ± 0.1[b]	9.38 ± 0.2[b]

[a] Mean ± SEM.
[b] Significantly different from control, $p<0.01$.
[c] Significantly different from control, $p<0.05$.

Organ Weights

30 Days. The weights of several organs were affected by CF_3I exposure. Statistically significant concentration-related decreases in absolute and relative (to body weight) mean thymic weights occurred in male and female rats exposed to 4 or 8% CF_3I. Mean testes weights of the 8% CF_3I-exposed male rats were decreased approximately 30% compared to control males. The means of relative thyroid weights were greater than control values in the 8% and 4% (female rats only) CF_3I groups. The relative mean weights of liver and brain were increased mildly in male and female rats of the high-exposure CF_3I group. Relative weight values for the kidneys, spleen, heart, adrenals, lungs, and ovaries (female rats) of CF_3I-exposed animals did not differ from control values. Details of the 30-day organ weight results are available in a technical report (Kinkead et al., 1996).

90 Days. Relative mean thymic weights of CF_3I-exposed female rats (2, 4, and 8% groups) and male rats (4 and 8% groups) were statistically significantly lower than the control means (Table 4.3-4 [data for female rats are not shown]). Mean testes weights of the 8% CF_3I-exposed male rats were decreased compared to control males. The relative mean thyroid weights of male CF_3I-exposed rats (2, 4, and 8% groups) and female rats (8% only) were increased compared to respective control values. The relative mean weights of liver and brain were increased mildly in male and female rats of the high-exposure CF_3I group. Relative weight values for the kidneys, spleen, heart, adrenals, lungs, and ovaries (female rats) of CF_3I-exposed animals did not differ from control values.

TABLE 4.3-4. ABSOLUTE (G) AND RELATIVE ORGAN WEIGHTS[a] OF MALE F-344 RATS TREATED WITH CF_3I FOR 90 DAYS

ORGANS	CONTROL[e]	LOW[f]	MEDIUM[g]	HIGH[e]
Body Weight	269.84 ± 10.25	261.56 ± 11.62	250.32 ± 7.91	222.77 ± 3.44[c]
Brain	1.91 ± 0.03	1.83 ± 0.05	1.78 ± 0.04[c]	1.82 ± 0.06[c]
Ratio[b]	0.71 ± 0.03	0.71 ± 0.03	0.72 ± 0.02	0.82 ± 0.02[d]
Liver	8.78 ± 0.40	8.53 ± 0.44	8.23 ± 0.34	7.40 ± 0.19
Ratio	3.25 ± 0.06	3.26 ± 0.05	3.28 ± 0.05	3.32 ± 0.05[d]
Kidneys	1.95 ± 0.07	1.82 ± 0.08	1.81 ± 0.06	1.66 ± 0.05[c]
Ratio	0.72 ± 0.02	0.70 ± 0.01	0.72 ± 0.01	0.74 ± 0.01
Spleen	0.58 ± 0.03	0.70 ± 0.18	0.48 ± 0.01[d]	0.44 ± 0.03[d]
Ratio	0.21 ± 0.01	0.26 ± 0.06	0.19 ± 0.01	0.20 ± 0.01
Thymus	0.25 ± 0.02	0.22 ± 0.02[c]	0.21 ± 0.01[d]	0.18 ± 0.02[d]
Ratio	0.087± 0.01	0.086 ± 0.01	0.083 ± <0.01[c]	0.081 ± 0.01[c]
Heart	0.87 ± 0.03	0.85 ± 0.04	0.89 ± 0.10	0.77 ± 0.04
Ratio	0.32 ± 0.01	0.33 ± 0.02	0.36 ± 0.05	0.35 ± 0.02

TABLE 4.3-4.(CONT'D) ABSOLUTE (G) AND RELATIVE ORGAN WEIGHTS[A] OF MALE F-344 RATS TREATED WITH CF_3I FOR 90 DAYS

	CONTROL[e]	LOW[f]	MEDIUM[g]	HIGH[h]
Adrenal Gland	0.11 ± 0.02	0.10 ± 0.03	0.07 ± 0.01	0.09 ± 0.02
Ratio	0.04 ± 0.01	0.04 ± 0.01	0.03 ± <0.01	0.04 ± 0.01
Lungs	1.71 ± 0.06	1.62 ± 0.05	1.52 ± 0.07	1.42 ± 0.05[d]
Ratio	0.63 ± 0.01	0.63 ± 0.05	0.61 ± 0.02	0.64 ± 0.02
Thyroid[h]	18.30 ± <0.01	20.12 ± <0.01	18.70 ± <0.01	17.22 ± <0.01
Ratio	0.0067 ± <0.01	0.0080 ± <0.01[c]	0.0076 ± <0.01[c]	0.0078 ± <0.01[c]
Testes	2.85 ± 0.04	2.59 ± 0.12	2.52 ± 0.08	2.06 ± 0.14[d]
Ratio	1.07 ± 0.03	1.00 ± 0.06	1.01 ± 0.02	0.92 ± 0.06[d]

[a]Mean ± SEM.
[b]Organ weight/body weight x 100.
[c]Significantly different from control, p<0.05.
[d]Significantly different from control, p<0.01.
[e]N=9.
[f]N=8.
[g]N=10.
[h]Expressed as mg.

Bone Marrow Micronuclei Induction

30 Days. The results of the micronuclei examination of bone marrow polychromatic erythrocytes of male and female rats indicate that CF_3I at concentrations of 4 and 8% increased the micronuclei frequency in male rats by 2.7- and 3.9-fold, respectively, and in female rats, 3.5- and 4.9-fold, respectively (Table 4.3-5). Rats administered the positive control agent, cyclophosphamide, increased micronuclei frequency 7- to 8-fold above the air-only control values. Female rats examined from the 2% CF_3I group did not show an increase in the mean percentage of micronucleated cells compared to the control value (Table 4.3-5). Bone marrow toxicity (indicated by a statistically significant reduction in the ratio of number of polychromatic erythrocytes to number of normochromatic erythrocytes; PCE/NCE) was observed in male and female rats exposed to 4 or 8% CF_3I and in female rats exposed to 2% CF_3I (data not shown).

TABLE 4.3-5. MICRONUCLEI INDUCTION BY CF_3I IN RAT BONE MARROW ERYTHROCYTES

GROUP	MICRONUCLEATED CELLS (%)[a]	RATIO
	Male Rats 30 Days	
Control (3)[b]	0.23 ± 0.05	1.00
4 % (5)	0.64 ± 0.10	2.74
8% (4)	0.90 ± 0.12	3.86
	Male Rats 90 Days	
Control (2)	0.25 ± 0.05	1.00
2 % (5)	0.60 ± 0.09	2.40
4 % (5)	1.06 ± 0.17	4.24
8 % (5)	1.38 ± 0.27	5.52
	Female Rats 30 Days	
Control (3)	0.20 ± 0.08	1.00
2 % (5)	0.20 ± 0.06	1.00
4 % (5)	0.70 ± 0.13	3.50
8 % (4)	0.98 ± 0.18	4.88
	Female Rats 90 Days	
Control (2)	0.30 ± 0.10	1.00
2 % (5)	0.58 ± 0.16	1.93
4 % (5)	0.78 ± 0.16	2.60
8 % (5)	1.30 ± 0.24	4.33

[a] **Mean ± SD.**
[b] **(N)**

<u>90 Days.</u> CF_3I exposure increased the micronuclei frequency in polychromatic erythrocytes of male rats by 2.4-, 4.2- and 5.5-fold and of female rats by 1.9-, 2.6- and 4.3-fold at concentrations of 2, 4 and 8%, respectively (Table 4.3-5). The PCE/NCE ratios, compared to control values, were reduced by 39, 51, and 57% in male rats and by 50, 56, and 60% in female rats of the 2, 4, and 8% CF_3I groups, respectively (data not shown).

Pathology

<u>30 Days.</u> At necropsy, there were no exposure-related or clinically significant gross lesions. Microscopically, the prevalence of rhinitis was 100% in the 8% CF_3I-exposed male and female rats (data not shown). This lesion was described as mild mucopurulent rhinitis, characterized by abundant intraluminal accumulation of mucus admixed with degenerate neutrophils. Two of five male rats of the 4% group had similar, but milder lesions in the nasal turbinates, while two of five female rats (4% group) had minimal olfactory epithelial necrosis. CF_3I-exposed male rats of the 8% group showed a marked degeneration and loss of spermatogonia, with dramatic loss of spermatids (data not shown). Aspermia was observed and

shrunken seminiferous tubules were lined by a single layer of Sertoli cells, which appeared normal. Epididymal lumina were filled with sloughed spermatogonia admixed with a few degenerative spermatids. Testicular lesions were similar, but milder in the 4% CF_3I group of male rats. No additional exposure-related microscopic lesions were observed.

90 Days. At necropsy, there were no exposure-related or clinically significant gross lesions. The rhinitis noted at 30 days was no longer present. However, 56% of the high-exposure male rats exhibited minimal multifocal olfactory epithelial necrosis (data not shown). Similar changes were present in the 8% CF_3I-exposed female rats, though at a lower incidence. This lesion was characterized by sloughing of scattered, small groups of epithelial cells, with shrunken cytoplasm and pyknotic nuclei; however, there was no intraluminal mucopurulent material.

Mild testicular degeneration and atrophy were noted in 78% of the high-exposure male rats (data not shown). The severity of this lesion was less when compared to the observations noted at Study Day 30. Minimal degeneration of the testes was noted also in the 2 and 4% CF_3I exposure groups. Epididymal sperm granulomas were present in 14% of the low-level CF_3I-exposed rats and 40% of the mid-level and; however, this lesion was not present in the 8% CF_3I or control male rats.

All male and female rats from the 8% CF_3I exposure group exhibited a mild, diffuse increase in thyroid follicular colloid content. No follicular lining cell hypertrophy or hyperplasia was observed. Similar changes were noted, but with much lower incidence, in low- and mid-exposure animals. Computerized morphometric image analysis of the observed follicular colloid confirmed that there was a treatment-related increase in follicular lumen area of CF_3I-exposed rats (Table 4.3-6). However, the increase was not concentration-related, and statistical significance was observed in the high-exposure female rats only.

For the remaining tissues examined, histologic lesions noted were considered to be commonly observed incidental findings unrelated to CF_3I exposure. In some animals, there was mild to minimal degenerative cardiomyopathy consisting of scattered bundles of degenerative myocytes, occasionally infiltrated by low numbers of macrophages and lymphocytes. Male rats exhibited some histologic evidence of renal tubular hyaline droplet formation and occasional mild tubular mineralization.

TABLE 4.3- 6. FOLLICULAR AREA[a] OF THYROIDS OF MALE AND FEMALE F-344 RATS FOLLOWING 90-DAYS INHALATION OF CF_3I

	CONTROL	LOW	MEDIUM	HIGH
Male	1395 ± 63	1515 ± 53	1607 ± 52	1587 ± 59
Female	1388 ± 47	1427 ± 45	1405 ± 49	1922 ± 67[b]

[a] (Microns)^2, Mean ± SEM, N = 993 to 1248.
[b] Significantly different from control, $p<0.05$.

DISCUSSION

In this study, male and female F-344 rats were exposed to 0 (air only), 2, 4, or 8% CF_3I vapor, 2 h/day, 5 days/week for 13 weeks. Because of the high cost of the CF_3I test material, and to keep study expenses reasonable, exposures were conducted in nose-only chambers where animals were placed in restraining tubes during the period of CF_3I inhalation. Exposure systems requiring animal restraint may cause animal stress (reviewed by Phalen et al., 1984), such as excessive heat, decreases in body weight (Klimisch et al., 1987), alterations in pulmonary function (Landry et al., 1983) or suppression of pulmonary defenses (Jakab and Hemenway, 1989). The deaths observed in the present study were attributed to accidents for the restraint system employed. Rationale for this explanation include the observation of deaths in male rats only (the heavier gender), lack of concentration-related effect (deaths occurred in the low and high CF_3I concentration groups only), and absence of mortality upon the use of larger (and presumably less stressful) holding tubes for rats weighing more than 225 g. Further, all deaths occurred between Exposure Days 9 and 13. In the two-week nose-only exposure range-finding study with CF_3I (Kinkead et al., 1995), no animal deaths were observed at vapor concentrations as high as 12%.

The testicular effects observed in the present study are suspect, in part, to be due to the heat stress associated with the use of nose-only exposure chambers. It is well recognized that in rats, the testis is exquisitely sensitive to hyperthermia-induced degeneration and atrophy (Haschek and Rousseaux, 1991). Recent studies at Haskell Laboratories with hydrofluorocarbon-143a (Frame et al., 1992) compared the results of toxicity observed by either nose-only or whole-body exposure systems and concluded exposure design was a factor in the testicular lesions that were observed only in the nose-only chamber system. Further, the finding in the current study, that the severity of testicular degeneration and atrophy was considerably less in male rats sacrificed at 90 days compared to male rats sacrificed at 30 days was associated closely with the size of the restraining tubes used and the timing of their use. However, the absence of both testicular atrophy and degeneration of spermatogonia in male rats of the low CF_3I concentration group (at 90 days) or air-only control group (at 30 and 90 days) suggest a CF_3I-induced effect at higher exposure concentrations.

Observations of lower body weights and mild to minimal inflammation in the nasal turbinates of male and female rats exposed to 4 or 8% CF_3I are common signs of toxicity indicative of inhalation studies conducted with mildly adverse chemicals at very high exposure concentrations. The measured endpoints of greatest concern, but not unexpected, are the lesions observed in the bone marrow and thyroid. CF_3I was positive in the Ames (Mitchell, 1995a) and mouse bone marrow erythrocyte micronucleus tests (Mitchell, 1995b), but was negative in the mouse lymphoma test (Mitchell, 1995c). The finding of induction of micronuclei in bone marrow PCEs in the present study extends the observation of an *in vivo* chromosomal effect to two mamalian species. However, the lack of histopathology, including any cell proliferative-type lesions in the numerous tissues observed in this study, places the concern of this genotoxic effect produced by CF_3I as equivocal.

The thyroid gland was a target organ in this study. Serum chemistry analysis provided support for the mechanistic hypothesis that CF_3I inhibits the enzyme iodothyronine 5'-deiodinase that is responsible for the conversion of T_4 to T_3, resulting in a decrease in T_3, a corresponding marked increase in rT_3, and a compensatory increase in TSH. The response of the thyroid gland to increased TSH levels is to increase T_4 production by the follicular cells (Capen, 1995). This physiologic event is manifested histologically by a marked decrease in follicular colloid content and increased height of follicular lining cells . Histologic alterations observed in the thyroid follicles in this study did not follow this histologic scenario, and in fact

exhibited the opposite condition, with follicles being slightly increased in area (statistically significant in female rats of the 8% CF_3I group), and lined by flattened rather than columnar follicular cells. Similar observations were observed in studies with excess iodide in drinking water (Kanno et al., 1994) and with FD&C Red No. 3 dye (Capen and Martin, 1989), and recent evidence (Capen, 1996) suggests that the presence of iodinated lipid molecules in the plasma membrane of thyroid follicular cells may modify receptor sensitivity to TSH, thereby decreasing the proliferative response of the cells to elevated TSH levels. In contrast, non-iodinated goitrogens (e.g., propylthiouracil) appear to decrease the formation of iodolipids, resulting in the classic proliferative response by follicular cells. In this study, the clinical and histopathologic results provide support for the expected response to a highly iodinated compound which inhibits 5'-deiodinase and results in the formation of iodolipids; however, further studies are required to confirm either hypothesis. Though toxicity concerns, including thyroid goiter and tumor development, exist for chemicals inducing a sustained increase in the secretion of pituitary TSH, rats and mice are highly sensitive species compared to humans to these pathological phenomena in the thyroid gland (Capen, 1996; McClain et al., 1988).

Subchronic inhalation toxicity of CF_3Br, a chemical used widely for its fire extinguishant properties, was investigated in rats and dogs more than 40 years ago (Comstock et al., 1953). Daily exposures for 18 weeks at an average concentration of 2.3% CF_3Br showed no signs of adverse effects and no pathological changes at necropsy. The present ACGIH TLV-TWA value of 1000 ppm (0.1% v/v) for CF_3Br was recommended to minimize the potential central nervous system and cardiovascular involvement observed in controlled studies performed in laboratory animals and humans (ACGIH, 1986). The epinephrine-induced cardiac sensitization NOAEL in dogs is approximately 5% for CF_3Br (reviewed by Trochimowicz, 1975) compared to a NOAEL of 0.2% for CF_3I (Kenny et al., 1995).

The results from this investigation showed definite signs of multiple organ toxicity in rats of the 8% group. Mild to minimal toxicity extended into rats of the 2 and 4% CF_3I exposure groups. Though NOAELs were observed for select target organs (e.g., testes and nasal turbinates), NOAELs were not apparent in all target organs examined (e.g., thyroid and bone marrow).

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4.4 GAS UPTAKE KINETICS OF 1,1,1,3,3,3-HEXAFLUOROPROPANE (HFC-236fa) AND IDENTIFICATION OF ITS POTENTIAL METABOLITES

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INTRODUCTION

As part of the Navy's efforts to eliminate ozone depleting substances, a new refrigerant, 1,1,1,3,3,3-hexafluoropropane (HFC-236fa), has been developed through a joint EPA/Navy effort. This refrigerant is a replacement for refrigerant CFC-114, which is used in centrifugal chillers aboard surface ships and submarines. NAVSEA, the EPA Air and Energy Research Laboratory, and the NMRI Toxicology Detachment have developed the toxicity information needed for approval under EPA's Significant New Alternative Policy (SNAP) program and the Clean Air Act. A two-year rodent bioassay was proposed to be conducted as a part of the toxicology profile for HFC-236fa. Uptake of this compound is low and indications are that the material is weakly metabolized. In order to assess the need for the bioassay, pharmacokinetic and metabolism studies were conducted to determine the extent of uptake and metabolism. The results of these studies are reported herein.

The aim of these studies was to measure tissue:air partition coefficients and to describe the kinetics of HFC-236fa, via recirculating gas uptake exposure methods, and to look for and identify potential metabolites in blood and urine after inhalation exposure to HFC-236fa.

Inhalation pharmacokinetics were determined experimentally in Fischer 344 (F-344) male rats. A physiologically based pharmacokinetic (PBPK) model was used to describe mathematically the disposition and metabolism of HFC-236fa employing chemical-specific parameters and apparent whole-body metabolic constants calculated from these experiments.

Samples from gas uptake studies were collected from rats for the determination of potential metabolites of HFC-236fa in blood, urine, and feces. Chamber air samples and moisture trap samples for differing exposure levels and durations were collected and analyzed. Possible metabolites that were considered were hexafluoroacetone, hexafluoropropanol, and fluorocarboxylic acids. In order to accommodate the range of volatility of these compounds, both headspace-GC/MS and liquid injection-GC/MS were used. Also both the direct extraction of samples and the derivatization/extraction (for carboxylic acid conversion to methyl esters) were used.

METHODS

Test Chemical (provided by DuPont)

1,1,1,3,3,3-hexafluoropropane	HFC-236fa
CAS # 690-39-1	
Mol. Weight	152
Empirical formula	CF3-CH2-CF3
Boiling point (°C)	-0.7

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Animals

Male F-344 (200 to 350 g) rats (*Rattus norvegicus*) were obtained from Charles River Breeding Laboratories (Kingston, NY). Animals received Purina Formulab #5008 and softened water *ad libitum*. They were housed in plastic cages (2 to 3/cage) with hardwood chip bedding prior to exposure, and were maintained on a 12-h light/12-h dark light cycle at constant temperature (22 ± 1 °C) and humidity (40 to 60%). Cages were changed twice per week. Animals were marked for identification with a tail tattoo.

Determination of Partition Coefficients

Partition coefficients were determined by using a modified version of the vial-equilibration technique described by Gargas et al. (1989). Whole tissue was harvested and minced into a tissue slurry versus prepared as a tissue homogenate in saline. Rats used to determine partition coefficients were euthanatized with CO_2. Blood was collected from the posterior *vena cava* using a heparinized syringe. Liver (L), muscle (M, quadriceps), fat (F, epididymal and perirenal), and gastrointestinal tract (G, stomach and small intestine) also were removed for analysis. Blood samples (2.0 mL) were placed in 12.4 mL glass vials and incubated/mixed for 3 h at 37 °C with 800 ppm of chemical in the vial headspace. Incubation time was determined by initially exposing samples for 1, 3, 5, or 7 h and observing that no change was seen after 3 h. Chemical concentration was determined by initially using 80, 400, or 800 ppm and observing no difference between 400 and 800 ppm. Whole tissue samples (1.0 g of L and M; 0.50 g of F and G) were minced and incubated/mixed under the same conditions as for blood.

The chemical concentrations in the headspace were analyzed using a HP19395A headspace sampler (Hewlett-Packard, Avondale, PA) connected to a HP5890 gas chromatograph (GC) (Hewlett-Packard, Palo Alto, CA) equipped with a hydrogen flame ionization detector (FID). A 30 m x 0.53 mm Supelco Vo Col™ 3.00 µm Film column was used. Gas chromatography conditions were set with the detector temperature at 250 °C, injection temperature 125 °C, helium carrier gas at 16.0 mL/min column flow plus 24.0 mL/min make-up flow, and oven temperature held constant at 150 °C.

Gas Uptake and Metabolic Constants

A closed chamber recirculating gas uptake system with a volume of 8.0 L was used for the estimation of whole animal metabolic constants (V_{maxc}, K_m, and K_{fc}) (Gargas et al., 1986). Initially loss runs without rats were completed to determine the loss rate of the chemical from the system alone. Then loss runs were performed with dead rats to determine if there was any additional loss by adherence to the surface of the animals. Next the actual uptake runs were performed in which three F-344 rats were exposed to the study chemical. Five exposure concentrations were performed for 6 h each (HFC-236fa concentrations were 100, 530, 2350, 7300, and 18000 ppm). Barilyme (75 g) was used as the CO_2 absorber. Oxygen concentrations were maintained at (21% ± 1) during the exposures. The system flow was maintained at 2.5 L/min with the flow to the sample loop of the GC at 100 mL/min.

The chemical concentrations in the chamber atmosphere were monitored every 5 min for the first 30 min, and every 15 min thereafter using a gas sampling valve connected to a HP5890 GC. Chromatography was performed on a 6ft x 1/8in 3% OV-17, 60/80 Chromosorb column. The GC was equipped with a hydrogen FID with temperature set at 250 °C, helium carrier flow at 35.4 mL /min, injection temperature of 100 °C, and oven temperature held constant at 105 °C.

Model Development

SIMUSOLV (DOW Chemical Co., Midland, MI), a Fortran-based continuous simulation language with optimization capabilities, was used on a VAX/VMS 8530 mainframe computer (Digital Equipment Corp., Maynard, MA). The general form of the PBPK model (Figure 4.4-1) followed that of Ramsey and Andersen (1984). The codes that made up the PBPK models are given in the Appendices. Parameters were optimized by SIMUSOLV which used the log likelihood function as the criterion, and either the generalized reduced gradient method for single parameter optimization or the Nelder-Mead search method for multiple parameters optimization to adjust the values.

Physiological constants for calculating volumes of the compartments are shown in Table 4.4-1. Tissue volume constants are scaled to the actual body weight (BW) of the rats under study (fat volume was derived from Anderson et al. [1993]); other constants were according to Linstedt (Physiological Parameters Working Group, ILSI Risk Science Institute, unpublished data). Blood flows are expressed as a percentage of cardiac output which was scaled to BW to the exponent 0.75. Alveolar ventilation is also scaled to BW to the exponent 0.75. Cardiac output and alveolar ventilation, based on those described by Gargas et al. (1986) for resting animals, are summarized in Table 4.4-1.

TABLE 4.4-1. KINETIC CONSTANTS AND PHYSIOLOGICAL PARAMETERS USED IN PBPK MODELING IN RATS

DESCRIPTION	[UNITS] PARAMETER
Tissue Volumes	[Fraction of Body Weight:BW]
Liver	$V_LC = 0.037$
Fat	$V_FC = 0.01*(35*BW+2.1)$
GI Tract	$V_GC = 0.033$
Slowly Perfused	$V_SC = 0.558$
Rapidly Perfused	$V_RC = 0.031$
Flow Rates	[L/h/kg]
Alveolar Ventilation	$Q_PC = 14.0$
Cardiac Output	$Q_CC = 14.0$
	[Fraction of Cardiac Output]
Liver	$Q_LC = 0.032$
Fat	$Q_FC = 0.058$
GI Tract	$Q_GC = 0.183$
Slowly Perfused	$Q_SC = 0.255$
Rapidly Perfused	$Q_RC = 0.472$

Blood:air and tissue:air partition coefficients were obtained as described in table 4.4-1. Metabolic constants were determined using the model to obtain a simultaneous fit to the closed chamber gas uptake data. The constants are scaled to BW using the allometric relationship described by Andersen et al. (1987).

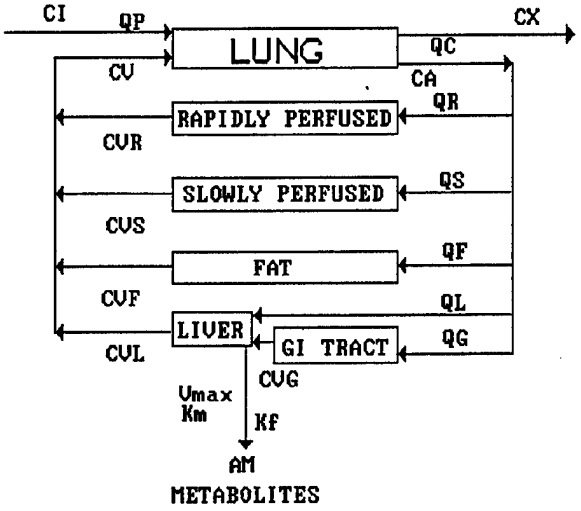

Figure 4.4-1. A scheme of PBPK model used for computer simulations of HFC-236fa disposition and metabolism in rats.

PBPK Model Construction

Figure 4.4-1 shows the scheme of the PBPK model, essentially as described by Ramsey and Andersen (1984). An additional compartment was added to describe the gastrointestinal (GI) tract.

Mass transfer differential equations describing each compartment of the PBPK model for both chemicals (schematically shown in Figure 4.4-1) are presented below.

For simple, well-stirred compartments in which neither metabolism nor other losses occurred (rapidly and slowly perfused tissues, fat, and gut), the change in the amount of chemical (A_i) over time (t) was described as follows:

$$dA_i/dt = Q_i(CA - CV_i)$$

where subscript $_i$ represents the "i-th" compartment; Q_i represents the blood flow through the "i-th" compartment; CA represents the arterial concentration; CV_i represents the venous concentration leaving the "i-th" compartment ($CV_i = C_i/P_i$; where C_i is a concentration in the tissue in the "i-th" compartment, and P_i is the tissue/blood partition coefficient for "i-th" compartment. $C_i = A_i/V_i$, where V_i represents the volume of the "i-th" compartment).

For the liver compartment, a loss term (RAM) was added to the well-stirred compartment description to account for rate of metabolism ($RAM = V_{max}CV_L/(K_m + CV_L) + K_fCV_LV_L$; where V_{max} is apparent-maximal velocity rate of the metabolism, CV_L is venous concentration leaving the liver, K_m is apparent Michaelis-Menten constant, K_f is the first order rate of metabolism, and V_L is the volume of liver):

$$dA_L/dt = Q_L(CA - CV_L) + Q_G(CV_G - CV_L) - RAM$$

where Q_G is the blood flow through the portal circulation (from the GI tract) and CV_G is a concentration of the chemical that reaches the liver via portal circulation (from the GI tract). Units for the above variables are as follows: amounts - mg, concentrations - mg/L, flows - L/h, and rates - mg/h.

Metabolite Identification

Extraction and Esterification/Extraction GC/MS Analyses

Simple extractions of either 0.5 mL or 0.5 g of samples with 1.0 mL hexane or cyclohexane were vortexed for 1 h at 40 °C. Both solvents were used in order to permit examination of the chromatographic regions obscured by either solvent. The organic layer was then transferred to autosampler vials for analysis.

Esterification of potential carboxylic acids present in the samples was achieved by taking 0.1 mL or 0.1 g of sample, adding 0.5 mL sulfuric acid and 0.1 mL dimethyl sulfate, and vortexing the samples for 30 min at 60 °C. Extraction of these samples was achieved by adding either 1.0 mL hexane or cyclohexane, vortexing the samples for 60 min at 37 °C, and then centrifuging the samples for 12 min at 2500 rpm. The organic layer was then transferred to autosampler vials for analysis.

Samples were analyzed using either the full scan or the selected ion monitoring modes of operation with the following analytical instruments:

Hewlett-Packard 5971A Series Mass Selective Detector

Hewlett-Packard 5890A Series II Gas Chromatograph

Column:	DB-624, 30 m X 0.25 mm ID, 1.4 μm film thickness
Program:	35 °C - 11.7 min/ 15 °C per min/ 180 °C - 5.0 min
Scan Range:	35-200 amu
SIM:	51, 69, 99, 147 and 59, 63, 69, 100 ions

Headspace GC/MS Analyses

After some experimentation to determine optimum conditions for the headspace autosampler, 0.5 mL (0.5 g) or less aliquots of samples were transferred to 9 mL headspace vials and analyzed using the full scan mode of operation with the following analytical instruments:

Hewlett-Packard 5970B Series Mass Selective Detector

Hewlett-Packard 5890A Gas Chromatograph

Tekmar 7000 Headspace Analyzer & Cryofocusing Module

Column: Poraplot Q, 30 m X 0.32 mm ID, 10 μm film thickness

Program: 45 °C - 10.0 min/ 12 °C per min/ 175 °C - 5.0 min

Scan Range: 47-500 amu

Reagents and Standards

The dimethyl sulfate, methyl trifluoroacetate, methyl pentafluoropropionate, pentafluoropropionic acid, 1,1,1,3,3,3-hexafluoro-2-propanol, 1,3-difluoro-2-propanol, 2,2,3,3,3-pentafluoro-1-propanol, and hexafluoroacetone•trihydrate were obtained from Aldrich Chemical Co. (Milwaukee, WI). The sulfuric acid, cyclohexane, and hexane were obtained from Fisher Scientific (Fair Lawn, NJ).

Equipment

All samples were prepared and analyzed using the following instrumentation:

Hewlett-Packard 5971A Series Mass Selective Detector, Serial No. 3324A04160

Hewlett-Packard 5890A Series II Gas Chromatograph, Serial No. 3310A49086

Hewlett-Packard Vectra 486/66U Data System, Serial No. 3328A52882

Hewlett-Packard 7673 Autosampler/GC Injector, Serial No. 3329A35598

Hewlett-Packard 18596B Sample Tray, Serial No. 3329A32579

Tekmar Cryofocusing Module, Serial No. 92176014

Tekmar 7000 Headspace Analyzer, Serial No. 92063014

Hewlett-Packard 5970B Series Mass Selective Detector, Serial No. 2623a01335

Hewlett-Packard 5890A Gas Chromatograph, Serial No. 2623A07142

Hewlett-Packard Vectra 386/25 Data System, Serial No. 312A091695

Haake-Buchler HBI Vortex-Evaporator, Model 4322000

IEC Centra-8R Centrifuge, Serial No. 24780720

RESULTS

Partition Coefficients

The rat tissue:air partition coefficients determined for HFC-236fa, which were used in the PBPK model optimization, are shown in Table 4.4- 2

TABLE 4.4-2. PARTITION COEFFICIENTS FOR HFC-236fa IN RATS

Partition Coefficients		Ratio ± S.D. (N= 12)
Blood:air	PB	0.49 ± 0.04
Liver:air	PLA	0.56 ± 0.06
Fat:air	PFA	3.69 ± 0.56
Gut:air	PGA	0.56 ± 0.06
Rapidly perfused/air	PRA	0.56 ± 0.06
Slowly perfused/air	PSA	0.87 ± 0.08

Loss Runs

Results of loss runs with and without animals are shown in Table 4.4-3. When the percent loss per hour for a 20,000 ppm HFC-236fa 6-h exposure of 3 live rats was calculated, a loss rate of 1.59% per hour resulted. This was nearly four times the average percent loss per hour for loss runs at this concentration (0.40 ± 0.06% per hour), either with or without dead rats. This increase in loss rate was also true for 10,000 ppm HFC-236fa. Lower concentrations however, such as 100 ppm and 500 ppm, yielded a larger increase in loss rate.

TABLE 4.4-3 SUMMARY OF LOSS RATES[A]

Nominal Chamber Concentration of HFC-236fa	Percent Loss per Hour During Loss Runs	Percent Loss per Hour During Exposures
100 ppm	2.85 ± 1.40% per hour	11.77% per hour
500 ppm	0.67% per hour	8.93% per hour
5,000 ppm	n/a	1.57% per hour
10,000 ppm	0.38 ± 0.07% per hour	2.00% per hour
20,000 ppm	0.40 ± 0.06% per hour	1.59% per hour

[a] Percent loss per hour calculated for loss runs includes loss runs with and without dead rats as there was no significant difference in rates. For 100 ppm and 20,000 ppm, n=3. For 10,000 ppm, n=4. For 500 ppm, n=1.

Gas Uptake Studies

The inhalational uptake of HFC-236fa by the rat showed two discernible phases: a rapid equilibration phase that lasted up to 30 minutes followed by a slow linear uptake phase (Figure 4.4-2). The continuous lines represent the simulations with the assumption of no metabolism and with appropriate chamber loss rates included. The individual points represent the actual gas uptake data collected with live animals in the chamber.

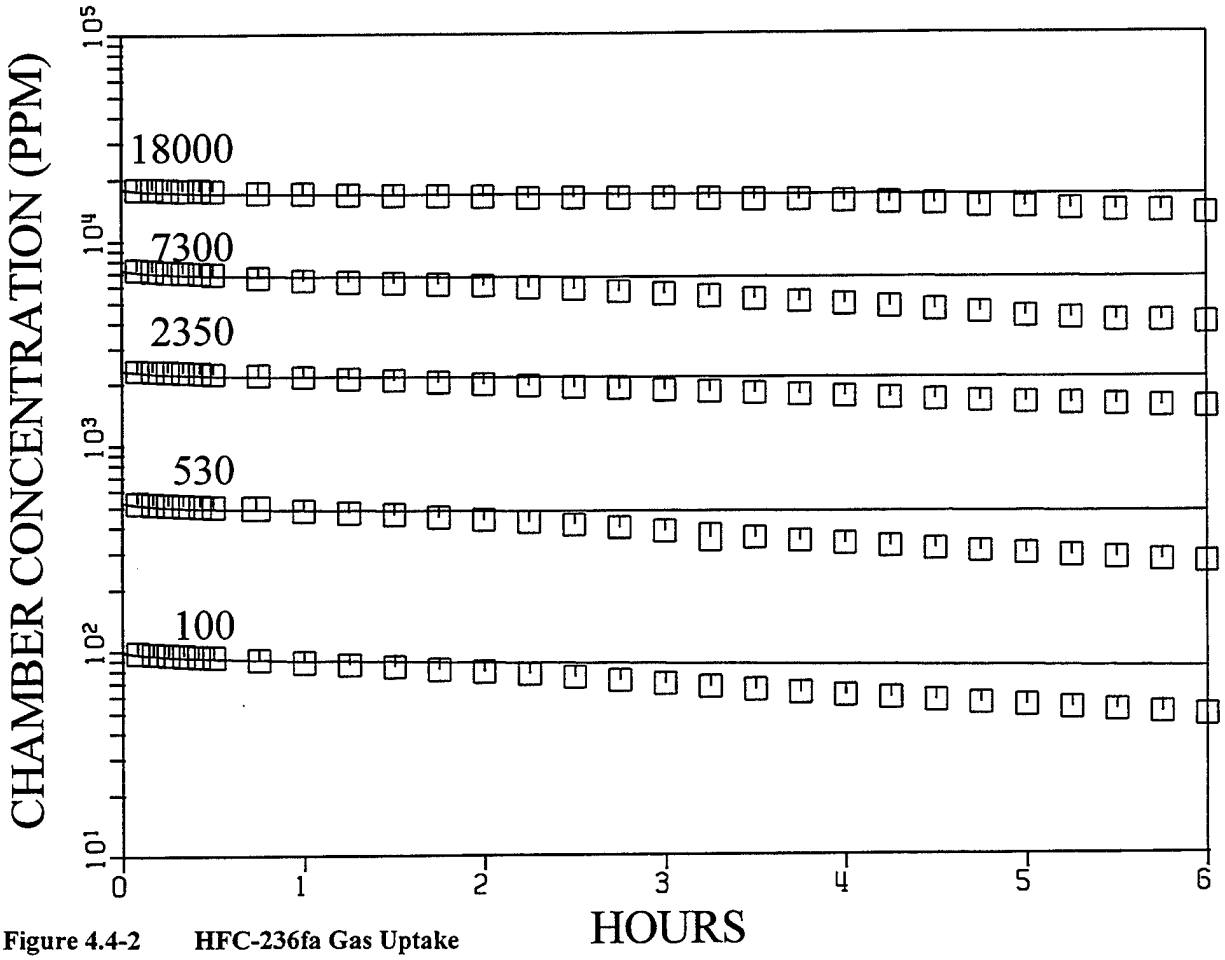

Figure 4.4-2 HFC-236fa Gas Uptake

Metabolite Identification

Samples were designated as being from either 6 h of exposure or 24 h postexposure. Sample extractions of one 24-h rat blood, one 24-h rat urine, one 24-h rat feces, one rat blood control, and one rat urine control, were extracted with hexane and again with cyclohexane as the solvents. One 6-h rat blood, three 24-h rat bloods, three 24-h rat urines, three 24-h rat feces, one rat blood control, one rat urine control, and one water blank were esterified and extracted with hexane as the solvent. In addition, one 24-h rat blood, one 24-h rat urine, one 24-h rat feces, one rat blood control, one rat urine control, and one water blank were esterified and extracted with cyclohexane as the solvent.

No potential fluorocarbon metabolites were detected in these samples, and no response to a single target ion, such as the 69 ion, was detected in these samples that could not also be found in a blank or control sample. In addition, standards of related fluorocarbons were obtained and solutions of these were analyzed in order to "map out" the chromatographic ranges and provide typical mass spectra likely for any metabolites. See Table 4.4-4.

TABLE 4.4-4. FLUOROCARBON STANDARDS DATA

STANDARD	Retention time DB-624	MAJOR IONS
Methyl Trifluoroacetate	3.7 min	50 59 69 99
Methyl Pentafluoropropionate	4.45 min	59 69 100 119
1,3-difluoro-2-propanol	15.2 min	43 63
Pentafluoropropanol	12.9 min	43 69 89 100
1,1,1,3,3,3-hexafluoro-2-propanol	16.6 min	51 69 79 99
Hexafluoroacetone •trihydrate	----	50 69 97 147
Pentafluoropropionic acid	-----	45 69 100 119
HFC-236fa	2.5 min	64 69 113 133

Analytical single ion monitoring runs of solutions of the standards yielded detection limits of 0.5 μg/mL for methyl trifluoroacetate and 0.2 μg/mL for methyl pentafluoropropionate. Curves that were generated for the fluoro-alcohols indicated a quantitation limit of 0.5-5 μg/mL. Since the esterified sample represents a ten-fold dilution (0.1 mL sample → 1 mL solvent), the effective detection limit would be in a range of 5-50 μg/mL.

Samples of chamber air, rat blood, rat urine, rat feces, and moisture trap water from various exposure levels were analyzed by headspace GC/MS. The only fluorocarbon compound detected was the HFC-236fa itself at a retention time of 17.2 min, as verified with air samples from the inhalation system. Examples of the results from four particular exposures are given in Table 4.4-5 , as follows:

TABLE 4.4-5. HEADSPACE SAMPLE RESULTS

Exposure date	Exposure concentration	Samples	HFC-236fa Area counts
26-Apr-95	20380 ppm	Feces sample collected postexposure	0
4-May-95	24460 ppm	Blood sample collected postexposure	74,963
4-May-95	24460 ppm	Blood sample collected postexposure	117,561
24-May-95	17820 ppm	Chamber air sample collected postexposure	6,848,743
24-May-95	17820 ppm	Water collected in moisture trap during exposure	1,989,869
24-May-95	17820 ppm	Blood sample collected postexposure	298,344
24-May-95	17820 ppm	Blood sample collected postexposure	246,587
24-May-95	17820 ppm	Urine sample collected postexposure, postmortem via bladder puncture	0
24-May-95	17820 ppm	Urine sample collected postexposure, postmortem via bladder puncture	0
24-May-95	17820 ppm	Water collected in moisture trap during exposure	2,193,195
26-May-95	2335 ppm	Urine sample collected postexposure, postmortem via bladder puncture	112,112
26-May-95	2335 ppm	Urine sample collected postexposure, postmortem via bladder puncture	0
26-May-95	2335 ppm	Headspace sample from above urine sample	36,884

It should be noted that for the results of the 26-May-95 exposure, only urine #1 was analyzed on 26-May-95. In order to check on the feasibility of storing samples, on 29-May-95 the frozen sample of 26-May-95 was reanalyzed and yielded nothing. A 3 mL aliquot of headspace air above the urine was transferred to a 9 mL headspace vial and yielded a peak of HFC-236fa close to 1/3 the previously found area. The samples had been kept frozen at -20 °C after the initial analysis, but there appears to be problems associated with storage and transferring to the analytical vials.

DISCUSSION

Gas Uptake Studies for Describing Metabolism and System Losses

This simulation approach for analysis of gas uptake data has been shown to distinguish between single and multiple metabolic pathways of several previously studied dihalomethanes and numerous other volatile organic compounds. In the case of HFC-236fa, the gas uptake results suggested a large uptake by the animals implying a high rate of metabolism for a chemical in which it is suspected there is little, if any, metabolism taking place.

The pattern of loss shown by the data in Figure 4.4-2 is inconsistent with the assumption of first-order or saturable metabolism. With first-order metabolism, the slopes of the data lines should be parallel, and with saturable metabolism the higher concentration data lines should approach the horizontal, at some intermediate concentration the slope should start curving downward and at lower concentrations the lines should be straight, parallel, and sloping downward. The data, as collected, show a random pattern of slope with concentration of exposure. Thus, the losses shown by the gas uptake data are assumed due to something other than metabolism. Furthermore, if metabolic constants were fitted to the data,

the rates of metabolism would be extremely high and not consistent with a chemical that is fluorinated at the end carbons, with the inability to find metabolites, and with the low tissue solubility of the chemical.

It was suspected that the humidity level in the chamber played a role in the high loss rates observed during uptake determinations. During a live animal exposure, the humidity level is maintained at approximately 77 to 80% with the moisture trap in place. Under normal conditions for the loss runs, the chamber humidity level was equal to the laboratory humidity level. In order to test this theory, the humidity level was increased in the chamber during loss runs by one of two methods.

The first method of increasing the chamber humidity level involved placing a beaker of water into the chamber during a loss run equal to the average weight of three rats, or approximately 550 grams. In addition, during an average exposure approximately 8 - 10 mL of water is collected in the moisture trap. Therefore eight milliliters of deionized water was placed into the moisture trap prior to addition of chemical into the chamber. A 20,000 ppm HFC-236fa loss run without animals was then completed. The humidity in the chamber averaged 64.64% ± 1.98% over the six-hour period and resulted in a loss rate of 0.30% per hour.

The second method of increasing the chamber humidity level included placing four Petri dishes containing 310 grams of deionized water into the chamber. Throughout the 20,000 ppm HFC-236fa loss run without animals, the chamber was warmed with a heating pad from under the chamber. In addition, periodically throughout the loss run the chamber was warmed with a heat gun. The humidity in the chamber averaged 78.72% ± 6.29% over the six-hour exposure and resulted in a loss rate of 0.28% per hour. Neither of these two methods showed an increase in the percent loss of chemical per hour, thus rejecting the possibility of humidity levels influencing the loss of HFC-236fa concentration levels.

In addition, it was also suspected that the difference in loss rates was due to carbon dioxide that is added to the system during an exposure from the expired breath of the rats. However, a 500 ppm HFC-236fa loss run without dead rats was completed under the original system configuration, with supplemental carbon dioxide at a rate of 0.88 mL/g/h -- the same rate at which rats expire carbon dioxide. A loss rate of 1.50% per hour resulted. This experiment showed an increase in loss rate, although it did not increase the loss rate to the same rate at which it was being lost during a live animal exposure.

Metabolite Identification Studies

No metabolites of HFC-236fa were present at concentrations sufficiently high enough to be detected by the previously described methods. The detection limit for the esterified methyl trifluoroacetate was 0.49 μg/ml and for methyl pentafluoropropionate the detection limit was 0.19 μg/ml. Approximate detection limit for the 1,3-difluoro-2-propanol was 0.3 μg/ml and for the 2,2,3,3,3-pentafluoro-1-propanol was 3.0 μg/ml. Further analyses involving extraction with other solvents, solvent-exchange, and volume reduction might be useful in identifying metabolites with low volatility. For compounds of high volatility, such as hexafluoroacetone, the headspace analysis is still probably the best approach. However, the results of the 26-May-95 exposure samples suggest that additional measures may need to be taken in handling these samples. In the future, no more than 0.5mL of blood or urine should be placed directly into a sealed analytical vial for headspace GC/MS in order to avoid possible losses of the parent compound or other highly volatile materials

due to transferring the sample from one vial to another.

CONCLUSION

1. The PBPK model could not adequately describe the disappearance of HFC-236fa from the chamber atmosphere during gas uptake experiments with the assumption that metabolism was the cause of chemical disappearance. The pattern of loss was, in fact, inconsistent with either first-order or saturable metabolism.

2. Possible cause of the unexplained loss may be associated with carbon dioxide exhalation during the exposure although final confirmation would require further investigation.

3. HFC-236fa had low solubility (partition) in blood and tissues and had minimal, if any, enzymatic metabolism in rats.

4. Explicit analyses for potential metabolites in blood, urine, and feces failed to detect anything but HFC-236fa itself.

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SECTION 5

NITRATE EXPLOSIVES AND PROPELLANTS PROJECT

5.1 DOSE (AND TIME DEPENDENT) BLOCKADE OF PREGNANCY IN SPRAGUE-DAWLEY RATS ADMINISTERED AMMONIUM DINITRAMIDE IN DRINKING WATER

E.R. Kinkead, R.E. Wolfe, and M.L. Feldmann

ABSTRACT

Ammonium dinitramide (ADN) is a class 1.1 explosive oxidizer that is being considered for use in solid rocket propellant mixtures and explosives. Previously, a 90-day general toxicity/reproductive screen performed on this compound at oral (drinking water) doses of 162, 103, 29, and 0.0 mg ADN/kg/day resulted in a treatment-related adverse effect on litter production. Incidences of animals producing litters (1/11, 3/12, 12/12 and 11/12, respectively) and mean numbers of pups per litter (7, 7, 16, and 15, respectively) were both statistically significantly less than controls. In a follow-up study, mated dams treated with ADN at the same doses and examined at Gestation Days 10 and 20 showed a similar effect in fetus loss as those in the reproductive screen. A pre- versus post-implantation dosing regimen indicated that implantation is vulnerable to ADN effects during the preimplantation period (Gestation Days 1-3). No implantation sites were found in the rats treated with 2000 mg ADN/L drinking water during Gestation Days 1-3. The number of implantation sites found in rats treated during Gestations Days 4-8 was similar to that observed in the control group. The pituitary was not specifically identified in this study as the site of primary action, but serum progesterone was reduced by 27%, which may have resulted in reduced fertility.

INTRODUCTION

Ammonium dinitramide (ADN) is being considered for use in solid rocket engine propellant mixtures and explosives. A minimal amount of acute toxicity data are available on ADN. Rabbits treated dermally at the EPA limit dose of 2 g/kg body weight survived a 14-day observation period. The oral (gavage) LD_{50} value for ADN in male Fischer 344 rats is 832 mg/kg (Kinkead and Wolfe, 1994a). Mortality occurred within an hour of treatment and death was preceded by convulsions. Field reports on exposed personnel indicate that the compound is readily absorbed by the skin, resulting in numbness of the fingers (Koppes, 1993).

A general toxicity and reproductive screen has been performed in which male and female Sprague-Dawley rats received ADN in drinking water, both before and during pregnancy (Kinkead et al., 1995). Treatment-related hemolytic anemia was noted in female rats; treatment-related hypokalemia was noted in both sexes. The major effect noted in the study was a dose-dependent blockade of pregnancy. Only 9 and 25% of female rats treated with 162 or 103 mg ADN/kg/day, respectively, produced litters. Additionally, litter sizes were significantly smaller than those produced by the low-dose (29 mg ADN/kg/day) and control groups. Histopathologic examination of reproductive organs of ADN-treated rats revealed no lesions which would preclude production of live litters.

Continuous treatment with ADN has toxic effects on the female reproductive system. Although the reproductive organs appear to be unaffected, the majority of the rats treated at the high levels failed to produce litters. Because the dams gained weight in the normal pattern of gravid rats for the first seven days following mating, it appeared as though a decidual

response occurred and that fetal death and/or resorption were occurring sometime after Gestation Day 7. To determine if the fetuses were being resorbed during early pregnancy, mated female rats were treated with similar doses of ADN as the original general toxicity reproductive study, then necropsied following 10 or 20 days of gestation. Fetuses, resorption sites, and corpora lutea were counted at necropsy. In a follow-up study, to assess the effect of ADN on either development of uterine receptivity or maintenance of pregnancy, a pre- versus post-implantation protocol was performed following the method of Cummings (1990). Following mating, dams received ADN-treated drinking water daily, or during days 1-3 or 4-8 of pregnancy. Assessments were performed at necropsy on Gestation Day 9.

MATERIALS AND METHODS

Test Compound

The ammonium dinitramide [$NH_4N(NO_2)_2$] was supplied by SRI International, Menlo Park, CA. Because ADN is a DOT explosive class "A" compound, only limited quantities were stored and no archive sample was maintained. The ADN sample was reported to be contaminated with 1 to 2% ammonium nitrate (Koppes, 1993). The test compound, a water-soluble powder, was maintained in an enclosed cabinet due to light sensitivity (Koppes, 1993).

Preparation Of Drinking Water Solutions

Solutions were prepared by weighing a specific quantity of ADN and adding it to a known volume of animal drinking water (wt/vol). The animal drinking water was supplied by a commercial water conditioning system (Osmotics Incorporated, Minnetonka, MN). This system consists of an activated carbon filter, softener, and reverse osmosis (RO) filtration. The pH of the RO water prior to the addition of ADN ranged from 6.5 to 7.0. Analyses of drinking water solutions are described in Kinkead et al., 1995.

STUDY I

GROUP ASSIGNMENTS AND DOSE LEVELS

Group	Number of Female Rats	Concentration of Administered ADN (mg/L Drinking Water)	Target Doses of ADN (mg/kgBody Wt/Day)[a]
Control	20	0	0
Low	20	200	16
Middle	20	1000	80
High	20	2000	160

[a]**Assumed daily water consumption of 40 mL/500 g rat.**

Test Animals and Clinical Measurements

Forty male and 80 female Sprague-Dawley derived outbred albino rats [Crl:CD®BR] known as Charles River CD rats, were purchased from Charles River Breeding Laboratories, Raleigh, NC. Male rats were used for the purpose of mating. The rats were 9 weeks of age upon arrival and 12 weeks of age at initiation of the treatment. All rats were identified by tail tattoo and were acclimatized for three weeks. ADN-treated water or control drinking water and feed (Purina Formulab #5002, St. Louis, MO) were available *ad libitum.* Rodent drinking water was supplied via glass bottles equipped with stainless steel sipper tubes and neoprene stoppers. Bottles, stoppers, and sipper tubes were changed weekly. Animal room temperatures were targeted at 21 to 25 °C, and the light/dark cycle was set at 12-h intervals. Parental rats were single housed (except during the mating period) in clear plastic cages with hardwood-chip bedding (Bettachip®, Northeastern Products Corp., Warrensburg, NY). During the mating period the animals were housed in clear plastic cages with stainless steel wire bottoms.

General Design

Female rats began receiving ADN-treated drinking water 14 days prior to mating, and continued through mating and gestation until necropsy. Mating occurred following procedures outlined in Kinkead et al., 1995. Serial sacrifice of gravid dams (10 per group) occurred on Gestation Days 10 and 20.

Body weights of female rats were measured weekly until confirmation of mating, after which they were weighed daily. Water consumption was measured daily except during the period of cohabitation with male rats. Dose levels were calculated from water consumption and expressed as mg ADN/kg body weight/day. At necropsy, uterine contents were examined and the number of implantations and/or resorptions was recorded. Corpora lutea were counted and the ovaries and uterus were sampled for histopathologic examination. Blood was sampled via the vena cava for clinical chemistry evaluations and whole livers were weighed at necropsy.

STUDY II

GROUP ASSIGNMENTS AND ADN TREATMENT

Group	Number of Female Rats	ADN Treatment Period (Gestation Days)	Untreated Water Period (Gestation Days)
Preimplantation	10	1-3	4-8
Postimplantation	10	4-8	1-3
Control	10	None	1-8

Test Animals and Clinical Measurements

Thirty male and 30 female Sprague-Dawley derived outbred rats {Crl:CD®BR] known as Charles River CD rats, were purchased from Charles River Breeding Laboratories, Raleigh, NC. Male rats were used for the purpose of mating. The animals were maintained under the conditions mentioned in Study I.

General Design

Mating occurred on the basis of one male to one female with the date of observation of a copulatory plug being designated Gestation Day 0. Mated female rats were divided into groups (N=10) and then administered ADN (2000 mg/L drinking water) on a daily basis during Gestation Days 1 through 3 (preimplantation period), or Gestation Days 4 through 8 (postimplantation period). A group of control dams (N=10) was maintained on untreated water during Gestation Days 1 through 8. All dams were necropsied on Gestation Day 9. Body weights were measured daily, beginning on Gestation Day 0, and water consumption was measured daily during the time period the dam received treated water. At necropsy, the ovaries and uterus were weighed; uterine contents were examined, and the number of implantations and/or resorptions was recorded. The number of corpora lutea present was also recorded. Blood was sampled via the vena cava for measurement of prolactin, progesterone, and estradiol.

Sera for clinical chemistry evaluations were assayed on an Ektachem 250 Analyzer (Eastman Kodak, Rochester, NY). Kit materials for the radioimmunoassays of prolactin, progesterone, and estradiol were obtained from Amersham Corp., Chicago, IL.

Statistics

Maternal body weights, organ weights, organ-to-body weight ratios, serum chemistry, hematology, and ADN dose calculations were analyzed for statistical significance using a one-factorial analysis of variance with Bonferroni multiple comparisons (Rosner, 1990). A one-factorial repeated measures analysis of variance with Bonferroni multiple comparisons was used for water consumption (Barcikowski, 1983). Tissue lesion severity data were analyzed using the Kruskal-Wallis analysis of variance (Rosner, 1990). Corpora lutea and fetus numbers were analyzed for statistical significance using a one-factorial analysis of variance (Barcikowski, 1983).

RESULTS

Study I

Water consumption decreased significantly ($p<0.01$) in the high-dose rats when treatment began and continued to be statistically significantly less than controls throughout the gestation period. The mid-dose rats showed a decreased consumption ($p<0.05$) during the gestation period only. Water consumption of the low-dose rats compared favorably with consumption measured in the control group. Maximum water consumption occurred during the gestation period resulting in calculated mean doses of 186, 116, and 26 mg ADN/kg/day for the high-, mid-, and low-dose groups, respectively.

No mortality occurred during the study. Treatment-related differences in mean body weights were not evident during the first 10 days of gestation. However, treatment-related differences were statistically significant beginning on Gestation Day (GD) 13. A similar effect was noted in absolute liver weights. However, when the difference in body weights is accounted for, the relative weights do not show any treatment-related differences.

The only treatment-related clinical chemistry effects noted in the GD10 group of rats were an increase in blood urea nitrogen (BUN) and total bilirubin in the high-dose group only. Clinical pathology evaluations measured at GD20 showed numerous treatment-related effects. BUN values were increased at all ADN-treatment levels. Calcium, magnesium, total protein, and albumin were increased in the high- and mid-dose groups. Serum potassium levels were significantly decreased at the high- and mid-dose levels. No differences in total bilirubin values were noted in any of the treated groups examined at GD20.

Mean values of corpora lutea present in the ADN-treated rats at necropsy did not differ significantly from mean values noted in the control rats at either GD10 or GD20 (Table 5.1-1). The numbers of fetuses present in the uterus were greatly reduced in the female rats in the high- and mid-dose groups. Only four of the nine high-dose rats necropsied on GD10 contained fetuses. Two dams had one fetus each, one dam had six, and one dam had 11 fetuses. Four of the 10 mid-dose dams had fetuses present in the uterus. Three had one fetus each, and the fourth rat had 10 fetuses (Appendix 5.1-A).

TABLE 5.1-1. CORPORA LUTEA AND FETUS COUNTS[a] FROM RATS TREATED WITH AMMONIUM DINITRAMIDE BEFORE AND DURING GESTATION

Rats were maintained through either 10 or 20 days gestation

	Gestation Day 10		Gestation Day 20	
Treatment	**Corpora lutea**	**Fetuses**	**Corpora lutea**	**Fetuses**
High	17.2 ± 0.9	2.1 ± 1.3[b]	21.3 ± 2.2	0.4 ± 0.2[b]
Medium	16.0 ± 1.0	1.3 ± 1.0[b]	19.6 ± 1.8	4.6 ± 1.8[b]
Low	16.1 ± 1.0	15.1 ± 1.8	19.5 ± 1.0	14.4 ± 0.8
Control	17.2 ± 0.7	17.1 ± 0.7	17.7 ± 0.8	14.1 ± 0.8

[a] **Mean ± SEM.**
[b] **Significantly different from control at $p<0.01$.**

Two of 11 high-dose rats necropsied at GD20 had two fetuses each present in the uterus. Seven of 10 mid-dose rats had fetuses ranging in numbers from two to 17. All low-dose and control rats had fetuses at necropsy averaging 14 per animal. Overall, six of 20 high-dose, 11 of 20 mid-dose, 19 of 20 low-dose, and 20 of 20 control animals had one or more fetuses present at necropsy. Only two resorption sites were found, both in the high-dose GD20 group of rats.

At necropsy, all animals were in good general condition. Two rats (one low-dose and one control) had enlarged spleens with slight modularity, and a low-dose rat had a flat discolored spot on the liver. None of the gross observations appeared to be treatment related.

Study II

Mean water consumption was 29.7 mL/day and 30.4 mL/day for the preimplantation and postimplantation groups, respec-

tively. Control animal water consumption was not measured in this study, but historically averaged 35 mL/day (Kinkead et al., 1994b). Mean doses for the two groups were 211 and 199 mg/kg/day. Treatment with ADN during Gestation Days 1 through 3 resulted in complete abolition of implantations (Table 5.1-2). ADN had no significant effect on implantations when administered during Gestation Days 4 through 8. Similarly, the implantation index (calculated as number of implantation sites/number of corpora lutea × 100) showed a significant effect in the preimplantation group, but the postimplantation group was unaffected. The relative uterus weights of the preimplantation group showed a similar pattern; however, no effects on ovarian weights were noted. ADN also had no effect on the number of corpora lutea, regardless of when the rats were treated.

TABLE 5.1-2. EFFECTS OF ADN TREATMENT DURING EARLY PREGNANCY[a]

Treatment Period	N	Ovarian Weight (mg)	Uterus Weight (g)	Number of Corpora Lutea	Number of Implantation Sites	Implantation Index
1 - 3	10	109.1 ± 17	0.38 ± 0.15[b]	19.0 ± 1.6	0.0[b]	0.0[b]
4 - 8	10	107.0 ± 19	1.79 ± 0.13[c]	17.6 ± 0.8	16.4 ± 0.5	93.2
None	9	118.4 ± 28	1.44 ± 0.38	17.3 ± 1.0	15.2 ± 1.5	87.9

[a] ADN was administered to groups of rats on either Gestation Days 1-3 or 4-8, or not at all (control). Parameters were assessed following euthanasia on Gestation Day 9. Data are presented as Mean ± SEM except for Implantation Index.

[b] Significantly different from control at $p<0.01$.

[c] Significantly different from control at $p<0.05$.

Serum progesterone, prolactin, and estradiol were sensitive to ADN treatment and showed a significant reduction in both ADN-treated rat groups (Table 5.1-3). All three hormones were statistically significantly ($p<0.01$) less than control values, while progesterone showed a significant ($p<0.01$) difference between the preimplantation and post implantation groups.

TABLE 5.1-3. EFFECTS OF ADN TREATMENT ON SERUM PROGESTERONE, PROLACTIN, AND ESTRADIOL LEVELS I IN RATS AT SACRIFICE

Treatment Period	N	Estradiol (pg/mL)	Progesterone (ng/mL)	Prolactin (ng/mL)
1 - 3	10	4.43 ± 0.16^{a}	111.11 ± 2.40^{ab}	79.99 ± 2.04^{a}
4 - 8	10	3.46 ± 0.06^{a}	128.80 ± 1.49^{a}	75.34 ± 1.70^{a}
None	9	5.27 ± 0.11	140.65 ± 1.19	92.82 ± 2.41

[a] **Significantly different from control at $p<0.01$.**
[b] **Significantly different from postimplantation group at $p<0.01$.**

Additional Experimental Data

A technical report entitled "Dose (and Time Dependent) Blockade of Pregnancy in Sprague-Dawley Rats Administered Ammonium Dinitramide in Drinking Water" by Kinkead, Wolfe, and Feldmann (AL/OE-TR-1995-0181) contains details of assessment data not included in this annual report.

DISCUSSION

Rats examined at Gestation Days 10 and 20 showed a similar pattern of dose-dependent blockade of pregnancy as was noted in the initial general toxicity/reproductive screen (Kinkead et al., 1995). Fertility rates in the present study were comparable to those of the general toxicity/reproductive screen. The increase in mean body weight of all dams during the first seven days was similar to what was noted in the general toxicity/reproductive screen. This would indicate that normal decidualization occurred; however, implantation was impaired in most instances.

In the second study, toxic effects occurred during the preimplantation period as none of the dams treated during Gestation Days 1-3 had implantation sites. Preimplantation failure can be caused by ova/embryolethality, failure of the blastocyst to form, or failure of the uterus to decidualize normally following blastocyst formation. Since normal weight gains of decidualization occurred, the latter explanation can be ruled out. It is possible that ADN effects the pituitary, thereby preventing the prolactin surges which normally occur during early pregnancy. The prolactin surges are necessary for the normal rise in serum progesterone secreted by the developing corpora lutea (Morishige and Rothchild, 1974). Adequate levels of progesterone are necessary for normal uterine function and the decidual growth of early pregnancy (Cummings et al., 1991). Low serum progesterone concentration prevents implantation. Serum prolactin and progesterone concentrations were also depressed in the postimplantation rat group; however, no embryolethality occurred in these animals. It is possible that implant failure could be due to other factors, i.e., oviduct motility which affects sperm, ova, and embryo transport; uterine

receptivity; and ova/embryolethality. Because of the ambiguity of the hormone levels in the pre- and postimplantation rats, it is possible that the lack of implantation was due to one of these other factors. Follow-up studies are presently being conducted to determine the specific causes of the toxic effect.

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APPENDIX 5.1- A

STUDY I-INDIVIDUAL ANIMAL RESULTS

Dose Group (mg/kg/day)	Animal Number	Corpora lutea	Fetuses
High	307	16	0
160 (10 days)	331	22	11
	317	15	6
	338	20	0
	305	16	0
	311	19	1
	356	16	0
	372	18	0
	363	13	1
Medium	306	15	0
100 (10 days)	325	20	0
	339	16	0
	328	21	0
	335	12	0
	346	19	1
	340	11	0
	364	14	10
	358	16	1
	362	16	1
Low	316	14	0
30.0 (10 days)	309	17	16
	327	9	18

	301	15	15
	321	15	15
	330	18	17
	350	17	17
	355	19	18
	348	16	15
	373	21	20
Control	323	20	17
0.0 (10 days)	304	16	16
	314	15	15
	332	19	21
	310	19	17
	360	16	19
	365	18	18
	374	14	13
	380	15	18
	379	0	17

Dose Group (mg/kg/day)	**Animal Number**	**Corpora lutea**	**Fetuses**
High	324	16	0
160 (20 days)	320	13	2
	354	28	0
	359	19	0
	366	21	0
	367	15	2

	371	30	0
	377	27	0
	361	34	0
	302	16	0
	349	15	0
Medium	313	19	3
100 (20 days)	326	14	3
	318	16	12
	333	27	0
	336	31	0
	352	18	3
	351	22	0
	353	14	2
	378	19	17
	344	16	6
Low	308	16	15
30.0 (20 days)	319	25	16
	329	17	15
	322	18	16
	347	19	16
	343	16	16
	337	23	12
	370	23	11
	376	17	17
	368	21	10

Control	334	17	11
0.0 (20 days)	312	14	13
	315	21	11
	341	23	16
	357	17	15
	342	19	18
	345	17	17
	369	17	16
	375	16	12

5.2 PREIMPLANTATION EFFECTS OF AMMONIUM DINITRAMIDE ADMINISTERED IN THE DRINKING WATER OF SPRAGUE-DAWLEY RATS

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ABSTRACT

The Department of Defense is considering the replacment of ammonium perchlorate (AP) with ammonium dinitramide (ADN) in rocket propellants and explosives. Previous studies performed at the Tri-Service Toxicology Laboratory have shown that ADN is a female reproductive toxicant, causing implantation failure in Sprague-Dawley rats when it was administered during the preimplantation period of gestation. The purpose of this follow-up study was to identify the mechanism(s) associated with implantation failure following exposure to ADN. In Phase I, mated female rats were treated with 2.0 grams/liter (g/L) ADN in their drinking water for 24, 48, 72, or 96 hours before preimplantation embryos were harvested from the oviducts or uterine horns. On gestation day one (GD-1), comparable numbers of morphologically normal 2-cell embryos were harvested from the oviducts of the treatment and control groups. On GD-2, the development of the embryos harvested from the treated animals was either slowed or halted when compared to the control embryos. By GD-4, 98% of the embryos harvested from the control group had developed to the morula or blastocyst stage; these were collected from the uterine horns. On GD-4 in the treated group, 41% of the harvested embryos remained at the 2- to 6-cell stage and 59% were degenerate; 82% of these embryos were collected from the oviducts. In Phase II, 2-cell embryos were harvested from the oviducts of superovulated mated B6C3F1 mice. The embryos were cultured for 72 hours in medium supplemented with 0 mM, 1 mM, 4 mM, 6 mM, 10 mM, or 20 mM ADN. The development of embryos cultured at < 4 mM ADN was not affected when compared to the control group. Culture in medium containing ≥ 4 mM ADN either slowed or arrested development of the embryos. These data suggest that the implantation failure seen in animals treated with ADN was due to embryolethality.

INTRODUCTION

The Department of Defense (DoD) is working towards compliance with international standards in minimizing the use of ozone depleting substances. The DoD is considering replacing ammonium perchlorate (AP) with ammonium dinitramide (ADN) in rocket propellants and explosives. Ammonium perchlorate leaves a heavy hydrochloric acid/ smoke trail upon burning. The chlorine has been identified as an ozone depleting substance, and the smoke trail makes rockets more vulnerable to detection. ADN is a cleaner burning, more efficient alternative compound. Preliminary data have shown that ADN is a female reproductive toxicant in rats. Further *in vivo* studies, and possible further *in vitro* studies, are required to determine the mechanism(s) associated with the compound's reproductive toxicity. The data obtained from these studies will be valuable to military industrial hygiene officers, as well as to hygiene personnel at production facilities.

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Background Reproductive Toxicity Data of ADN

Previous studies have shown that ADN is a female reproductive toxicant. The 90-day reproductive toxicity screen of ADN administered in the drinking water of Sprague-Dawley rats showed that only 3 of 12 mid-dose dams and 1 of 12 high-dose dams produced live litters, compared to 9 of 12 in the control group and 11 of 12 in the low-dose group (Kinkead et al., 1994). A pre- vs postimplantation study indicated that ADN exerts its effects during the preimplantation period in rats, or gestation days 1-3 (Kinkead et al., 1995). If ADN is administered in drinking water during this period at a dose of 2.0 g/L, implantation is totally blocked. If the compound is administered only on gestation days 4-8, the number of implantation sites is comparable to the control group. Data from this study also indicated a significant increase in free radical formation in uterine tissue of the preimplantation group, compared to the control and postimplantation groups. The number of corpora lutea was consistent in each group, implying that ovarian function was not affected by ADN treatment. The cause of implant failure could occur at a number of points during the reproductive process: 1) oviduct motility which affects sperm, ova, and embryo transport, 2) uterine receptivity, and 3) ova/embryolethality.

MATERIALS AND METHODS

Test Compound

The ADN [$NH_4N(NO_2)_2$] was supplied by SRI International, Menlo Park, CA. ADN is a white soluble powder. Because it is light sensitive, the test compound was stored in protective vials in an enclosed cabinet. The test compound is known to be contaminated with 1-2% ammonium nitrate.

The test compound drinking water solution was prepared by adding a specific amount of ADN to a known amount of reverse osmosis-treated water (wt/vol), as described by Kinkead et al. (1995). The test solution was light-protected during use.

Animals

Phase I: Male and female Sprague-Dawley rats, 11 weeks of age, were purchased from Charles River Breeding Laboratories, Raleigh, NC. All animals were single-housed, identified by tattoo, and allowed a 14-day acclimation period. Water and feed (Purina Formulab #5002, St. Louis, MO) were available *ad libitum* prior to treatment. The females' drinking water was supplied via glass water bottles equipped with stainless steel sipper tubes and neoprene stoppers. The animal room temperature was maintained at 21-25 °C and the light/dark cycle was set at 12-h intervals. During the mating period, the animals were housed in clear plastic cages with stainless steel wire bottoms.

Phase II: Female B6C3F1 (23 days of age) and male CD-1 (42 days of age) mice were purchased from Charles River Breeding Laboratories, Raleigh, NC. All animals were single-housed, identified by tattoo, and allowed a 14-day acclimation period. Water and feed (Purina Formulab #5002, St. Louis, MO) were available *ad libitum*. The animal room was maintained at 21-25 °C and the light/dark cycle was set at 12-h intervals.

METHODS

Phase I: The embryo transport technique developed by Cummings (1990) was employed in Phase I. The animals were mated one pair/cage; the observance of a copulatory plug was designated as Gestation Day 0 (GD-0). Treatment of the females was begun immediately following observation of a copulatory plug. The mated females were randomly assigned to one of 8 groups (n= ≥ 6/group): Control or treatment GD-1; control or treatment GD-2; control or treatment GD-3; and control or treatment GD-4. The animals were sacrificed via CO_2 inhalation on the gestation day corresponding to their group assignment. The oviducts and uterine horns were removed to separate culture dishes and their lumens flushed with phosphate-buffered saline to expel the embryos. The embryos were collected, counted, their location (oviduct or uterus) recorded, and the stage of development evaluated via phase contrast microscopy. A sample of oviduct and uterine tissue was harvested from each animal for free radical analysis. At necropsy, these tissue samples were homogenized in DBNBS (Sigma, St. Louis, MO), flash frozen, and lyophilized.

Phase II: The females were injected intraperitoneally (IP) with 10 International Units (IU) of pregnant mare's serum gonadotropin (Sigma, St. Louis, MO) followed 48 h later by an IP injection of 10 IU human chorionic gonadotropin (HCG) (Sigma, St. Louis, MO). The animals were mated one pair/cage at the time of HCG injection. The females were sacrificed 38 h later by cervical dislocation. Under aseptic conditions, the oviducts were removed to culture dishes and flushed with medium. The harvested 2-cell embryos were placed into culture in control medium or medium supplemented with ADN as described above. The development of the embryos was monitored via phase contrast microscopy daily for 72 h. The dose response experiment was performed three times.

RÉSULTS

Phase I: There was neither a significant difference in the water consumption nor in the body weights of each treatment group when compared to the respective control group (data not shown). The embryo harvest data is presented in Table 5.2-1. Overall, 9.1 ± 0.35 (SEM) embryos were harvested/dam. There was not a significant difference in the number of morphologically normal 2-cell embryos harvested from the treatment group on GD-1 when compared to its control group. On GD-2, 77% of the control embryos had reached the 3- to 6-cell stage; 16% remained at the 2-cell stage. In the GD-2 treated group, 21% of the embryos had reached the 3- to 6-cell stage, while 52% were at the 2-cell stage. This trend continued on GD-3. By GD-4, 98% of the control embryos had developed to the morula or blastocyst stage; these were harvested from the uterine horns. In the treatment group, 59% of the embryos were degenerate and 41% remained at the 2- to 6-cell stage; 82% of these embryos were harvested from the oviducts. The analyses for free radical identification in samples of uterine and oviduct tissue are in progress.

TABLE 5.2-1 PHASE I: GRADING OF HARVESTED EMBRYOS

	GROUP							
EMBRYO STAGE	**GD-1 C**	**GD-1 T**	**GD-2 C**	**GD-2 T**	**GD-3 C**	**GD-3 T**	**GD-4 C**	**GD-4 T**
N=	**6**	**6**	**6**	**7**	**7**	**8**	**6**	**7**
2-CELL	59	57	10	38	1	10		7
3-6 CELL	3		47	15	15	2		14
7-12 CELL			3		36			
MORULA					9		12	
BLASTOCYST					2		49	
DEGENERATE		4	1	20	3	46	1	30

Values represent the total number of embryos harvested/group.
9.1 ± 0.35 (SEM) embryos were harvested per/dam.
C=CONTROL
T=ADN TREATED

Phase II: The embryo grading for the *in vitro* cultured embryos is shown in Table 5.2-2. Overall, 10.6 ± 0.70 (SEM) 2-cell embryos were harvested/dam. These were randomly distributed in culture between the doses. In the first experiment the embryos were monitored for 96 h; no changes in embryonic development were observed between 72 and 96 h. In the control cultures, 61% of the embryos had reached the morula stage after 24 h; 100% of the control embryos developed to the blastocyst stage after 72 h in culture. There were no degenerate embryos found in the control cultures. There was no significant difference in the development of the embryos cultured in 1 mM ADN. After 72 h in culture, 68% of the embryos in the 4-mM group were blastocysts, 29% were morulas, and 3% were degenerate. After 72 h in 6 mM ADN, 50% of the embryos remained at the 3- to 16-cell stage; 31% were morulas or blastocysts and 19% were degenerate. After 72 h in the high-dose group, 20 mM ADN, 86% remained at the 2- to 16-cell stage and 14% were degenerate. Immunofluorescent staining of nuclear components and cytoskeletal organization of control and treated embryos is in progress.

DISCUSSION

The implantation process is a complex blend of events leading to the successful implantation of a blastocyst in the uterine lining. A normal estrus cycle must be followed by timely fertilization, normal development and transport of the zygotes through the oviducts, and the mounting of a decidual response by the uterus. Disruption of these events at any point may lead to implantation failure. In the rat, ova are fertilized within hours of mating; the embryos develop to the blastocyst stage in the oviducts during GD 0-4. The hatching blastocysts enter the uterine horns on GD 4 and implant between GD 4 and 5 (DeFeo, 1967).

TABLE 5.2-2. PHASE II: GRADING OF *IN VITRO* CULTURED EMBRYOS

ADN CONCENTRATION	HOURS IN CULTURE 24	48	72
O mM	12 (3-16 Cell) 19 Morula	31 Blastocysts	31 Hatching Blastocysts
1 mM	11 (3-16 Cell) 17 Morula	1 (3-16 Cell) 1 Morula 26 Blastocysts	1 (3-16 Cell) 27 Blastocysts
4 mM	18 (3-16 Cell) 15 Morula 1 Degenerate	11 Morula 22 Blastocysts 1 Degenerate	10 Morula 23 Blastocysts 1 Degenerate
6 mM	25 (3-16 Cell) 1 Morula	17 (3-16 Cell) 7 Morula 1 Blastocyst 1 Degenerate	13 (3-16 Cell) 5 Morula 3 Blastocyst 5 Degenerate
10 mM	24 (3-16 Cell) 1 Morula	16 (3-16 Cell) 5 Morula 4 Degenerate	11 (3-16 Cell) 8 Morula 6 Degenerate
20 mM	22 (2-16 Cell)	21 (2-16 Cell) 1 Degenerate	19 (2-16 Cell) 3 Degenerate

Values represent the total number of embryos cultured/dose.
10.6 ± 0.70 2-cell embryos were harvested/dam.

In the pre- vs postimplantation study, none of the dams treated during the preimplantation period had implantation sites, but the numbers of corpora lutea and the weight gain accompanying decidualization were comparable to the control group. These data imply that ovarian and uterine function were not affected by ADN treatment (Kinkead et al., 1995). In the same study, serum progesterone, prolactin, and estradiol were significantly reduced ($p < 0.01$) at necropsy on GD-9 in the group that was treated with ADN during the preimplantation period (GD-0 to GD-3) implying that ADN may affect pituitary function, but normal levels of these hormones are also driven by maternal-embryonic signals and a normal implantation process (Weitlauf, 1988). Further investigation would be required to determine if ADN does affect the pituitary loop. This study indicates that embryolethality is in part responsible for the implantation failure seen following treatment with ADN. In the control group, morphologically normal blastocysts were recovered from the uterine horns on GD-4. In the treated groups, embryonic development was slowed or halted as early as GD-2. No embryos that recovered from the treated groups developed past the 4-cell stage. ADN is known to be a genotoxin (Zhu et al., 1994). ADN may be genotoxic to the developing embryo in the oviduct, or the hormonal/biochemical milieu in the oviductal fluid may in some way be affected by ADN.

In Phase II, the development of embryos *in vitro* was not affected by concentrations of ADN < 4 mM. The development of the embryos *in vitro* was slightly delayed in this group, but 97% did reach the morula or blastocysts stage. At ADN concentrations > 4 mM, embryonic development was significantly delayed and halted completely in the high dose groups (10 and 20 mM). These results correlate with viability studies done with hepatocytes cultured in similiar concentrations of ADN (Dean and Channel, 1995). The results of that study suggested that ADN is a cytotoxic genotoxin, possibly indirectly damaging DNA through an oxidative challenge mechanism. Additional *in vitro* studies would be necessary to determine if the embryolethality reported in this study is the result of genotoxicity/oxidative stress.

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5.3 PREPARATION OF BOVINE TESTICULAR TISSUE SLICES: EVALUATION OF 1,3,5-TRINITROBENZENE TOXICITY

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ABSTRACT

Recent toxicity studies with 1,3,5-trinitrobenzene (TNB) have demonstrated testicular injury in rats (depletion of sperm count and degeneration of seminiferous tubules). A new animal model to evaluate testicular toxicity has been developed and has been used to assess the effects of TNB. This model is noteworthy because it is a novel application of tissue slice technology, it uses tissues which normally would be discarded, and does not require the use of laboratory animals. Preparation of slices of testes was successful using bovine tissue, which was obtained from bulls used for meat processing purposes. Testicular cell relationships (interstitial cell and seminiferous tubules) were maintained and slices remained viable for more than 24 h, as measured by histopathological examination, cellular enzyme (AST) and potassium leakage, and the rate of protein synthesis. The level of toxicity observed for TNB during a 24-h incubation was proportional to the dose. Protein synthesis was the most sensitive indicator of toxicity; a dose/response relationship was also observed for release of intracellular potassium. As compared to control values, protein synthesis was inhibited throughout the incubation period at a concentration of 100 μM TNB, whereas inhibition was detected only after 24 h at a concentration of 10 μM. Protein synthesis was completely stopped by TNB (1000 μM) and cycloheximide (100 μM). TNB was a more potent testicular toxicant than other toxicants evaluated (ethane-1,2-dimethanesulfonate [4.6 mM] and cadmium acetate [1000 μM]). Lipid peroxidation, as measured by release of thiobarbituric acid reactive substances (TBARS), was high in control slices. TNB prevented the release of TBARS in a dose/response fashion. An explanation of this finding is provided. This model offers a novel approach for studying male reproductive toxicity, reduces experimental costs and replaces the need for laboratory animals.

INTRODUCTION

Detection of toxicants which specifically target the testes is best performed *in vivo* since establishing a substance as a testicular toxicant requires a complete organism with integrated tissues. Once testicular toxicity is established, however, numerous investigations with many animal preparations will be required to determine the mechanism of toxicity. Therefore, using whole animals in mechanistic investigations is generally inappropriate, expensive, and poor management of animal resources. More importantly, evaluation of chemical insult to human testes, which is most often the objective of testicular toxicity research, is generally not possible except with isolated cell populations. Since these isolated cell populations do not closely resemble the original testicular tissue, interpretation and extrapolation of results for assessment of risk is difficult.

During the past ten years, tissue slicing techniques which allow the evaluation of chemical insults to a variety of tissues from multiple species have been refined. Using the Krumdieck tissue slicer (Krumdieck et al., 1983) or the Brendel/Vitron slicer, slices can be reproducibly prepared. The viability of tissue slices has been greatly extended through the development of the

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dynamic roller culture method (Sipes et al., 1987). Tissue slice methods allow multiple preparations (50 to 1000/tissue/animal, depending on the tissue and animal), maintain an integrated cellular anatomy and physiology, are relatively inexpensive, and allow interspecies comparisons, including humans. Moreover, essentially all of the analytical methods which can be used for *in vivo* studies are also applicable to slice preparations. Although a few reports on the use of testicular slices appear in the scientific literature (Daehlin et al., 1985; Wettemann and Dsjardins, 1979), use of precision cut testicular slices with the dynamic roller culture methods to evaluate testicular toxicity has not previously been described.

Recent toxicity studies with 1,3,5-trinitrobenzene (TNB), a by-product in the manufacture of trinitrotoluene (TNT), have demonstrated testicular injury in rats (depletion of sperm count and degeneration of the germinal epithelium of the seminiferous tubules [Kinkead et al. 1995]). TNB, along with other munitions products, has been found as a contaminant of soil and groundwater at numerous U.S. Army installations (Walsh and Jenkins, 1992).

The objective of this study was to characterize the mechanism of TNB testicular toxicity and simultaneously demonstrate that testicular slices can be prepared, maintained for extended periods of time, and used to characterize the toxicity of xenobiotics. We found that a ready source of fresh and slicable testicular tissue was bull testes obtained from a local meat processing company.

In addition to TNB, other known testicular toxicants evaluated in this study were cadmium acetate, an ill-defined toxicant and testicular carcinogen (Gunn et al., 1963), and ethane dimethanesulfonate, a Leydig cell toxicant (Kelce et al., 1991; Kelce, 1994).

MATERIALS AND METHODS

Chemicals

1,3,5-Trinitrobenzene was provided by Dr. Gunda Reddy, Army Biomedical Research and Development Lab, Ft. Detrick, Frederick, MD. Ethane dimethanesulfornate (EDS) was generously provided by Dr. William R. Kelce, Health Effects Research Laboratory, U.S. EPA, Research Triangle Park, NC. All other chemicals used in this study were of reagent grade quality or higher.

Preparation of Slices

NOTE: Initial developmental studies focused on the laboratory rat. This model was selected because the data base for testicular toxicants has been developed primarily using rats. However, rat testes presented mechanical challenges because the testicular contents are "semi-solid" and do not allow slicing. To overcome this difficulty, low temperature agarose suspensions of testes were developed. Rat testicular slices suspended in agarose were prepared; however, this approach was unsatisfactory because the presence of the gel would influence the uptake and distribution of toxicants and did not allow *in situ* correlation The solution to this slicing problem was the selection of an alternative species, the bull. Bovine testes possess sufficient intertubular connective tissue to allow slices to be prepared.

Bovine testicles were provided by Landes Meats, Inc., Dayton, OH. Warm and cold ischemic times were approximately 10 and 50 min, respectively. The tunic was stripped from the testicle and the tissue was cored (8 mm diameter) and sliced (300 μ thickness) in ice-cold Sacks buffer using a Brendel/Vitron rotary slicer.

Slice Incubations

Tissue slice incubations were carried out using Teflon/viton/titanium rollers and a dynamic organ culture incubator as previously described (Sipes et al., 1987; Brendel et al, 1993). Slices were placed on rollers, loaded into glass scintillation vials, and pre-incubated for 30 min or 2 h (depending on the experimental design) prior to specific exposures. The incubation medium was Waymouth's medium (1.7 mL/vial) supplemented with 10% fetal calf serum, gentamicin (84 μg/mL), L-glutamine (3.5 mg/mL), and sodium bicarbonate (2.4 mg/mL). Specific toxicants (dissolved in 50 μL DMSO) were added to the medium prior to inserting the slice and roller. Incubation temperatures were 34 °C with an incubator atmosphere of O_2/CO_2 (95%/5%).

Indices of Toxicity

***AST Leakage*:** Aliquots of the culture medium and slice homogenate were assayed for aspartate aminotransferase (AST) using a Kodak Ektachem Analyzer (Model 700XR). Results are expressed as percent of total AST released. **NOTE:** ALT and LDH were also assayed, but were found to be unsatisfactory indicators of viability/toxicity for this particular tissue.

***Protein Synthesis*:** An assay of the incorporation of ^{3}H-leucine (55 mCi/mmol) into acid-preciptable protein was used as a measure of the rate of protein synthesis. For each concentration and time point, three separate slices were incubated. Following preincubation, slices were transferred to Waymouth's medium which had received ^{3}H-leucine (approximately 1 μCi/slice) and a known amount of toxicant. At the end of the incubation period, slices were removed from the medium, washed in Sach's buffer, blotted dry and then homogenized in 1 mL distilled water [dis$H_2$0]. Testicular protein was precipitated by addition of an equal volume of trichloroacetic acid (6%) followed by centrifugation (2,500 X g) for 10 min. The protein pellet was washed by resuspension (1 mL dis$H_2$0) and collected by centrifugation. After resuspension in 1 mL dis$H_2$0, an aliquot (0.5 mL) of washed protein was removed, solubilized in Soluene^R (Packard Instruments Co.), and incorporated radioactivity was determined by scintillation counting (Hionic Fluor^R scintillant). The amount of precipitated protein was determined using a BCA protein assay (Pierce). Results are expressed as DPM/mg protein. Cycloheximide (100 μM) was assayed as a positive control.

***Intracellular K+ Content*:** Individual tissue slices were removed from incubation, homogenized, and protein precipitated with 5% 5-sulfosalicylic acid (50 μL into 250 μL homogenate). After centrifugation, the supernatant was assayed for K+ content using an electrolyte analyzer (model AVL 982-S, AVL Scientific Corp., Roswell, GA). Results are expressed as μmol K+/g slice wet weight.

Histopathology: Bovine testicular slices were fixed in Bouin's solution, paraffin embedded, and stained with Hematoxylin and Eosin (H&E). Slices were evaluated to assess the histologic effects from exposure to 1,3,5-trinitrobenzene (TNB), as well as time-related degenerative changes in controls associated with the tissue slice and media incubation procedure itself.

***Lipid Peroxidation*:** Whether TNB would produce lipid peroxidation in testicular slices was evaluated by measuring release of thiobarbituric acid reactive substances (TBARS) as previously described (Janero, 1990). Aliquots of slice homogenates were added to ice-cold Dulbeco's phosphate buffered saline containing GSH (20 mg/100 mL) and EDTA (48 mg/100 mL). Malondialdehyde (MDA) equivalents were measured by fluorescence spectrophotometry of butanol homogenate extracts.

Electron Paramagnetic Resonance Spectrometry: To detect the production of free radicals in TNB-treated slices, electron paramagnetic resonance spectrometry was used. α-*Tert*-butylphenyl nitrone (PBN) was included as a spin-trap to the incubation medium. At specific times, slices were removed from the medium, weighed, returned to the medium, homogenized, frozen in liquid nitrogen, and freeze dried. The dried homogenate was resuspended in a concentrated form by adding back only 200 μL disH_2O. A 20 μL aliquot of this solution was assayed using an EMS 104 EPR Analyzer (Steel-Goodwin et al., 1994) which had a limit of detection of at least 2 X 10^{10} spins/10^{-4} T. It was linked to a microprocessor (XPS P90 Dell) via a RS232 interface which can be used to start remote acquisition and data collection for post processing using the WIN-EPR® 3.0 program (Bruker, Billerica, MS). Using hyperfine values obtained with this instrument, a computer simulation of a matching EPR signal was performed by Dr. Alasdair J Carmichael's laboratory at Armed Forces Radiobiology Research Institute, Bethesda MD.

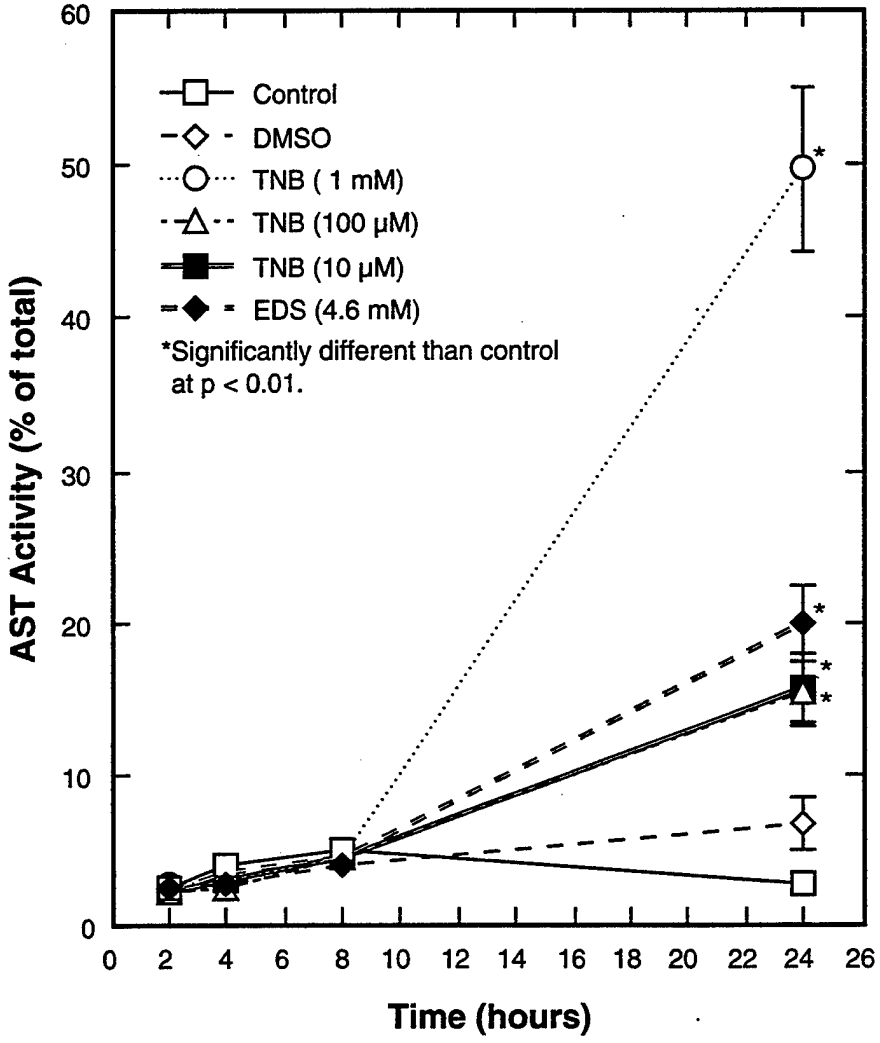
***Statistical analysis*:** A two factorial multivariate analysis of variance, with the two factors being dose (at 3 levels) and time (at 4 levels) was performed. Levine's test for homogeneity of variance was applied, using a two factorial multivariate analysis of variance. The Wilk-Shapiro test was used to test for normality. If either normality or homogeneity were violated, an appropriate transformation such as log or rank was used.

RESULTS

Biochemical Analyses: Assay of testicular insult by TNB was measured by cellular enzyme (AST) and potassium leakage, changes in the rate of protein synthesis, and histopathological examination. For some of these assays, a comparison of TNB toxicity with testicular toxicants cadmium acetate and/or EDS was performed.

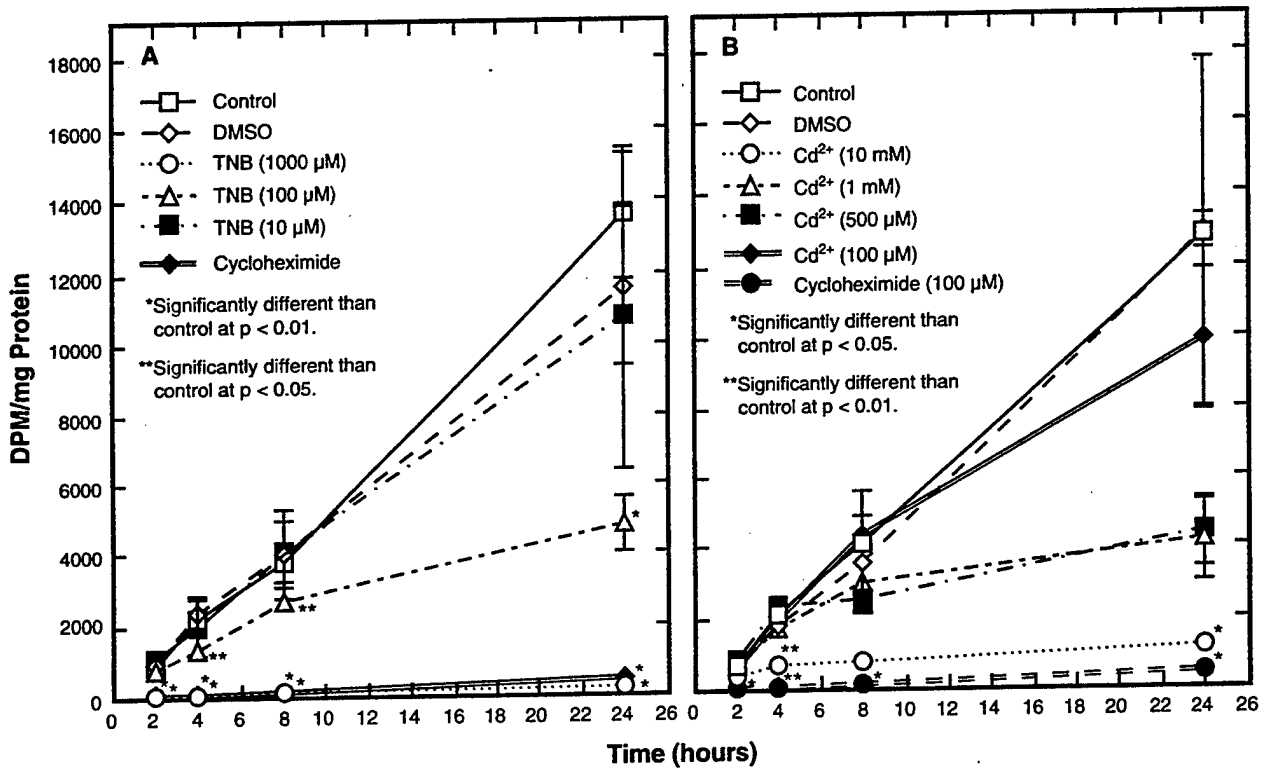
Figure 5.3-1 depicts the release of cellular AST above control values for treatment groups of TNB and EDS. The percent of total AST enzyme released was significantly increased ($p<0.01$) for all toxicant treatments, but not until after 8-h incubation. No increase in AST release was measured in both control treatments (naive and DMSO).

TNB (1000 μM) completely blocked protein synthesis in testicular slices (Figure 5.3-2a); a comparable effect was produced by cycloheximide (100 μM) at a tenfold lower concentration. The effect of TNB (100 and 1000 μM) on protein synthesis is discernible as early as 2-h incubation time. No inhibition of protein synthesis was produced, even at 24-h incubation, at a TNB concentration of 10 μM. Protein synthesis in control slices was linear over the 24-h incubation period (naive, $r=0.996$, DMSO, $r=0.998$). The effect of varied concentrations of Cd^{2+} on protein synthesis is shown in Figure 5.3-2b. Although protein synthesis inhibition appeared to be proportional to the concentration of Cd^{2+}, the extent of inhibition was considerably less than that observed with TNB. Even at a concentration of 10 mM, Cd^{2+} did not completely block protein synthesis, as was observed with TNB (1 mM).



Values represent the mean ± S.D. of three determinations

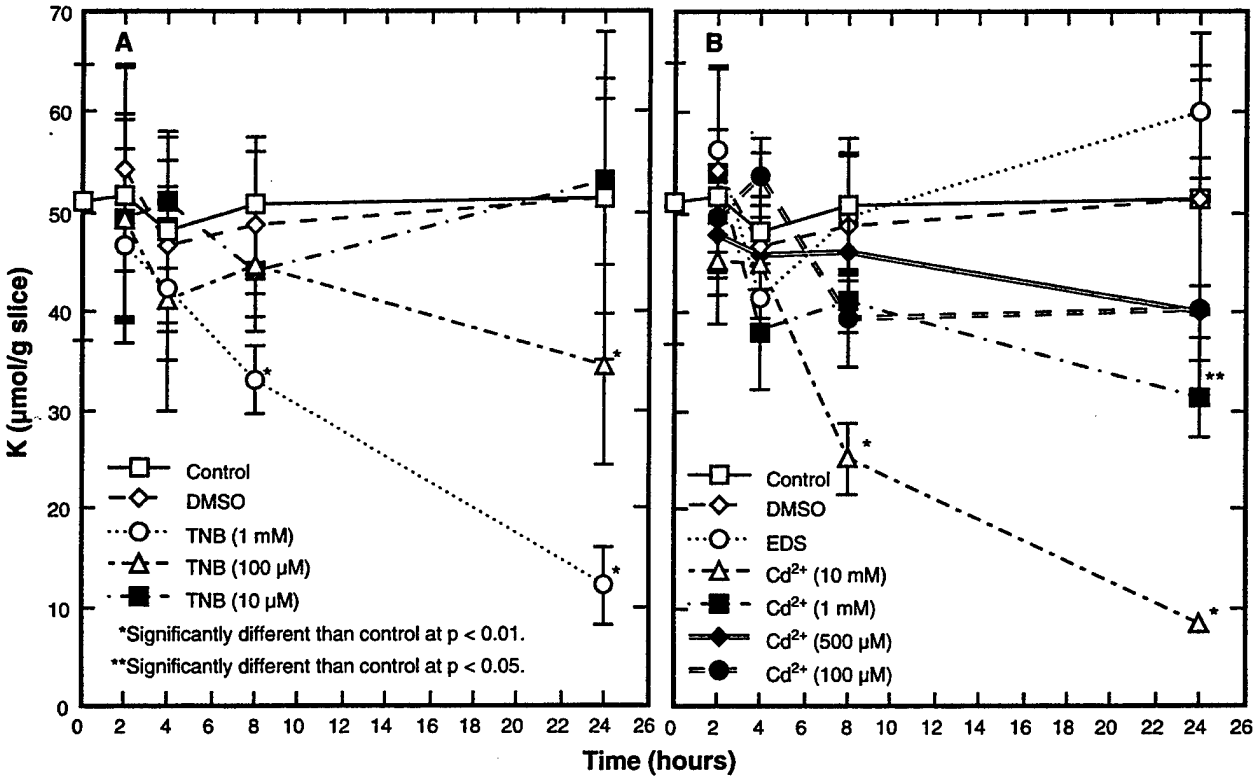
Figure 5.3-1 Tempural Release of AST from Bovine Testicular Slices Exposed to TNB and EDS



Values represent the mean ± S.D. of at least three determinations.

Figure 5.3-2. Temporal Incorporation of 3H-Leucine Into Protein of Bovine Testicular Slices Exposed toVaried Concentration of 1,3,5-Trinitrobenzene (a) and Cadmium Acetate (b).

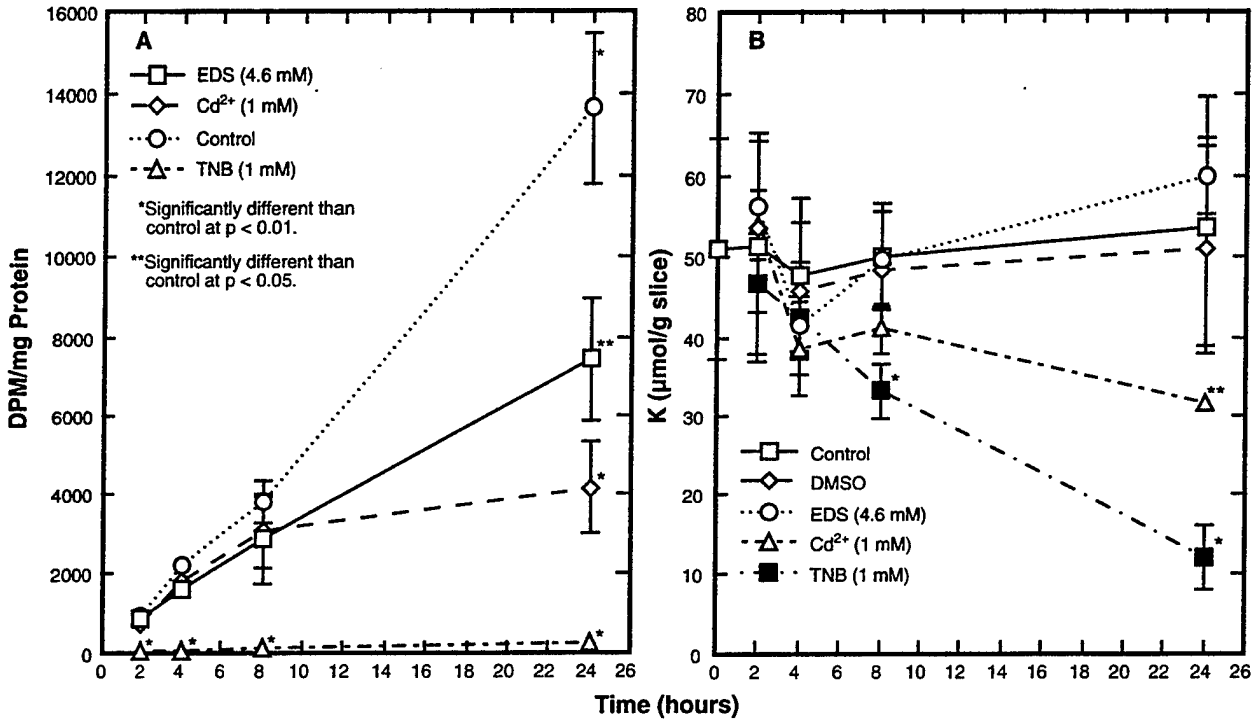
The release of potassium ion over time in response to TNB and Cd^{2+} is depicted in Figures 5.3-3a and 5.3-3b, respectively. A significant decrease in intracellular K^+ was determined for both TNB (1 mM) and Cd^{2+} (10 mM), but not until after 8-h incubation. EDS was non-toxic as measured by K^+ leakage. Intracellular K^+ concentration for control slices remained constant (50 to 60 µmol/g slice) throughout the 24-h incubation period.



Values represent the mean ± S.D. of at least three determinations

Figure 5.3-3 Temporal Release of Potassium Ion from Bovine Testicular Slices Exposed toVaried Concentration of TNB (A) and Cadmium Acetate (B).

The relative toxicity of toxicants, as measured by inhibition of protein synthesis and loss of intracellular potassium (Figures 5.3-4a and 5.3-4b, respectively) was TNB > Cd^{2+} >> EDS. Use of DMSO as a vehicle produced no significant changes in viability parameter as compared to control slices for all biochemical indices measured (Figures 5.3-1 through 5.3-4).



Values represent the mean ± S.D. of at least three determinations.

Figure 5.3-4. Comparative Inhibitory Effect of Testicular Toxicants on Protein Synthesis (a) and Release of Potassium Ion (b) in Bovine Testicular Slices

Results of Histopathology: The following is a narrative description of the histomorphology of bovine testicular slices from both control and the high-dose (1000 μM) TNB-exposed tissue during a 24 hr incubation. A treatment- related effect was seen in all dosage groups of TNB-treated slices, but was most evident in the high dose group. Because artifact damage from tissue preparation and handling was most prominent around the outer edge of the testicular slices, one or two seminiferous tubules from the central portion of each section were used to evaluate histologic changes.

Preincubation Timepoint: Preincubation testicular slices were compared histologically with larger (non-sliced) tissue sections taken from the same testicle to evaluate changes associated with the tissue slice procedure itself. Both samples were fixed and processed identically. The preincubation testicular slice showed minimal procedure-related artifact compared with the larger sections. As shown in Figure 5.3-5a, seminiferous tubules displayed normal cellularity and synchronous, orderly progression of the germ cells composing the germinal epithelium. Low numbers of sloughed, but viable spermatids were present in the tubule lumen. Interstitial (Leydig) cells in preincubation samples had abundant eosinophilic, finely granular cytoplasm and round open-faced nuclei. The clear space present around many seminiferous tubules is an artifact associated with fixation and processing.

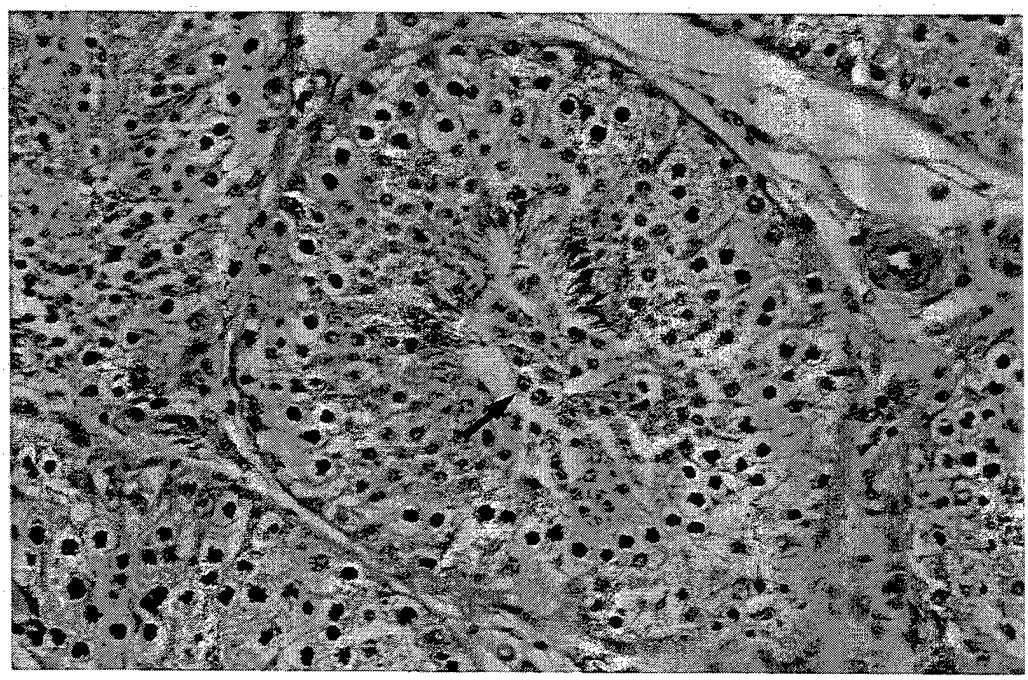


Figure 5.3-5a

Figure 5.3-5a - 5.3.-5i Time Sequence of Histopathology for Control and TNB-Treated Testicular Slices

CONTROL

2 Hour B

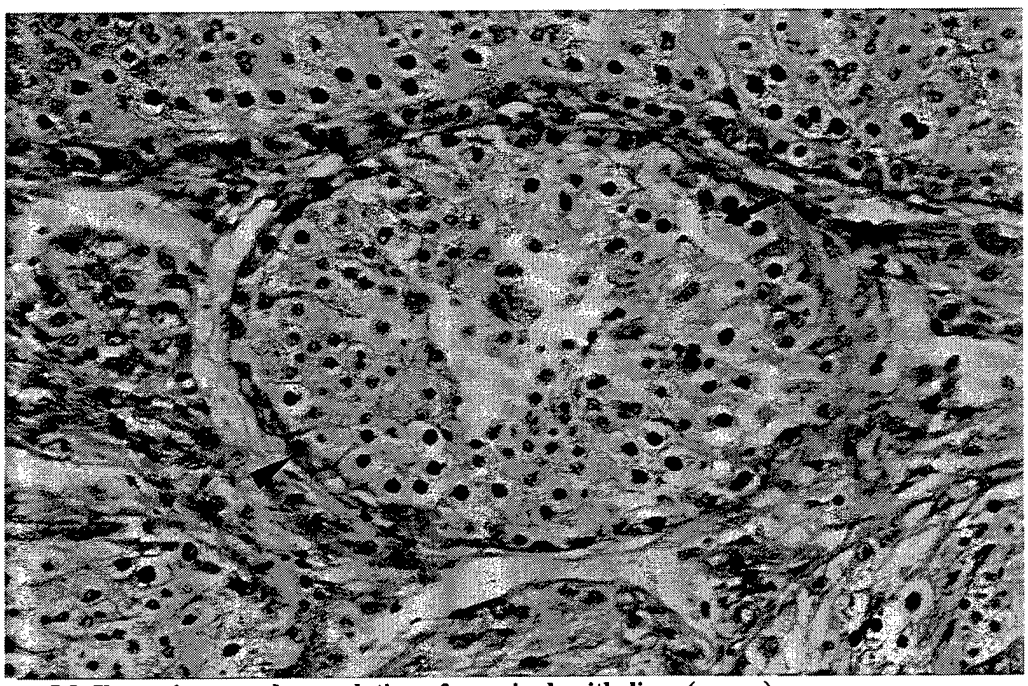


Figure 5.3-5b - increased vacuolation of germinal epithelium (arrow)
- mild grm cell disorganization
- no change in Sertoli cells (arrowhead)

2 Hour C

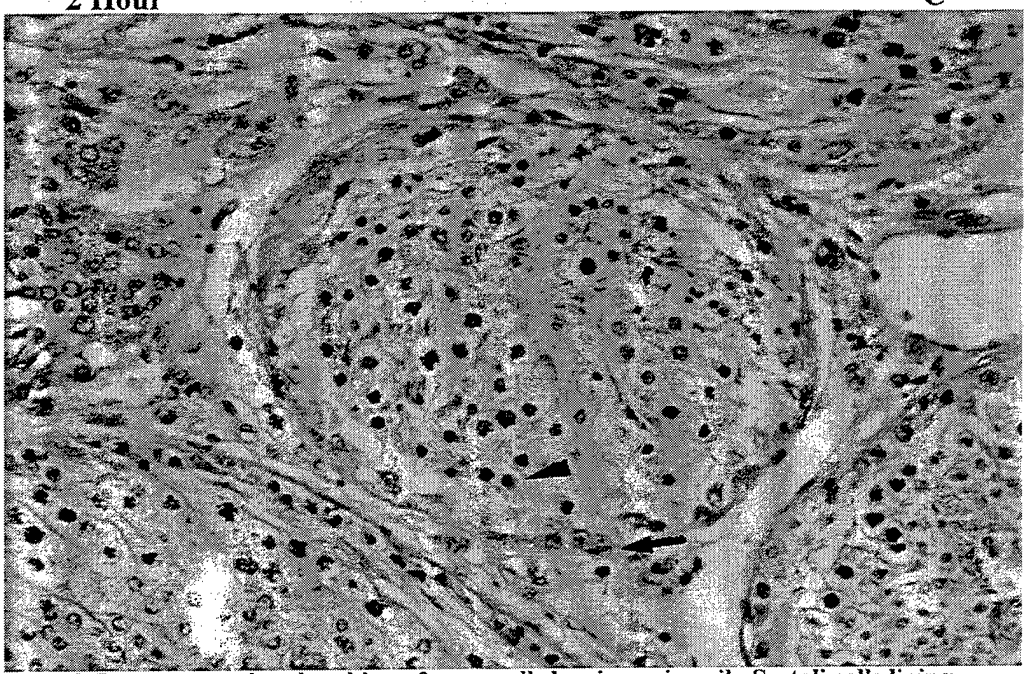


Figure 5.3-5c - extensive sloughing of germ cells leaving primarily Sertoli cells lining tubules (arrow)
- a few necrotic cells in lumen (arrowhead)

CONTROL

4 Hour D

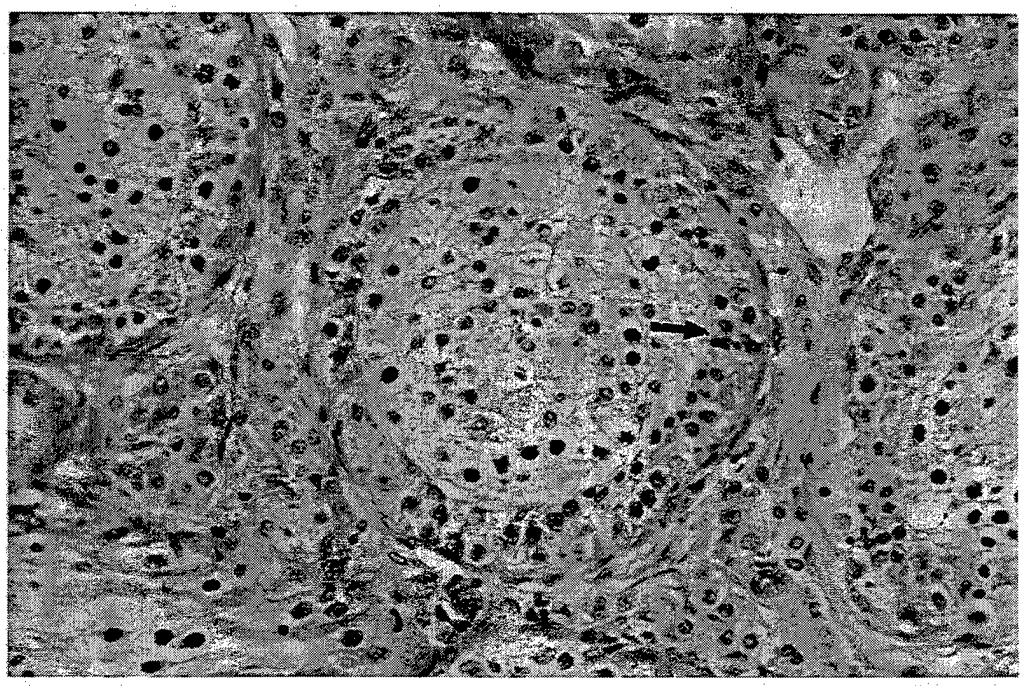


Figure 5.3-5d - germinal epithelium thinned to 3-4 cells thick (arrow)
- many sloughed, viable spermatocytes and spermatid in lumen

4 Hour TNB Treated (1000 μM) E

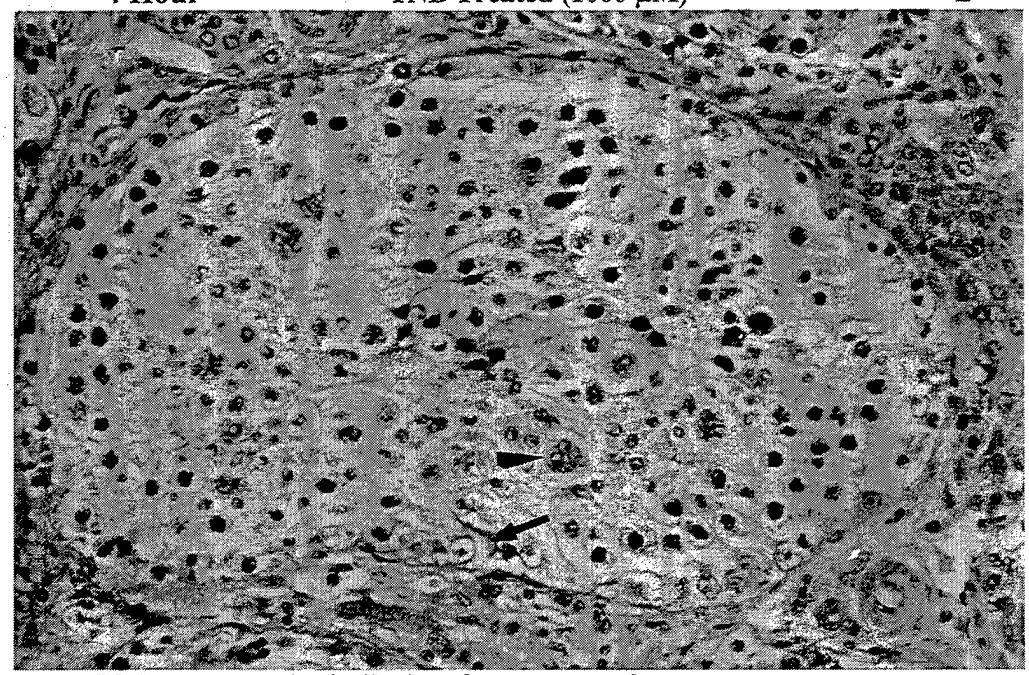


Figure 5.3-5e - necrosis of cells along basement membrane
- large, vacuolated nuclei of degenerate Sertoli cells (arrow)
- multinucleate giant cells from degenerating spermatid (arrowhead)

CONTROL

8 Hour F

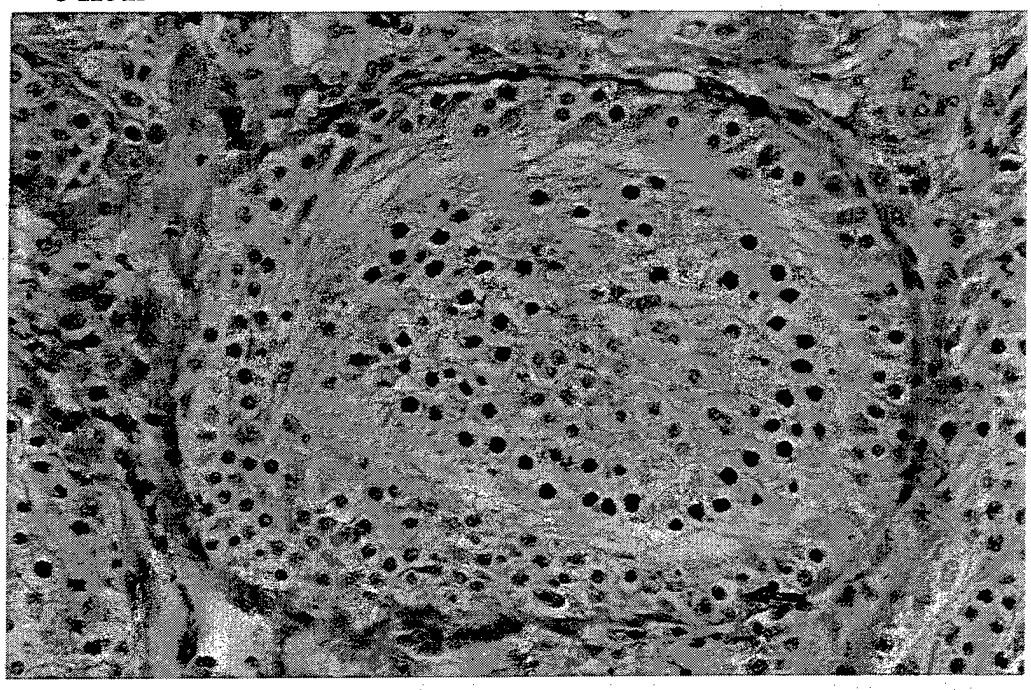


Figure 5.3-5f - changes similar to 4 h controls
- a few necrotic germ cells now present (arrow)

8 Hour TNB Treated (1000 μM) G

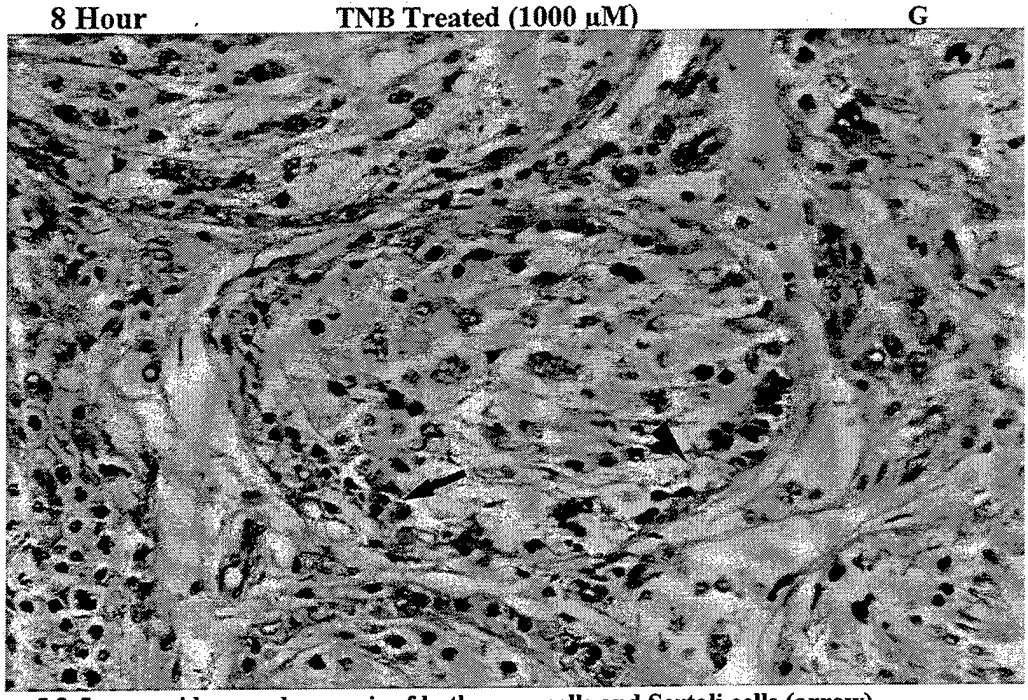


Figure 5.3-5g - widespread necrosis of both germ cells and Sertoli cells (arrow)
- thickened, irregular basement membrane and myoid cell degeneration (arrowhead)
- decreased cellularity compared with previous timepoints

CONTROL

24 Hour H

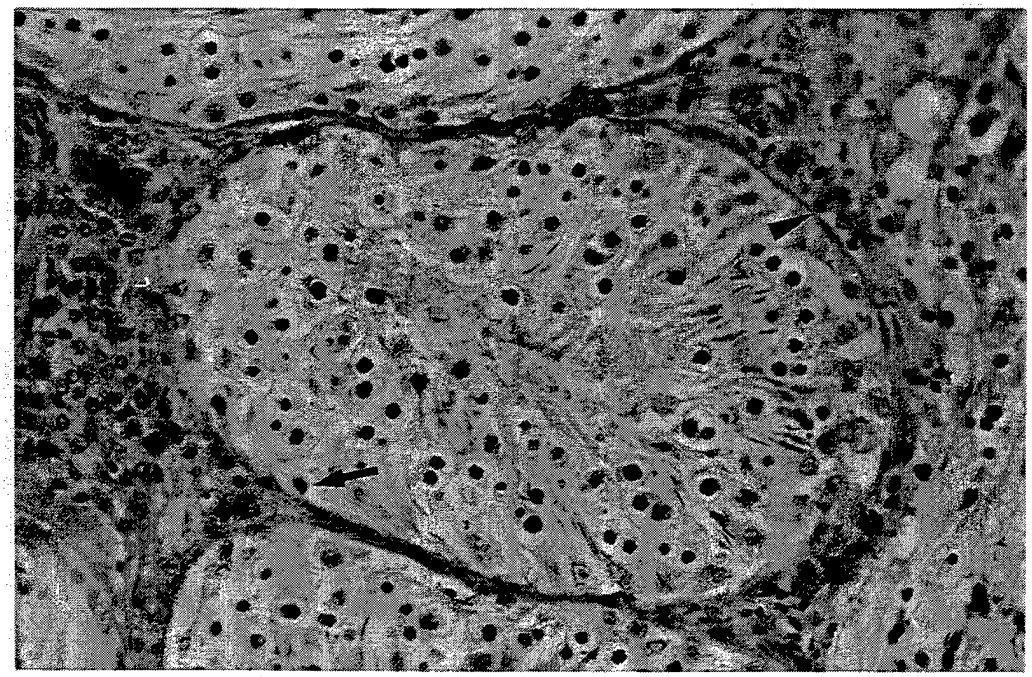


Figure 5.3-5h
- decreased cellularity from previous timepoints
- extensive sloughing of germ cells
- degenerate, but viable germ cells and Sertoli cells still present (arrow)
- intact basement membrane/myoid cell complex (arrowhead)

24 Hour TNB Treated (1000 µM) I

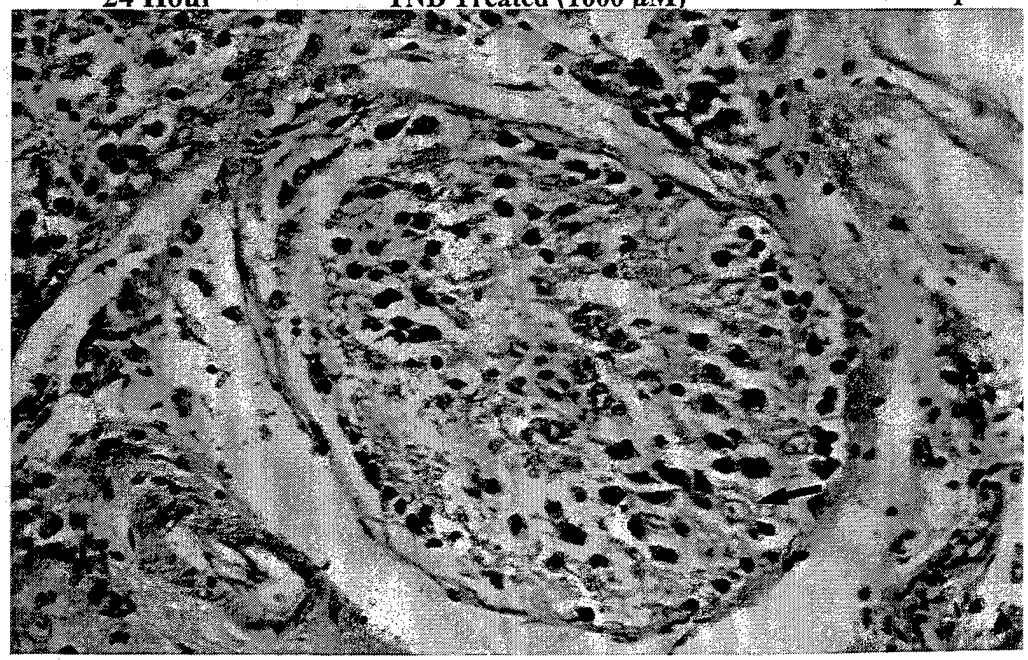


Figure 5.3-5i
- widespread necrosis
- basement membrane devoid of cells, few remaining Sertoli cells sloughed into lumen (arrow)

2-Hour Timepoint: Mild degenerative changes were observed in control tissue slices (Figure 5.3-5b) following 2 h incubation, including intercellular vacuolation of germinal epithelium, and mild sloughing and disorganization of germ cells. There was little or no change detected in Sertoli cells. As shown in Figure 5.3-5c, seminiferous tubules incubated with TNB (1000 μM) demonstrated extensive sloughing and disorganization of germinal epithelium by 2 h. Only Sertoli cells and a few spermatogonia remained attached to the tubular basement membrane. Sertoli cell degeneration was characterized by swollen, vacuolated nuclei.

4-Hour Timepoint: Control slices (Figure 5.3-5d) showed a decrease in overall cellularity of the seminiferous tubules compared with preincubation slices. The germinal epithelium was thinned to a 3-4 cells thickness, while the enlarged tubular lumen contained a mixture of sloughed spermatocytes and spermatids. Overall cell viability remained good. For TNB-treated slices (Figure 5.3-5e), the germ cells had almost completely exfoliated by 4 h incubation. Pyknotic and possibly apoptotic nuclei seen along the basement membrane were evidence of Sertoli cell degeneration and death. The remaining viable Sertoli cells had large, vacuolated, pale nuclei. Multinucleated giant cells derived from degenerating spermatids or spermatocytes were present in some tubules.

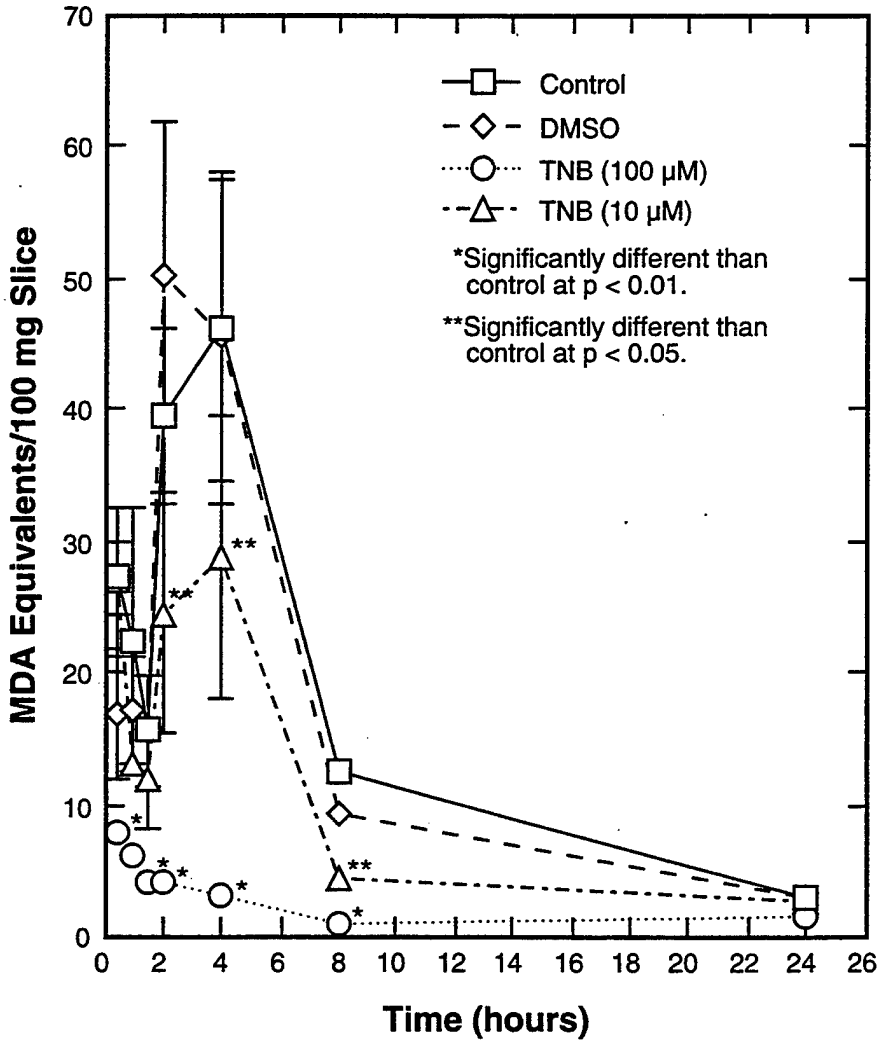
8-Hour Timepoint: There was little progression of tissue degeneration observed in the seminiferous tubules at 8 h (Figure 5.3-5f) compared to 4-h controls. Although the majority of the germ cells were still viable, a few degenerate and necrotic cells were visible. Low numbers of interstitial cells have become shrunken and hypereosinophilic (degeneration). By 8h, the seminiferous tubules for TNB-treated slices were increasingly shrunken and lined by a thickened, irregular basement membrane and degenerate myoid cells (Figure 5.3-5g). Tubular cellularity was markedly decreased compared with 8 hour controls, and widespread necrosis of germ cells and Sertoli cells was present. Most interstitial cells were shrunken and rounded with hypereosinophilic cytoplasm, but only scattered interstitial cell necrosis was evident.

24-Hour Timepoint: By 24 h, the cellularity of tubules in control slices was decreased compared with controls at previous timepoints (Figure 5.3-5h). Most germ cells had exfoliated. Swollen, yet viable spermatocytes and spermatids were still present in the tubular lumen, admixed with fewer necrotic germ cells. The basement membrane was lined primarily by degenerate, but viable Sertoli cells, and the basement membrane/myoid cell complex remained intact. Interstitial cells had shrunken cytoplasm and darkened nuclei compared with preincubation slices. Numerous degenerate and scattered necrotic interstitial cells were present. Changes in seminiferous tubules and interstitial cells in TNB-treated slices were similar to those described in TNB-treated slices at 8 h (Figure 5.3-5i). Tubules were lined only by a swollen and sometimes fragmented basement membrane as remaining Sertoli cells had sloughed into the tubular lumen.

Slices incubated in DMSO were not consistently different from controls. Attempts to quantify the degenerative lesions for statistical comparison between different treatment groups and timepoints was considered impractical due to the variability of tubular degeneration present within a given section.

Production of Reactive Intermediates: Whether TNB toxicity involved peroxidation of lipid through the formation of reactive intermediates was assayed by measuring the release of thiobarbituric reactive substances (TBARS). Rather than causing lipid peroxidation, TNB blocked it and prevented the release of TBARS from testicular slices (Figure 5.3-6), as compared to control values. This effect was proportional to the TNB dose. Lipid peroxidation is typically caused by

oxygen-centered radicals. It is possible that an oxygen centered radical (superoxide), which was naturally produced by the testes(Ahotupa and Huhtaniemi, 1992), was trapped by a radical(s) derived from TNB metabolism.



Values represent the mean ± S.D. of at least three determinations.

Figure 5.3-6 Inhibition of Release of TBARS by TNB in Bovine Testicular Slices

To determine if free radicals of TNB were produced during incubation of testicular slices, EPR analysis of incubated slices incubated in the presence of PBN spin-trap was performed. Figure 5.3-7 shows the EPR signal derived from slices incubated with 1000 μM TBN, hyperfine values and a computer simulated signal.

Cd^{2+} has previously been shown to cause lipid peroxidation of membranes in multiple tissues, including testes (Kojima et al., 1990; Koizumi and Li, 1992; Manca et al, 1991). However, no increase in release of TBARS was observed in slices treated with Cd^{2+} even though the length of exposure was comparable (data not shown).

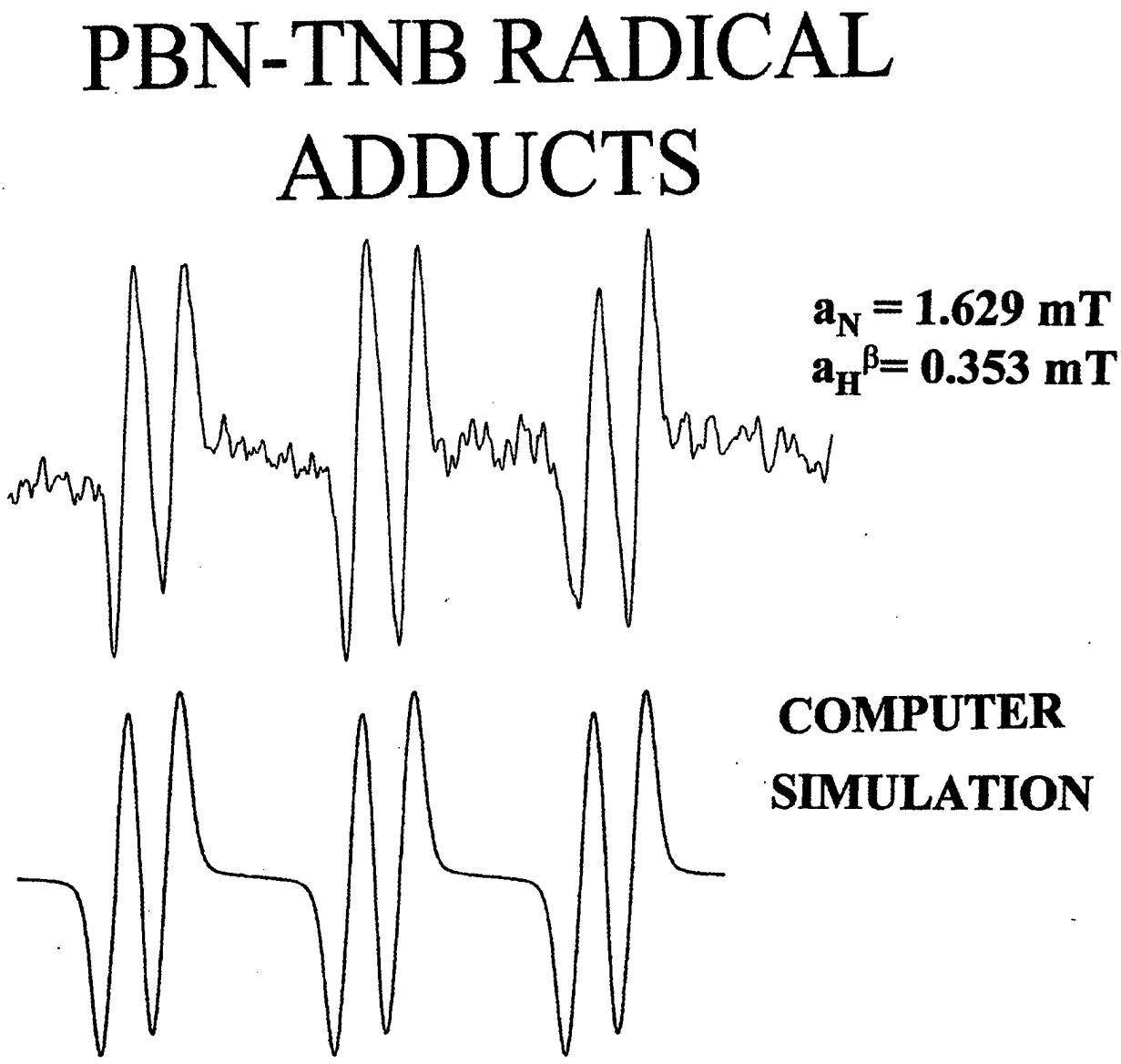


Figure 5.3-7. EPR Signal for PBN-TNB adical Adducts

DISCUSSION

This study represents the first report of the application of tissue slice dynamic culture methods for evaluation of testicular function. The adaptability of testicular slice assays for biochemical, histopathological, and EPR spectrometry was quite good and allowed a rapid means of characterizing TNB toxicity to the testes. Of the indices of toxicity evaluated, the most sensitive marker of testicular toxicity was a change in the rate of protein synthesis, which was visible for both high and medium doses of TNB as early as 2 h. In comparison, the effect of any of the toxicants tested were not visible until 8-h incubation for both K^+ and AST leakage.

The results of histopathological exam of testicular slices allowed the most complete characterization of the testicular effects of TNB. Although all sections of testicular slices demonstrated some degree of degenerative change progressing over the time periods evaluated, both the quality and severity of the changes observed were clearly different between the control and TNB-treated testicular slices. Histologically, the degenerative changes in control tissues were considered relatively minor, and cellular viability remained good up through the 8-h timepoint. By 24 h, seminiferous tubules from control slices experienced loss (sloughing) of most of the germinal epithelium, and although many cells remained viable, normal cellular morphology was significantly altered by this time.

In contrast, seminiferous tubules incubated in TNB-treated media showed an accelerated rate of degenerative change as compared to time-matched controls. This treatment-related effect was evident as early as 2 h, and became more pronounced with each successive timepoint examined. Degeneration and necrosis of both germ cells and Sertoli cells was common in TNB-treated slices. Histologically, these cells appeared to be the primary targets of TNB's testicular toxicity. Interstitial (Leydig) cells were visibly more resistant to TNB-associated effects. The rate of interstitial cell degeneration and necrosis appeared to be only moderately exacerbated by TNB treatment when compared with controls at similar timepoints.

Pathological changes observed *in vivo* by Kinkead, et al., 1995 were comparable to those determined *in vitro* for bovine testis. Degeneration of the germinal epithelium, formation of multinucleated giant cells, and a relative sparing of interstitial Leydig cells. These findings suggest that the toxicity of TNB is directly attributable to the parent compound or its metabolite(s), and is not a result of extra-testicular interactions (endocrine effects). Moreover, if TNB toxicity is a result of metabolite formation, then the prerequisite metabolism is accomplished within testicular tissue and not at an extra-testicular site.

The TNB-induced damage visible through histopathology was not closely correlated with changes in biochemical markers over time. By 8 h incubation, results of histopathology indicated that testicular cells incubated with TNB (1000 μM) were severely compromised and dying. The results of AST and K^+ leakage for identically incubated slices do not suggest that the tissues were in serious distress.

How TNB produces damage to the germinal epithelium is not known. A structural analog to TNB, 1,3-dinitrobenzene (DNB), has been postulated (Cave and Foster, 1990) to produce Sertoli cell and germ cell damage through a reactive intermediate, 1,3-nitrosonitro-benzene (*in vitro* co-culture studies). Formation of 3-nitrosonitrobenzene from DNB has also been observed in rabbits (*in vivo,* Rickert, 1987). In rat brain, rather than testes, a somewhat similar characterization of DNB toxicity suggested that damage to nerve cells may be linked to production of free radicals from DNB (Romero et al, 1995). In the present study, the detection of a free radical using EPR spectroscopy suggests that TNB also produces reactive

intermediates. The inhibition of lipid peroxidation, observed in TNB-treated slices, may be an indirect result of a TNB intermediate serving as a spin-trap for endogenously produced oxgen-centered radicals. Nitroso-containing compounds are in common use as spin-traps for free radical capture (Buettner, 1982). It seems plausible that TNB could be metabolized in the same manner as DNB, with formation of a nitroso-derivative responsible for testicular insult. Characterizartion of the TNB radical species is currently underway.

Further development of this *in vitro* assay, with the application of biomarkers that reflect specific functions of testicular cells, such as Leydig cell production of testosterone and Sertoli cell release of inhibin and/or zinc may provide more sensitive markers for measuring insult. These studies and the evaluation of TNB toxicity in human testicular slices are being pursued in this laboratory.

SUMMARY

This research did not require the sacrifice of laboratory animals. Testicular slices were prepared and remained viable for periods up to 24 h. This represents a new application of dynamic roller culture tissue slice technology. As measured by inhibition of protein synthesis, release of potassium ion and AST, and histopathological exam, TNB produced a dose-related insult to testicular slices. Inhibition of protein synthesis was the most sensitive viability assay for detecting xenobiotic effects on the testicular slices. Qualitatively, histopathologic exam revealed degeneration of the testis over time. TNB appeared to selectively target Sertoli cells over Leydig cells. Degenerative changes in control slices were present after 24-h incubation. The relative toxicity of xenobiotics examined was TNB >Cd^{2+} >>EDS. TNB prevented the release of TBARS from testicular slices and a free radical produced from TNB was detected.

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5.4 RANGE-FINDING STUDY FOR A REPRODUCTIVE SCREEN OF MODULAR ARTILLERY CHARGE SYSTEM (XM231/XM232) ADMINISTERED IN THE DIET OF SPRAGUE-DAWLEY RATS

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ABSTRACT

An artillery propellant under development by the US Army is a granular mixture of nitrocellulose, nitroglycerin, nitroguanidine, ethylcentralite, cryolite, and graphite. The propellant, Modular Artillery Charge System (MACS; XM231/XM232), consists of a single increment of propellant charge contained within a rigid combustible casing. Nitrocellulose, nitroglycerin, and nitroguanidine make up greater than 98% of the total propellant mixture. As part of the process to develop environmental and health effects criteria, a 90-day modified Screening Information Data Set (SIDS) reproductive assay is planned. In order to provide information on clinical signs, methemoglobin formation, and possible target organs, as well as determine dose levels, a range-finding study was performed. Male and female Sprague-Dawley rats were treated with diet containing either 0.0, 0.25, 0.5, 1.0, or 2.0 g propellant/kg diet for a three-week period. No mortalities occurred and body weights were unaffected by treatment. Methemoglobin concentrations measured at the conclusion of the study indicated no differences between treated and control rats. Relative liver weights of the high-dose female rats were significantly ($p<0.1$) greater than the control group. No gross lesions were noted at necropsy.

INTRODUCTION

Modular Artillery Charge System (MACS; XM231/XM232) is a propellant under development by the US Army for autoloading howitzer artillery, consisting of a single increment of propellant charge contained within a rigid combustible casing. This technology will simplify autoloader requirements, result in a significant reduction in propellant production and logistics requirements, and eliminate propellant waste. The MACS propellant is a granular mixture of nitrocellulose (NC), nitroglycerin (GTN; glyceryl trinitrate), nitroguanidine (NQ), ethylcentralite (N,'N' diethyl, N,N'-diphenylurea), cryolite, and graphite. Since NC, GTN, and NQ make up >98% of MACS propellant, it is commonly referred to as "triple-based" propellant mixture.

Nitroguanidine is a primary component (approximately 47%) of the triple-based propellant mixture. Previously conducted acute oral and percutaneous studies on NQ found the compound to be relatively nontoxic. The compound was not irritating to either rabbit skin or eyes using the standard Draize technique (Hiatt et al., 1986; Morgan et al., 1986). Nitroguanidine did not kill rats by oral gavage at a limit test dose of 5 g/kg (Brown et al., 1988), while the LD_{50} values for male and female mice are reported as 5.0 g/kg and 4.3 g/kg, respectively (Hiatt et al., 1988).

Nitroguanidine did not produce treatment-related effects when administered to Sprague-Dawley rats at doses as high as 1000 mg/kg/diet for 14 days. However, after 90 days, that dose level produced a decrease in body weight gain and food con-

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sumption (Reddy and Korte, 1988). No evidence of developmental toxicity in rats or rabbits was reported at doses of 1000 mg/kg diet.

Nitroglycerin is also a component (approximately 22%) of the triple-based propellant. The toxic, physiologic, and pharmacologic effects of GTN have been studied extensively in animals and man (NIOSH, 1978). Nitroglycerin is readily absorbed by ingestion, inhalation, sublingual contact, and through the skin. When both inhalation and dermal exposure to GTN occur, dermal contact is thought to make the major contribution to the total amount absorbed by the body (NIOSH, 1978). Vasodilation following exposure to GTN can occur within minutes, regardless of route of exposure. This effect has led to sudden death and chronic cardiac disease (Carmichael and Lieben, 1963). Small amounts can cause headaches which can persist for hours or days. Larger doses can cause hypotension, cyanosis, and methemoglobinemia. These effects are potentiated by alcohol consumption (NIOSH, 1978). Symptoms also include nausea and vomiting, and in some instances, diarrhea. The mouse intraperitonal (IP) LD_{50} of GTN is 194 mg/kg (Kylin et al., 1964) and the rat IP LD_{50} has been reported as 93 mg/kg (Burginson et al., 1962).

The third major component (approximately 28%) of the triple-based propellant is nitrocellulose or cellulose nitrate. Cellulose nitrate is relatively nontoxic but is known for its extreme flammability (Montgomery, 1982). Dry NC may explode when subjected to heat or sudden shock; NC is therefore generally handled wet with either water or alcohol. Toxic combustion products are generated when the compound is ignited, primarily carbon monoxide and oxides of nitrogen (Montgomery, 1982).

Munition workers exposed to nitrate containing explosives have experienced skin irritation, liver damage, and anemia (Hathaway, 1977; Morton et al., 1976; Stewart et al., 1945). These compounds have also been found to cause methemoglobin formation, liver and spleen hypertrophy, and degeneration of the seminiferous tubules resulting in decreased spermatogenesis (Cody et al., 1981; Levine et al., 1983, 1984; Furedi et al., 1984a,b). A recently completed reproductive screen in this laboratory with 1,3,5-trinitrobenzene (TNB), resulted in testicular atrophy and decreased spermatogenesis in the male rats. Female rats displayed clinical signs of altered locomotion during the postpartum period and both sexes had brain lesions at necropsy (Kinkead et al., 1994a,b). Neurotransmitter analyses showed statistically significant increases in norepinephrine, epinephrine, seratonin, and dopamine in the TNB-treated female rats (Narayanan et al., 1995). Changes in neurotransmitter levels in specific regions may be one of the mechanisms responsible for TNB-induced neurological disorder. A reproductive screen on another nitrate containing explosive compound, liquid propellant XM46, resulted in hemolytic anemia (Kinkead et al., 1994c). Loss of embryos and/or death of fetuses were observed following treatment with ammonium dinitramide indicating that this compound is a reproductive toxicant (Kinkead et al., 1994d).

Nitrate-containing explosive compounds have produced adverse reproductive effects in both male and female rats. Testicular atrophy and decreased spermatogenesis are common in male rats; lack of live litter production has been noted in female rats. Brain lesions and loss of motor skills, methemoglobinemia, and hemolytic anemia are common findings in the reproductive screens that were performed within this laboratory with nitrate-containing explosives.

An objective of this range-finding study was to determine the dose levels in the diet that rodents could tolerate and to provide information on clinical signs, methemoglobin formation, and possible target organs. Data from this range-finding

study were used to determine dose levels for a 90-day modified Screening Information Data Set (SIDS) protocol that was used to evaluate the developmental and reproductive toxicity of MACS in rats.

MATERIALS AND METHODS

Test Agent

The MACS was provided by the U.S. Army, Picatinny Arsenal, NJ. The pellets were ground to granular form before receipt. Pertinent chemical and physical properties of the test compound are listed below:

MACS

Synonyms:	Smokeless powder
	Modular Artillery Charge System XM231/XM232
CAS#:	None assigned
Specific gravity:	1.5880
Vapor pressure:	Negligible
Appearance:	Hard cylindric pellet white to reddish orange, coated with graphite

Diet Preparation, Homogeneity, Stability, and Analysis

MACS was administered by the oral route, mixed appropriately in the diet. The test material was mixed in powdered Purina Formulab # 5002 (Ralston Purina, St. Louis MO) certified rodent diet meal. The high concentration diet was prepared by adding 20g MACS to 10 kg rodent diet to produce a diet concentration of 2g MACS/kg diet. Diet concentration of 1.0, 0.5, and 0.25 g MACS/kg were made by serial dilutions adding weighed amounts of feed to equal amounts of prepared diet. Each batch of diet was mixed for 30 minutes.

The MACS analysis was performed using a gas chromatographic method described in Appendix 5.4-A. Total MACS concentrations were based on nitroguanidine concentrations measured in five gram samples of diet.

To assess the efficiency of the mixing process, analyses were performed on samples taken from the top, middle, and bottom of the mixing bowl. Duplicate samples were taken for each measurement. Because the diets could be stored in polyethylene containers for as long as 3 weeks, or in the feed jars for up to 7 days, stability of the diets was measured over those time frames. Duplicate samples were measured for each.

Test Animals, Group Assignments, Clinical Measurements

Fifteen male and 15 female Sprague-Dawley derived outbred albino rats [Crl:CD®BR] known as Charles River CD rats, were purchased from Charles River Breeding Laboratories, Raleigh, NC. The rats were 9 weeks of age upon arrival and 11 weeks of age at initiation of the treatment period. All rats were identified by tail tattoo and were subjected to a two-week acclimatization period. Water from a reverse-osmosis system and feed were available *ad libitium* to the animals. Animal rooms were maintained on a 12-h light/dark cycle (fluorescent light) and targeted at a temperature of 23 ± 2 °C with a relative humidity of 55 ± 15%.

The rats were single housed in clear plastic cages with hardwood-chip bedding (Bettachip®, Northeastern Products Corp. Warrensburg, NY). There were five study groups with target diet concentrations of 0.0, 0.25, 0.5, 1.0, and 2.0 g MACS/kg diet. Feed jars were cleaned on a weekly basis at which time all leftover diet was discarded. Rats were assigned to groups of three per sex by means of a computer-generated randomization, stratified by body weight such that the mean body weight of all groups were homogeneous by statistical analysis at study initiation.

Rats were observed twice daily for signs of toxic stress. Body weights were measured weekly, food consumption was measured and doses were calculated as mg MACS/kg body weight/day.

All rats received treated or control diet for 21 days and were necropsied on day 22. Animals were not fasted prior to necropsy. Blood was collected via the vena cava for hemoglobin and methemoglobin determinations. Wet weights were recorded for liver, kidney, testes, brain, and spleen. Animals were examined carefully for gross lesions.

Body weights, organ weights, food consumption, hemoglobin, methemoglobin, and MACS dose calculations were treated for statistical significance using a one-factorial analysis of variance with Bonferroni multiple comparisons (Rosner, 1990). Due to the small size of the study groups, statistically significant differences were inferred when $p<0.1$.

RESULTS

Diet Preparation and Stability

Diet target concentrations were 2.0, 1.0, 0.5, and 0.25 g MACS/kg diet. Measured diet concentration were 1.98, 0.95, 0.47, and 0.2 g MACS/kg diet. Analyses of samples taken from the top, middle, and bottom of the diet preparation bowl for homogeneity determined that the test material was uniformly distributed throughout the diet in the high and moderate level concentrations (means per measurement level within 15%). The concentrations per level of the mid and low level showed a greater variation (Appendix 5.4-B).

Analysis of the diet taken from feed jars and polyethylene storage containers showed no degradation in MACS concentration over the 7- to 21-day time period. Samples were removed from the middle of the containers and represented time periods diet would be stored in either container.

General Toxicity

No mortality occurred during the three-week study. Food consumption of either sex did not differ between the treatment groups. Based on the diet consumption rate, male rats received 141, 71, 35, and 19 mg MACS/kg/day and female rats received 154, 81, 40, and 20 mg MACS/kg/day. Mean body weights of male and female treated rats did not differ significantly from their respective control groups at any of the measurement periods during the study.

Absolute and relative organ weights of male rats treated with MACS were compared with controls. There were no statistically significant differences (Table 5.4-1). Absolute organ weights of the female rats did not differ from controls; however, relative liver weights of the high-dose female group were significantly greater than those of the

TABLE 5.4-1. ABSOLUTE (G) AND RELATIVE ORGAN WEIGHTS[a] OF MALE RATS TREATED WITH MACS FOR THREE WEEKS

	Diet Concentrations				
Organs	**Control**	**0.25 g/kg**	**0.5 g/kg**	**1.0 g/kg**	**2.0 g/kg**
Body	466.3 ±30.7	504.2 ±30.9	476.1 ±23.1	474.0 ±12.5	465.8 ± 24.6
Brain	2.07 ± 0.03	2.13 ± 0.05	2.15 ± 0.08	2.11 ± 0.06	2.16 ± 0.06
Ratio[b]	0.45 ± 0.03	0.03 ± 0.02	0.06 ± 0.03	0.45 ± 0.02	0.46 ± 0.01
Liver	14.10 ± 1.42	16.78 ± 1.99	15.11 ± 1.73	15.66 ± 1.30	14.16 ± 1.07
Ratio	3.00 ± 0.12	3.31 ± 0.22	3.16 ± 0.22	3.29 ± 0.20	3.03 ± 0.07
Kidneys	3.75 ± 0.11	3.76 ± 0.13	3.59 ± 0.16	3.73 ± 0.07	3.47 ± 0.13
Ratio	0.81 ± 0.07	0.75 ± 0.02	0.75 ± 0.00	0.79 ± 0.02	0.75 ± 0.01
Spleen	0.87 ± 0.10	0.98 ± 0.15	0.92 ± 0.07	0.86 ± 0.06	0.96 ± 0.09
Ratio	0.19 ± 0.02	0.19 ± 0.03	0.19 ± 0.01	0.18 ± 0.01	0.21 ± 0.01
Testes	3.39 ± 0.27	3.59 ± 0.04	3.61 ± 0.08	3.63 ± 0.06	3.61 ± 0.37
Ratio	0.73 ± 0.07	0.72 ± 0.04	0.76 ± 0.04	0.77 ± 0.02	0.77 ± 0.05

[a]Mean ± SEM, N=3.

[b]Organ weight/body weight x 100.

respective control group (Table 5.4-2). Total hemoglobin and methemoglobin concentrations measured at necropsy indicated no differences between treated and control rats. At necropsy, all rats utilized in this study were in good general condition. No lesions were noted during the gross pathologic examination and no tissues were removed for further evaluation.

TABLE 5.4-2. ABSOLUTE (G) AND RELATIVE ORGAN WEIGHTS[a] OF FEMALE RATS TREATED WITH MACS FOR THREE WEEKS

	Diet Concentrations				
Organs	**Control**	**0.25 g/kg**	**0.5 g/kg**	**1.0 g/kg**	**2.0 g/kg**
Body	245.1 ± 7.6	262.5 ± 19.1	255.1 ± 9.9	256.7 ± 30.2	250.3 ± 2.0
Brain	1.93 ± 0.05	1.98 ± 0.03	1.96 ± 0.03	1.88 ± 0.04	1.86 ± 0.05
Ratio[b]	0.79 ± 0.01	0.76 ± 0.06	0.77 ± 0.02	0.75 ± 0.08	0.75 ± 0.02
Liver	6.68 ± 0.10	7.59 ± 0.60	7.39 ± 0.27	7.47 ± 0.75	7.82 ± 0.29
Ratio	2.73 ± 0.07	2.89 ± 0.02	2.90 ± 0.13	2.92 ± 0.05	3.13 ± 0.14[c]
Kidneys	1.89 ± 0.17	1.92 ± 0.12	1.82 ± 0.09	1.91 ± 0.20	1.79 ± 0.08
Ratio	0.77 ± 0.05	0.73 ± 0.02	0.72 ± 0.05	0.75 ± 0.03	0.71 ± 0.04
Spleen	0.57 ± 0.06	0.48 ± 0.04	0.42 ± 0.04	0.57 ± 0.09	0.49 ± 0.02
Ratio	0.23 ± 0.02	0.18 ± 0.00	0.17 ± 0.01	0.22 ± 0.01	0.20 ± 0.01

[a]Mean ± SEM, N=3.
[b]Organ weight/body weight x 100.
[c]Significant at p<0.10

DISCUSSION

Diet concentrations measured in the total mix were well within the expected values, less than ±6% of target concentrations. The homogeneity analysis showed a variation between the levels measured in the mixing bowl. One reason for this variation was because the test compound was of irregular granular form and had particles of varying sizes. A relatively large granule in a small feed sample (5 grams) may result in an abnormally high concentration value. The measurements of the total mix are more representative of the actual concentration of the diet the rodents received. However, to ensure complete mixing, the length of time for mixing of diet for the 90-day reproductive screening study will be increased from 30 to 90 minutes per batch.

The purpose of this study was to establish dose levels for use in a 90-day modified SIDS reproductive study. The only effect of note in this range-finding study was the increase ($p<0.1$) in the relative liver weights of the high-dose female rats.

Because the liver is naturally stressed during pregnancy and increases greatly in relative size, this effect was considered to be of potential toxicologic importance. Based on the increased liver weights, diet concentrations selected in the reproductive study were 2.0, 1.0, 0.2, and 0.0 g MACS/Kg diet. The high-dose concentration (2.0 g/kg) is approximate to a concentration level of 1.0 g nitroguanidine/kg diet, a level that resulted in decreased body weight gains in a 90-day feeding study (Reddy and Korte, 1988).

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APPENDIX 5.4-A

MACS composition as listed in the Hercules Material Safety Data Sheet dated December 15, 1992 is:

47.7% nitroguanidine

28.0% nitrocellulose

22.5% nitroglycerin

1.5% Ethylcentralite

0.3% Cryolite

0.2% Graphite

An analysis of the MACS material used to prepare the rat diets was 46.4% nitroguanidine. The main component, nitroguanidine, was detectable by spectrometry (UV absorption at 265 nm wavelength). Because two secondary components are not readily detected by absorption, the MACS analysis was based on the nitroguanidine analysis.

Standards were prepared by adding weighed amounts of MACS to 5.0 g rodent diet and 10 mL of methanol in a 20 mL scintillation vial. The standards were mixed for 20 min on a Haak-Buchler vortex mixer. (Haak-Buchler Instruments, Inc., Saddlebrook, NJ). After centrifugation for 5 min at 2000 rpm, 1-mL samples were pipetted into 2-mL autosampler vials. Control diet samples and treated diet samples were treated similarly.

Samples were analyzed using a Hewlett-Packard High Performance Liquid Chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a 4.6 mm X 220 mm Spheri 5 RP-8S, 5μ particle size, reverse phase column (Alltech Associates, Inc., Deerfield, IL). The carrier flow was set at 0.4 mL/min of 50/50 methanol and water with an injection volume of 1.0 μL. A variable wavelength detector set at 265 nm provided maximum sensitivity for nitroguanidine. All rodent diet analysis concentrations are expressed as mg MACS/g rodent chow.

APPENDIX 5.4-B

Diet Batch Analysis

Duplicate samples of approximately 20g each, were taken from the top, middle, and bottom of each prepared batch of diet. The samples were mixed thoroughly and analyzed in duplicate for MACS concentration.

High Concentration (Target: 2.0 mg/g diet)

Analysis: 2.04

2.71 2.38 ± 0.47^a

Moderate Concentration (Target: 1.0 mg/g diet)

Analysis: 0.95

1.02 $.99 \pm 0.05$

Mid Concentration 1 (Target: 0.5 mg/g diet)

Analysis: 0.38

0.28 0.33 ± 0.07

Low Concentration (Target: 0.25 mg/g diet)

Analysis: 0.21

0.11 0.16 ± 0.07

[a]mean ± S.D.

Diet Batch Homogeneity

Two samples each from the top, middle, and bottom of the mixing bowl of the high- and low-concentration diets were analyzed. In addition, one sample from the top, middle, and bottom of the moderate and mid levels were analyzed.

High Concentration (Target: 2.0 mg/g diet)

Top 1	1.95	
Top 2	1.48	
Middle 1	2.07	
Middle 2	1.94	
Bottom 1	1.79	
Bottom 2	1.87	1.85 ± 0.20^a

Moderate Concentration (Target: 1.0 mg/g diet)

Top	0.99	
Middle	0.88	
Bottom	0.89	0.92 ± 0.06

Mid Concentration (Target: 0.5 mg/g diet)

Top	0.41	
Middle	0.41	
Bottom	0.85	0.56 ± 0.25

Low Concentration (Target: 0.25 mg/g diet)

Top 1	0.19	
Top 2	0.27	
Middle 1	0.19	
Middle 2	0.17	
Bottom 1	0.26	
Bottom 2	0.18	0.23 ± 0.05

[a]**Mean ± S.D.**

Diet Batch Stability

Stability was determined for various time periods up to 21 days in the polyethylene storage containers and for 7 days in the open glass rodent feeders. All analyses were determined on high-concentration (2 mg/g) diet.

Polyethylene Storage Container

0 day sample 1	2.04	
0 day sample 2	2.71	2.38 ± 0.47[a]
7 days, sample 1	2.27	
7 days, sample 2	1.66	1.96 ± 0.43
14 days, sample 1	1.80	
14 days, sample 2	2.52	2.16 ± 0.51
21 days, sample 1	1.99	
21 days, sample 2	2.16	2.08 ± 0.12

Glass Rodent Feeder

0 day sample 1	2.04	
0 day sample 2	2.71	2.38 ± 0.47
7 days, sample 1	2.33	
7 days, sample 2	2.29	2.31 ± 0.03

[a]**Mean ± S.D.**

5.5 GENERAL TOXICITY / REPRODUCTIVE TOXICITY SCREEN OF MODULAR ARTILLERY CHARGE SYSTEM (XM231/XM 232) ADMINISTERED IN THE DIET OF SPRAGUE-DAWLEY RATS

E.R. Kinkead, M.L. Feldmann, R.E. Wolfe, C.D. Flemming, D.L. Pollard, D.J. Caldwell[1], J.S. Eggers[2]

ABSTRACT

An artillery propellant under development by the U.S. Army is a granular mixture of 98% nitrocellulose, nitroglycerin, nitroguanidine, and <2% cryolite and graphite. The propellant, Modular Artillery Charge System (MACS; XM231/XM232) consists of a single increment of propellant charge contained within a rigid combustible casing. As part of the process to develop environmental and health effects criteria, a 90-day modified Screening Information Data Set reproductive assay was performed. Male and female Sprague-Dawley rats were treated with diet containing either 0.0, 0.2, 1.0, or 2.0 g propellant/kg diet. No mortalities occurred and body weights were unaffected by treatment. Methemoglobin concentrations of the high-dose rats measured at 28 days and at the conclusion of the study were significantly elevated (23 to 25%) compared to control rats. Relative organ weights of treated animals did not differ from weights of their respective control groups. No adverse effects occurred in mating or fertility indices. No significant treatment-related differences were noted in length of gestation, sex ratio, gestation index, or mean number of pups per litter.

INTRODUCTION

Modular Artillery Charge System (MACS; XM231/XM232) is a propellant under development by the US Army for autoloading howitzer artillery, consisting of a single increment of propellant charge contained within a rigid combustible casing. This technology will simplify autoloader requirements, result in a significant reduction in propellant production and logistics requirements, and eliminate propellant waste. The MACS propellant is a granular mixture of nitrocellulose (NC), nitroglycerin (GTN; glyceryl trinitrate), nitroguanidine (NQ), ethylcentralite (N,N'diethyl, N,N'diphenylurea), and potassium sulfate. Since NC, GTN, and NQ make up approximately 99% of MACS propellant, it is commonly referred to as "triple-based" propellant mixture.

Nitroguanidine is a primary component (approximately 47%) of the triple-based propellant mixture. Previously conducted acute oral and percutaneous studies on NQ found the compound to be relatively nontoxic. The compound was not irritating to either rabbit skin or eyes using the standard Draize technique (Hiatt et al., 1986; Morgan et al., 1986). Nitroguanidine did not kill rats by oral gavage at a limit test concentration of 5 g/kg (Brown et al., 1988), while the oral LD_{50} values for male and female mice are reported as >5.0 g/kg and 4.3 g/kg, respectively (Hiatt et al., 1988).

Nitroguanidine did not produce treatment-related effects when administered in the diet of Sprague-Dawley rats at doses as high as 1000 mg/kg/day for 14 days. However, after 90 days that dose level produced a decrease in body weight gain and

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food consumption (Reddy and Korte, 1988). No evidence of developmental toxicity in rats or rabbits was reported following conditions of the above study.

Nitroglycerin is also a primary component (approximately 22%) of the triple-based propellant. The toxic, physiologic, and pharmacologic effects of GTN have been studied extensively in animals and man (NIOSH, 1978). Nitroglycerin is readily absorbed by ingestion, inhalation, sublingual contact, and through the skin. When both inhalation and dermal exposure to GTN occur, dermal contact is thought to make the major contribution to the total amount absorbed by the body (NIOSH, 1978). Vasodilatation following exposure to GTN can occur within minutes, regardless of route of exposure. This effect has led to sudden death and chronic cardiac disease (Carmichael and Lieben, 1963). Small amounts can cause headaches which can persist for hours or days. Larger doses can cause hypotension, cyanosis, and methemoglobinemia. These effects are potentiated by alcohol consumption (NIOSH, 1978). Symptoms also include nausea and vomiting, and in some instances, diarrhea. The mouse intraperitoneal (IP) LD_{50} of GTN is 194 mg/kg (Kylin et al., 1964), and the rat IP LD_{50} has been reported as 93 mg/kg (Burginson et al., 1962).

The third major component (approximately 28%) of the triple-based propellant is nitrocellulose or cellulose nitrate. Cellulose nitrate is relatively nontoxic but is known for its extreme flammability (Montgomery, 1982). Dry NC may explode when subjected to heat or sudden shock; NC is therefore generally handled wet with either water or alcohol. Toxic combustion products are generated when the compound is ignited, primarily carbon monoxide and oxides of nitrogen (Montgomery, 1982).

Munition workers exposed to nitrate-containing explosives have experienced skin irritation, liver damage, and anemia (Hathaway, 1977; Morton et al., 1976; Stewart et al., 1945). These compounds have also been found to cause methemoglobin formation, liver and spleen hypertrophy, and degeneration of the seminiferous tubules resulting in decreased spermatogenesis (Cody et al., 1981; Levine et al., 1983, 1984; Furedi et al., 1984a, b). A recently completed reproductive screen in this laboratory with a nitrate-containing explosive compound resulted in testicular atrophy and decreased spermatogenesis in the male rats. Female rats displayed clinical signs of altered locomotion during the postpartum period, and both sexes had brain lesions at necropsy (Kinkead et al., 1994, 1995a). Neurotransmitter analysis showed statistically significant increases in norepinephrine, epinephrine, seratonin, and dopamine in the TNB-treated rats (Narayanan et al., 1995). Changes in neurotransmitter levels in specific regions may be one of the mechanisms responsible for TNB-induced neurological disorder. A reproductive screen on other nitrate containing explosive compounds resulted in hemolytic anemia (Kinkead et al, 1995b), loss of embryos, and/or death of fetuses postmating, indicating that the compound is a reproductive toxicant (Kinkead et al., 1995c).

Nitrate-containing explosive compounds have produced adverse reproductive effects in both male and female rats. Testicular atrophy and decreased spermatogenesis are common findings in male rats; lack of live litter production has been noted in female rats. Brain lesions and loss of motor skills, methemoglobinemia, and hemolytic anemia have been common findings in the reproductive screens performed within this laboratory. Because of the above findings, a 90-day reproductive screen was performed on MACS to evaluate its potential to produce alterations in paternal fertility, maternal pregnancy and lactation, and growth and development of offspring.

A range-finding study was first performed in which MACS was administered in the diet of male and female Sprague-Dawley rats for a three-week period. The rats received diet containing either 0.0, 0.25, 0.5, 1.0, or 2.0 g propellant/kg diet (Kinkead et al., 1995d). No mortalities occurred during the study and body weights were unaffected by the treatment. Methemoglobin concentrations measured at the conclusion of the treatment period indicated no differences between treated and control animals. Relative liver weights of the high-dose female rats were significantly ($p<0.1$) greater than the control group.

MATERIALS AND METHODS

Test Agent

The MACS was provided by the U.S. Army, Picatinny Arsenal, NJ. The pellets were ground to granular form prior to receipt. Pertinent chemical and physical properties of the test compound are listed below:

MACS

Synonyms:	Smokeless powder Modular Artillery Charge System XM231/XM232
CAS#	None assigned
Specific gravity:	1.5880
Vapor pressure:	Negligible
Appearance:	Hard cylindric pellet white to reddish orange, coated with graphite

Diet Preparation, Homogeneity, Stability, and Analysis

MACS was administered by the oral route, mixed appropriately in the diet. The test material was mixed in powdered Purina Formulab #5002 (Ralston Purina, St. Louis, MO) certified rodent diet meal. The diet was prepared by adding the appropriate amount of MACS to 10 kg rodent diet and mixing for a minimum of 90 minutes. MACS analysis was performed using a gas chromatographic method described in Appendix A. Total MACS concentrations were based on nitroguanidine concentrations measured in five gram samples of diet.

To assess the efficiency of the mixing process, analyses were performed on samples taken from the top, middle, and bottom of the mixing bowl. Duplicate samples were taken for each measurement. Stability analyses were performed on diet stored in polyethylene containers for up to 21 days (Kinkead et al., 1995d).

Group Assignments and Dose Levels

Group	Number of Animals Male	Female	Target Dose Levels of MACS (mg/kg diet)[a]	Target Dose of MACS (mg/kg body weight/day)[b]
Control	12	12	0.0	0.0
Low	12	12	200.0	12.0
Middle	12	12	1000.0	60.0
High	12	12	2000.0	120.0

[a] Target dose levels based on range-finding results. Highest dose level produced relative liver weight increases in female rats (Kinkead et al., 1995d).

[b] Assume 500g rat consumes 30g feed/day.

Study Design and Experimental Evaluations

The study design, experimental evaluations, and animal assessments for this study are detailed in Section 6.1 of the 1994 Toxic Hazards Research Unit Annual Report (Kinkead et al., 1995e). A technical report entitled "General Toxicity/Reproductive Toxicity Screen of Modular Artillery Charge System Administered in the Diet of Sprague-Dawley Rats" by Kinkead et al. (in press), contains details of assessment data and figures not included in this annual report.

RESULTS

Food Consumption and Calculated Dose

Food consumption of the male rats remained relatively constant throughout the 90-day study. Food consumption of the female rats increased during the postpartum (lactation) period compared to the premating and gestation periods. Male rats consumed approximately 35 g diet/day which resulted in a mean dose of 127, 72, and 14 mg MACS/kg/day in the high-, mid-, and low-dose groups, respectively. Female rats received a median dose of 161, 93, and 19 mg MACS/kg/day for the high-, mid-, and low-dose groups, respectively.

General Toxicity

No mortality occurred in the parental animals during the study. Mean body weights of the treated rat groups did not differ from the mean body weights of their respective control groups. No clinical signs of motor skill loss were noted during the

study. This was confirmed by the Opto-Varimex tests in which no differences in locomotor skills were detected in treated or control rats.

Six male rats were necropsied following the mating period (28 day of treatment) and the remaining six/group were necropsied at study termination (90 days of treatment). No treatment-related differences were noted in absolute or relative organ weights in male rats at either of the evaluation periods. Likewise, no treatment-related differences were noted in absolute or relative organ weights of female rats examined after 90 days of treatment.

Male rats sacrificed following mating (28 days) and after 90 days of treatment showed no adverse effects on sperm function/activity. All measurements of sperm function/activity evaluated in the MACS-treated rats were similar to the sperm function of the control rats.

Methemoglobin concentration was significantly ($p<0.01$) increased in the high-dose male rats following 28 and 90 days of treatment. A similar increase in methemoglobin concentration was noted in the mid- and high-dose female rats examined following 90 days. All additional hematology measurements were within normal value ranges.

After 28 days of treatment, alanine aminotransaminase (ALT) values of the high-dose male rats were significantly ($p<0.05$) lower than control values. The ALT value of high-dose male rats was also statistically significantly ($p<0.05$) less than control values following 90 days of treatment, however this difference was not noted in female rats.

At necropsy, all animals were judged to be in good general condition. Renal cysts were observed in the left kidney of a low dose and a control female rat. Hydronephrosis was observed in the kidneys of two control male rats. Unilateral testicular atrophy was noted in a control rat and reticulated yellow mottling of the liver was observed in a mid-dose male rat.

Histopathology

Observed histopathologic lesions were considered to be incidental findings unrelated to treatment. Statistical analysis revealed a significantly ($p<0.05$) increased incidence of renal hyaline casts in the low-dose female rats. The finding was not dose-related and is not considered to be of biological significance. In all groups, minimal (early) nephropathy changes were a common finding in male rats, as was renal mineralization in female rats.

Reproductive Indices

The treatment produced no adverse effects on mating as 100% of the animals mated (Table 5.5-1). The fertility index was 100% in the mid-dose group and 92% in the other treated and control groups. No significant treatment-related differences were noted in length of gestation, sex ratio, gestation index, or mean number of offspring per litter. The mean body weights of male and female pups from the MACS-treated dams did not differ from the mean weights of their respective controls.

TABLE 5.5-1 LITTER DATA FOR RATS TREATED WITH MACS

PARAMETER	CONTROL	DOSE LEVEL LOW	MEDIUM	HIGH
No. of mated pairs	12	12	12	12
No. dams w/ pups born	11	11	12	11
No. dams w/ pups born live	11	11	12	11
Fertility index (%)	91.7	91.7	100.0	91.7
Gestation index[a] (%)	91.7	91.7	100.0	91.7
Live Birth Index[b] (%)	97.5	98.8	93.1	97.7
4-Day Survival Index	99.4	98.1	97.8	98.8
7-Day Survival Index	100.0	100.0	100.0	100.0
14-Day Survival Index	100.0	100.0	100.0	100.0
21-Day Survival Index	100.0	100.0	100.0	100.0
Lactation Index[c] (%)	100.0	100.0	100.0	100.0
Mean no. pups / litter	14.4	14.8	5.8	15.5
Mean no. gestation days	22	22	23	22
Sex ratio (M/F)	1.0/1.0	1.0/0.86	1.0/0.83	1.0/0.95

[a] **Number of females with live litters**
Number of females pregnant
[b] **Number of live pups at birth**
Total number of pups born
[c] **Number of pups surviving 21 days**
Number of pups surviving 4 days

DISCUSSION

Administering MACS in the diet of Sprague-Dawley rats at calculated dose levels of 127, 72, and 14 (male rats), or 161, 93, and 19 (female rats) mg MACS/kg body weight/day produced no adverse effects on reproductive performance or litter parameters. No treatment-related effects in mean pup body weights were noted during the weaning or postweaning time periods.

Anemia and increased methemoglobin production are common characteristics of nitrate poisoning. Anemia was not noted in this study but methemoglobin concentration was increased slightly in the high-dose male rats and the mid- and high-dose female rats. Although the increase in methemoglobin was statistically significant, the effects would be considered to be of little biological significance. In an oral study of guanidine nitrate (Mullen, 1988), the only treatment-related gross finding was multiple red foci in the thymus, presumably hemorrhage. Thymic hemorrhage was not a treatment-related finding in this study. There was also no treatment-related effects in body weight gain or food consumption, as reported by Reddy and Korte (1988) in a 90-day toxicity study of nitroguanidine in Sprague-Dawley rats.

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5.6 MEASUREMENT OF CARDIOVASCULAR RESPONSE OF MALE SPRAGUE-DAWLEY RATS TO MODULAR ARTILLERY CHARGE SYSTEM USING RADIOTELEMETRIC IMPLANTS

F. W. Abernathy and C. D. Flemming

ABSTRACT

The U.S. Army is developing an artillery propellant which is a granular mixture of 98% nitrocellulose, nitroglycerin, nitroguanidine, and 2% cryolite and graphite. The propellant charge is contained within a rigid, combustible case. As part of the process to develop health effect criteria, a reproductive toxicity screen was performed (Kinkead et al., 1994; Kinkead et al., 1995). Because of the presence of nitrates, it was also necessary to evaluate the cardiovascular effects of repeated exposure. The propellant was ground to a powder and mixed with powdered feed. Animals were allowed to eat *ad libitum*. Target dosage levels in the feed were 0.0, 0.2, 1.0, and 2.0 g/kg of propellant. Blood pressure and heart rates of Sprague-Dawley rats containing radiotelemetric implants were monitored 24 hours per day for 7 days. A baseline evaluation was performed 7 days prior to treatment and recovery was evaluated 7 days after treatment.

It appears that M30A1 propellant ingested in amounts of 117 mg per kg of body weight per day over a 7-day period may cause a significant reduction in systolic blood pressure followed by a partial recovery. During the recovery period the heart rate appears to be inversely proportional to dose. These data are confounded by other variables which may be eliminated in the future by performing an analysis of covariance to minimize baseline effects.

INTRODUCTION

The U.S. Army is developing a Modular Artillery Charge System (MACS) also known as M30A1 or Unicharge. It is a granular mixture of 98% nitrocellulose, nitroglycerin, nitroguanidine, and 2% cryolite and graphite. The propellant charge is contained within a rigid, combustible case. Components like nitroguanidine (Hiatt et al., 1986; Morgan et al., 1986, Brown et al., 1988; Hiatt et al., 1988; Reddy and Korte, 1988; Ho et al., 1988) and nitrocellulose (Montgomery, 1982) are relatively nontoxic. In contrast, the nitrate ester nitroglycerin lowers blood pressure in a dose-dependent manner (NIOSH, 1978; Stewart et al., 1974; Kylin et al., 1964). It is important to monitor the effects of unique blends of nitrated compounds like MACS to determine if they exert dose-dependent effects on blood pressure and/or heart rate that might adversely effect the performance of military personnel, munitions workers or constitute an environmental hazard.

In this study, cardiovascular monitoring was performed in male Sprague-Dawley rats as an extension of other MACS studies involved with reproductive toxicity (Kinkead et al., 1994; Kinkead et al., 1995). This data can be used in pharmacodynamic modeling studies to refine the rat model as a means for predicting toxicity of unique blends of nitrated explosives and propellants to humans. The method of choice was radiotelemetric monitoring which involves cannulation of a major artery with an implantable transmitter that is sewn in place inside a body cavity (Brockway et al., 1991). Typically, the lower abdominal aorta is cannulated with the transmitter body sewn to the peritoneal cavity wall. This method was chosen over tailcuff sphygmomanometry or direct cannulation with tethering because direct blood pressure readings are obtained, large amounts of data can be obtained over long periods of time with minimal intrusion in animal activity, and after recovery from

surgery, problems with patency, sterility and stress generated from conventional tethering are avoided. The technique has been validated against long-term, direct, tethered cannulation (Guiol et al., 1992; Depasquale et al., 1994) and against noninvasive tailcuff sphygmomanometry (Bidani et al., 1993; Abernathy et al., 1995a; Abernathy et al., 1995b). Disadvantages include the requirement for relatively expensive transmitters which must be returned to the manufacturer for battery replacement and refurbishment, electrical interference of transmitter signals from other instruments, and electronic "drift" of transmitters over time. Electronic drift can be minimized by keeping study times short (e.g., 1 month or less) and readjusting transmitter offsets prior to implantation.

METHODS

Animals

Male Sprague-Dawley rats, generally 8 weeks of age at receipt and 12 weeks of age at treatment, were purchased from Charles River Breeding Laboratories, Raleigh, NC, and provided with food pellets of Purina Rodent Chow #5008 (Ralston, Purina, St. Louis, MO) and water ad libitum. Animals were quarantined for a two-week period during which standard quality control procedures were performed. Rats were housed singly in clear, plastic shoe-box type cages with wood-chip bedding (Bettachip®, Northeastern Products Corp., Warrensburg, NH). All rats were identified by tail tattoo. Food pellets were replaced with feed jars containing powdered Purina Formulab #5002 (Ralston, Purina, St. Louis, MO) near the end of the week prior to the initiation of each study the following week.

Radiotelemetric Monitoring Equipment

Data Sciences Software (Version 2.22, 1994), a computer board Model DQ1188, a BCM 100 consolidation matrix module with a voltage regulating power plug, Model RA1010 flat receivers, Model RLA3000 cylindrical receivers, Model TA11PA-C40 radiotelemetric implants, and a Model C11PR ambient pressure monitor were purchased from Data Sciences, St. Paul, MN. The Data Sciences card was installed in a Zenith 386 computer containing 8 megabytes of RAM, an 80 megabyte hard drive, and a math coprocessor. The computer was loaded with OS/2 (Version 2.00). Included with the computer was a color monitor and a mouse. The computer was connected via the card to the BCM 100 consolidation matrix and the latter was connected to receivers.

Surgical Procedures

Transmitter offsets were adjusted 0 mm Hg $\pm$ 2 prior to implantation. Eight-week old rats were anesthetized by intraperitoneal injection with pentobarbital (50 mg/kg body weight). The abdomen was opened, the transmitter catheter tip was inserted into the abdominal aorta just above the lower bifurcation into the femoral arteries, and threaded into the aorta just beneath the renal artery junction. The catheter was glued into place with a cellulose patch and surgical glue. The transmitter body was looped around so it was lying next to the catheter tip and then sewn into the wall of the peritoneal cavity. The wound was sewn shut, skin stapled together, and the animals were allowed to recover for several days prior to use.

Preparation of Test Material

As previously described, test material was mixed with powdered certified Purina Formulab #5002 (Ralston Purina, St. Louis, MO) to prepare target doses of 2.0, 1.0, and 0.2 g of MACS per kg of powdered feed (Kinkead et al., 1995).

Experimental Protocol

Four rats were placed into individual cages in racks above 1 of 4 receiving units. Each rat represented a different dose group. The cages were separated by one space on the rack to avoid "cross-talk" between transmitters (Abernathy et al., 1995b). Each receiver relayed the transmitter signal to the BCM 100 consolidation matrix which relayed them to the computer for additional processing. Data acquired included blood pressure (systole, mean, and diastole) and heart rate. Ten-second scans were recorded every five minutes. Generally, data acquisition continued 24 hours a day. Mean blood pressure was calculated as a weighted average (minus outliers) from a series of 500 data points collected during a one second period. Baseline data were collected during the first week (7 days) of monitoring. The animals were dosed during week 2 and returned to regular food during week 3 (recovery). The next week the animals were terminated and replaced with a new lot of 4 animals. This continued until data were acquired from a total of 16 implanted rats, n = 4 animals per dose group. Body weights were recorded at the beginning of each week including the week the animals were terminated. Food consumption was recorded on Monday, Wednesday, and Friday.

Data Analysis

As previously described, body weights and food consumption data were used to calculate dose (Kinkead et al., 1995). The blood pressure and heart rate data were consolidated into Lotus spreadsheets and uploaded into RS1 for subsequent in-house analyses by BMDP and SAS statistical programs. Blood pressure and heart rate readings acquired for 7 consecutive days were pooled for each parameter within each dose group. A 2-factorial multivariate analysis of variance with Bonferroni multiple comparisons was performed to detect differences between dose groups and baseline, treatment, and recovery periods.

RESULTS

Transmitters removed from the animals were found to be within 5 mmHg from zero, which is within acceptable limits. In Table 5.6-1, slight but statistically significant differences were noted between the means of most of the blood pressure readings and heart rates in Groups 1 and 2 versus Group 4 ($p < 0.01$). Of particular note are the consistently high readings of Group 1 versus Group 4 during the week prior to exposure of animals to MACS. In Table 5.6-2, a similar pattern of differences is noted between Group 1 (high dose) and Group 4 (control) after dosing. However, additional significant elevations in the mean blood pressure and diastolic pressures in Group 2 (medium dose) were also noted ($p < 0.01$). Differences in Group 3 (low dose) and controls were slight, with heart rates in Group 3 reduced to approximately control levels. In Table 5.6-3, significant differences were noted between blood pressure and heart rate readings from high, medium, and low dose groups versus controls at $p < 0.01$. The high dose systolic and mean blood pressure readings were slightly elevated above readings acquired during dosing, but slightly lower than those recorded during baseline monitoring.

In Tables 5.6-4 through 5.6-7 the data shown in Tables 5.6-1 through 5.6-3 have been rearranged to demonstrate differences noted within each group as the animals progressed from the baseline to dosed and recovery periods. All significance levels were at $p < 0.05$. In the high dose group (Table 5.6-4), systolic and mean blood pressures were significantly greater during the baseline period than during the dosed or recovery periods. Systolic and mean blood pressures in the recovery period were slightly but significantly elevated above parallel readings acquired during dosing. Heart rate was lowered as the animals progressed from baseline to dosed to recovery periods. In the medium dose group (Table 5.6-5), a smaller but similar set of differences to those shown in Table 5.6-4 was noted together with a slight, but significant, flucuation in diastolic pressure. In the low dose group (Table 5.6-6), all blood pressure and heart rate readings decreased in a "dose-like" fashion as the animals progressed from baseline to dose and recovery periods. In the control group (Table 5.6-7), blood pressure and heart rate changes between periods were similar to those seen in the high dose group.

TABLE 5.6-1. BASELINE (WEEK 1)

No dose	Group 1	Group 2	Group 3	Group 4
Systole (mm Hg)	125 ± 0.13 [a]	119 ± 0.17	118 ± 0.13 [a]	119 ± 0.15
Mean blood pressure (mm Hg)	105 ± 0.13 [a]	101 ± 0.14	100 ± 0.13	100 ± 0.17
Diastole (mm Hg)	88 ± 0.12 [a]	85 ± 0.12	86 ± 0.11 [a]	85 ± 0.17
Heart rate (beats per minute)	378 ± 0.60 [a]	370 ± 0.52	380 ± 0.54 [a]	368 ± 0.57

Data are means ± SEM. Four lots of animals were used with 4 animals per lot. Animals in each lot were assigned to either group 1, 2, 3, or 4. Each lot of animals was monitored for 3 weeks, terminated, and replaced with the next lot of animals. Each group of data are total usable readings acquired over 7 consecutive 24 periods from 4 pooled animals (1 animal per lot). Readings ranged from 5635 to 7813. Each reading was the average of 5000 data points acquired every 5 minutes during a 10-second sampling period. Animals were allowed to eat only powdered feed

[a] Significantly different from control at $p < 0.01$.

TABLE 5.6-2. DOSED (WEEK 2)

Mean mg of M30A1 consumed per kg of body weight per day ± SEM	Group 1 (117 ± 4.86)	Group 2 (67 ± 6.04)	Group 3 (13 ± 0.46)	Group 4 (0)
Systole (mm Hg)	118 ± 0.11 [a]	116 ± 0.14	114 ± 0.15 [a]	116 ± 0.11
Diastole (mm Hg)	88 ± 0.12 [a]	86 ± 0.13 [a]	84 ± 0.12 [a]	81 ± 0.10
Heart rate (beats per minute)	370 ± 0.53 [a]	362 ± 0.44	361 ± 0.51 [b]	363 ± 0.52
Mean blood pressure (mm Hg)	101 ± 0.11 [a]	99 ± 0.13 [a]	98 ± 0.14 [b]	97 ± 0.10

Data are means ± SEM. Refer to Table 5.6-1 for additional details. Readings were acquired after baseline readings during a 7-day period when animals were exposed to M30A1 mixed with powdered feed.

[a] Significantly different from control at $p < 0.01$.

[b] Significantly different from control at $p < 0.05$.

TABLE 5.6-3. RECOVERY (WEEK 3)

No dose	Group 1	Group 2	Group 3	Group 4
Systole (mm Hg)	121 ± 0.13 [a]	117 ± 0.17	112 ± 0.22 [a]	118 ± 0.10
Mean blood pressure (mm Hg)	102 ± 0.12 [a]	99 ± 0.16 [a]	95 ± 0.19 [a]	98 ± 0.10
Diastole (mm Hg)	88 ± 0.13 [a]	84 ± 0.16 [a]	82 ± 0.16	82 ± 0.10
Heart rate (beats per minute)	343 ± 0.57 [a]	346 ± 0.48 [a]	353 ± 0.52 [a]	358 ± 0.53

Data are means ± SEM. Refer to Table 5.6-1 for additional details. Readings were acquired after dosed readings during a 7-day period when animals were allowed to eat powdered feed containing no M30A1.

[a] Significantly different from control at $p < 0.01$.

DISCUSSION

Dose effects of MACS on blood pressure of male Sprague-Dawley rats is confounded by the fact that blood pressure baseline readings for the high dose group (Group 1) were significantly higher than all other groups, including the controls (Table 5.6-1). A slight but discernable dose effect was observed in the mean blood pressure and diastolic pressure which gradually elevated as the dose was increased (Table 5.6-2). Recovery from this dose effect was also dose-related, e.g., the higher the dose, the poorer the recovery. The low dose group appeared to overcompensate for the elevated mean blood pressure during recovery (Table 5.6-3). Heart rate readings in Groups 1 and 3 were ignificantly higher than controls prior to

dosing (Table 5.6-1). The medium and low dose groups dropped slightly below control levels during dosing (Table 5.6-2), and together with the high dose group displayed a dose response inversely proportional to the heart rate during the recovery period (Table 5.6-3). The data presented in Table 5.6-4 suggests that systolic blood pressure in male Sprague-Dawley rats exposed to 117 mg of M30A1 propellant per kg of body weight per day over a 7-day period may be lowered as much as 7 mmHg with a slight rebound effect (3 mmHg) occurring during the recovery period. The diastolic pressure remained constant throughout baseline, dose, and recovery periods which is inconsistent with the dose response effect observed between groups. Heart rate dropped consistently within each group (including controls) as the animals progressed from baseline to dosed to recovery periods (Tables 5.6-4 through 5.6-7). This observation confounds any interpretation of dose effects on heart rates.

TABLE 5.6-4. THREE WEEK DATA FROM ANIMALS RECEIVING A HIGH DOSE OF M30A1

	Baseline (week 1)	Dosed (week 2)	Recovery (week 3)
Systole (mm Hg)	125 ± 0.13	118 ± 0.11	121 ± 0.13
Mean blood pressure (mm Hg)	105 ± 0.13	101 ± 0.11	102 ± 0.12
Diastole (mm Hg)	88 ± 0.12	88 ± 0.12	88 ± 0.13
Heart rate (beats per minute)	378 ± 0.60	370 ± 0.53	343 ± 0.57

Data are means ± SEM. Refer to Tables 1-2 for additional details.

All baseline means except diastolic were significantly different from dosed and recovery means at $p < 0.05$. All dosed means except diastolic were significantly different from recovery means at $p < 0.05$.

TABLE 5.6-5. THREE WEEK DATA FROM ANIMALS RECEIVING A MEDIUM DOSE OF M30A1

	Baseline (week 1)	Dosed (week 2)	Recovery (week 3)
Systole (mm Hg)	119 ± 0.17	116 ± 0.14	117 ± 0.17
Mean blood pressure (mm Hg)	101 ± 0.14	99 ± 0.13	99 ± 0.16
Diastole (mm Hg)	85 ± 0.12	86 ± 0.13	84 ± 0.16
Heart rate (beats per minute)	370 ± 0.52	362 ± 0.44	346 ± 0.48

Data are means ± SEM. Refer to Tables 1-2 for additional details.

All baseline means were significantly different from dosed and recovery means at $p < 0.05$. All dosed means except diastolic were significantly different from recovery means at $p < 0.05$.

TABLE 5.6-6. THREE WEEK DATA FROM ANIMALS RECEIVING A LOW DOSE OF MACS

	Baseline (week 1)	Dosed (week 2)	Recovery (week 3)
Systole (mm Hg)	118 ± 0.13	114 ± 0.15	112 ± 0.22
Mean blood pressure (mm Hg)	100 ± 0.13	98 ± 0.14	95 ± 0.19
Diastole (mm Hg)	86 ± 0.11	84 ± 0.12	82 ± 0.16
Heart rate (beats per minute)	380 ± 0.54	361 ± 0.51	353 ± 0.52

Data are means ± SEM. Refer to Tables 5.6-1 and 5.6-2 for additional details.

All baseline means were significantly different from dosed and recovery means at $p < 0.05$. All dosed means were significantly different from recovery means at $p < 0.05$.

In conclusion, it appears that MACS ingested in amounts of 117 mg per kg of body weight per day over a 7-day period may cause a significant reduction in systolic blood pressure from which the animal only partially recovers during the final week of monitoring. The apparent dose effects on mean and diastolic blood pressure indicated in Tables 5.6-2 and 5.6-3 are negated by the lack of change in diastolic pressure from baseline to dose to recovery periods shown in Table 5.6-4. The apparent dose effects on heart rate indicated in Table 5.6-3 are confounded by the fact that heart rates are decreasing in all groups as they progress from baseline to dose to recovery periods (Tables 5.6-4 through 5.6-7). It may be possible to minimize or eliminate these confounding variables by performing an analysis of covariance to eliminate baseline effects from dosed and recovery periods.

TABLE 5.6-7. THREE WEEK DATA FROM ANIMALS RECEIVING NO M30A1

	Baseline (week 1)	Dosed (week 2)	Recovery (week 3)
Systole (mm Hg)	119 ± 0.15	116 ± 0.11	118 0.10
Mean blood pressure (mm Hg)	100 ± 0.17	97 ± 0.10	98 0.10
Diastole (mm Hg)	85 ± 0.17	81 ± 0.10	82 0.10
Heart rate (beats per minute)	368 ± 0.57	363 ± 0.52	358 0.53

Data are means ± SEM. Refer to Table 5.6-1 for additional details.

All baseline means were significantly different from dosed and recovery means at $p < 0.05$. All dosed means were significantly different from recovery means at $p < 0.05$.

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SECTION 6

ENVIRONMENTAL INITIATIVE PROJECT

6.1 DEVELOPMENT OF PHYSIOLOGICALLY BASED PHARMACODYNAMIC MODEL FOR ETHANE EXHALATION

J.Z. Byczkowski and C.S. Seckel

ABSTRACT

A physiologically based pharmacodynamic (PBPD) model was developed to simulate the kinetics of ethane generation and disposition in experimental animals. Ethane exhalation is recognized as a reliable index of lipid peroxidation which may be stimulated by pro-oxidant chemicals. The PBPD model was written in Continuous Simulation Language (ACSL), and simulations were performed using SIMUSOLV software on a VAX/VMS mainframe computer. The PBPD model simulated formation of ethane over time, depending on the concentration of the pro-oxidant chemical inducer, and predicted the disposition and exhalation of ethane. The partition coefficients for ethane were determined in mouse tissues *in vitro* and ethane metabolism parameters were measured in mice *in vivo*, using a closed gas uptake chamber. In preliminary experiments, the PBPD model simulated the kinetics of ethane exhalation in mice treated with massive doses of either CCl_4 or TCE. It is suggested that the PBPD model, when systematically calibrated with experimental data for several pro-oxidant chemicals, may be useful for future pharmacodynamic descriptions of oxidative stress and dose-response characterization of pro-oxidant chemicals.

INTRODUCTION

Lipid peroxidation is a pathological process leading to the unique form of hepatocellular injury, implicated in the genesis of liver necrosis evoked by several pro-oxidant chemical hepatotoxicants such as carbon tetrachloride (CCl4), yellow phosphorous, and ethanol (reviewed by Kulkarni and Byczkowski, 1994), and it may be linked with carcinogenicity (reviewed by Byczkowski and Channel, in press). Lipid peroxidation is characterized by the formation of conjugated dienes, formation of thiobarbituric acid reactive substance (TBARS; mainly malondialdehyde), and the exhalation of hydrocarbons (e.g., ethane).

Several free radicals and reactive oxygen species can initiate the lipid peroxidation process by abstracting the hydrogen with its single electron (hydrogen atom) from unsaturated fatty acid molecules. Apparently, the most common initiators are: .OH, 'ΔgO2, alkoxyl, peroxyl, and perferryl (but not superoxide alone, Byczkowski and Gessner, 1988). Lipid peroxidation may be initiated also by carbon-centered free radicals (e.g., CCl3.) produced from certain pro-oxidant chemicals by cytochrome P450 system and redox cycling (Kulkarni and Byczkowski, 1994). During the initiation, lipienyl free radical (L.) is formed from unsaturated fatty acid (LH):

$$LH + X^{\cdot} \text{---> } L^{\cdot} + XH$$

where X. is a carbon-centered or peroxyl-type free radical formed from pro-oxidant chemical. The initiation reaction depends on the fresh supply of free radicals and may be terminated by free radical scavengers and antioxidants. Malondialdehyde and alkanes (e.g., ethane) are generated, among other stable products, during the propagation and termination of lipid peroxida-

tion process. The alkanes are formed in biological systems through peroxidation of the omega-3 (ethane) or omega-6 (pentane) fatty acids and the subsequent beta-scission decomposition of the intermediate hydroperoxides (Gardner, 1989).

Based on the findings of Riely et al. (1974) that mice treated with carbon tetrachloride have an increased amount of the exhaled ethane *in vivo*, numerous studies have been conducted in which ethane and/or pentane were measured as indices of lipid peroxidation or surrogate biomarkers of tissue damage by oxidative stress. Increased ethane exhalation was also found by Cojocel et al. (1989) as a consequence of lipid peroxidation in mice treated with trichloroethylene (TCE). It was also demonstrated, using a spin-trapping technique by Gonthier and Barret (1989), that further metabolism of trichloroethanol (TCOH), an intermediate product of TCE metabolism, may supply free radicals in the microsomal preparations *in vitro*. Larson and Bull (1992a; 1992b) observed that other metabolites of TCE increased production of TBARS in mice. They suggested that at least two TCE metabolites, TCA and DCA (at doses above 300 mg/kg), may supply carbon-centered free radicals produced as a result of reductive dechlorination. The carbon-centered free radicals under aerobic conditions tend to interact very fast with dioxygen, providing the reactive oxygen species.

The detection of volatile hydrocarbons (ethane and/or pentane) in expired air has been used for some time as a non-invasive technique to measure lipid peroxidation in whole animals or human subjects (Refat et al., 1991; Kazui et al., 1992; Arterbery et al., 1994; Guilbaud et al., 1994). Ethane exhalation is more reliable as an index of lipid peroxidation than pentane, because cytochrome P450-mediated metabolism of ethane is substantially slower than that of pentane (Smith, 1991). Typically, the exhaled air is probed for ethane by gas chromatograph equipped with flame ionization, photoionization, or the ion trap detectors (Kneepkens et al., 1994). Because of its direct relation to lipid peroxidation (Jeejeebhoy, 1991), we chose the ethane exhalation assay as an end point for development of a computer-aided PBPD model for simulation of the biological effects caused by certain pro-oxidant chemicals. The developed PBPD model may be linked with a PBPK model, that describes the local concentrations of chemical in the liver, and may be used for pharmacodynamic description of oxidative stress and dose-response characterization of pro-oxidant chemicals.

MATERIALS AND METHODS

PBPD Model

The PBPD model was written in Advanced Continuous Simulation Language (ACSL; Mitchell and Gauthier Associates, Inc. 1993). Simulations were performed using SIMUSOLV software with optimization capabilities (DOW Chemical Co., Midland, MI) on a VAX/VMS mainframe computer. Parameters were optimized by SIMUSOLV using the log likelihood function as the criterion, and either the generalized reduced gradient method for single parameter optimization or the Nelder-Mead search method for multiple parameters optimization to adjust the values (Steiner et al., 1990).

Partition coefficients for ethane were measured in our laboratory (Seckel and Byczkowski, 1995) by a modified method of Gargas et al. (1989). Metabolic parameters were estimated, fitted, and optimized with a PBPD model based on our experi-

mental measurements of ethane uptake in a closed chamber using the method for volatile chemicals, as described by Gargas et al. (1986).

Animals

B6C3F1 mice were used to conduct both the exhalation and partition coefficient experiments.

RESULTS

PBPD Model Structure

A general simplified scheme of the PBPD model for ethane exhalation is shown in Figure 6.1-1. The PBPD model was built

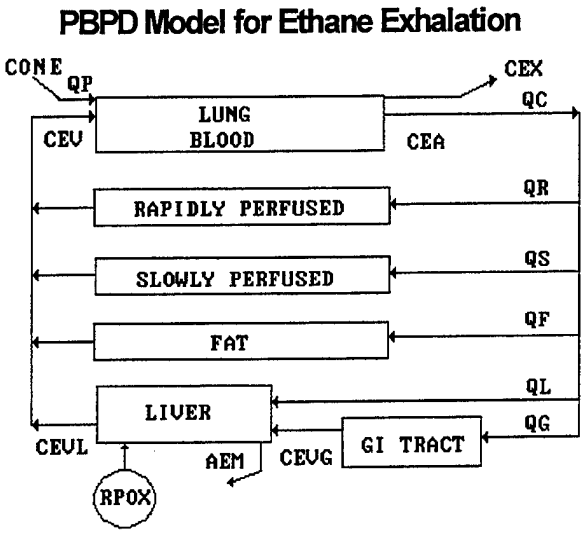

Figure 6.1-1. Conceptual scheme of physiologically based pharmacodynamic model for ethane exhalation. CONE - initial concentration of ethane in chamber [ppm]; QP - alveolar ventilation rate [L/h]; CEX - concentration of ethane in exhaled air [mg/L]; QC - cardiac output [L/h]; CEV - mixed venous blood concentration of ethane [mg/L]; CEA - arterial blood concentration of ethane [mg/L]; QR - fraction of blood flow to rapidly perfused tissues; QS - fraction of blood flow to slowly perfused tissues; QF - raction of blood flow to fat; QL - fraction of blood flow to liver; CVEL - concentration of ethane in liver vein [mg/L]; CVEG - concentration of ethane in portal vein [mg/L]; QG - fraction of blood flow to gut; RPOX - lipid peroxidation rate [mg/h]; AEM - amount of ethane metabolized [mg].

based on a template with a code for metabolism suppression and calibrated with experimental data collected that were in the closed gas uptake chamber from mice inhaling a known concentration of ethane (to determine V_{max} and K_m), or injected i.p. with CCl_4 (1.5 g/kg) or TCE (2.5 g/kg) and exhaling ethane (to determine rates of peroxidation, REOX). The rate of ethane

production (dAE/dt) was linked with a local concentration of pro-oxidant chemical in the liver by a square root function (McKenna et al., 1991; Byczkowski and Flemming, 1995) based on a peroxidation rate (RPOX [mg/h]):

PROOXIDANT CHEMICAL

k_i ↓ k_t

ETHANE + ETHANE <--<-- FR + FR ----> TERMINATION and QUENCHING

$$dAE/dt = REOX = \sqrt{RPOX}$$

$$RPOX = PXFE * C_L * V_L$$

where: C_L is a concentration of pro-oxidant chemical in the liver [mg/kg]; V_L is a volume of liver [kg]; and PXFE is an empirical, lumped factor [1/h] estimated from the experimental data (theoretical components of PXFE are listed below).

$$PXFE = K_{eff} * k_i / k_t * MEW/MCW$$

where: K_{eff} is a peroxidative yield of ethane [1/h]; k_i is the overall rate constant of lipid peroxidation initiation/propagation by the pro-oxidant chemical [1/h*μM]; k_t is the overall rate constant of its termination and quenching by the biosystem [1/h*μM]; and MEW and MCW are molecular weights of ethane and the pro-oxidant chemical, respectively.

The concentration of a pro-oxidant chemical in the liver (C_L) was either estimated by a biexponential equation for a classic two-compartment open pharmacokinetic model (Yang and Andersen, 1994), or determined by separate PBPK models for TCE (Fisher et al., 1991; Das et al., 1994) and CCl_4 (Gargas et al., 1986; Paustenbach et al., 1986a; 1986b). The biexponential equation was of the form:

$$C_L = C_{L0} * k_{1,0} * (e^{-\uparrow * t} - e^{-\rightarrow * t}) / (\rightarrow * - \uparrow)$$

$$C_{L0} = DOSE/V_{LC}$$

where: C_{L0} is an estimate of initial concentration of pro-oxidant chemical [mg/kg]; $k_{1,0}$ is a pharmacokinetic transfer micro-constant [1/h]; α and β are pharmacokinetic macro-constants; DOSE is a dose of pro-oxidant chemical [mg/kg]; t is time [h]; V_{LC} is a fraction of liver tissue [kg/kg].

The rate of ethane utilization by hepatic metabolism (REAM [mg/h]) was described by the Michaelis-Menten-type equation with an additional code for metabolism suppression and a linear term for first order metabolism:

$$REAM = V_{MEX}*CEV_L/(K_{EM}+CEV_L*(1+CEV_L/KS)) + KEF*CEV_L*V_L$$

where: V_{MEX} is a pseudo maximum velocity of ethane metabolism [mg/h]; CEV_L is an ethane concentration in venous blood leaving liver [mg/L]; K_{EM} is an apparent Michaelis constant for ethane metabolism [mg/L]; KS is a suppression constant for metabolism (KS=1.0 turns metabolism off, while KS set to a large number, e.g., 100,000, turns metabolism on); KEF is a first order metabolism rate constant [1/h]; V_L is a volume of liver [kg].

Compartment Description and Governing Equations

The basic assumption in constructing this PBPD model was that blood flow to the tissue was limiting the ethane delivery. Because ethane is retained by the tissue according to its tissue/blood partition coefficient (PE_i) which may be measured *in vitro*, the concentration of ethane in venous blood leaving the non-producing tissue (CEV_i) during the equilibration phase is lower than the concentration in arterial blood (CEA). Therefore, the rate of change of ethane amount in tissue (dAE_i/dt) is given by a simple difference between concentration in blood entering and exiting the tissue ($CEA-CEV_i$) multiplied by the blood flow (Q_i). Accordingly, for each well-stirred compartment "*i*" without metabolism or other losses or gains (fat tissue, gut, slowly perfused and rapidly perfused tissues), the rate of change in the amount "A" [mg] of ethane (dA_i over time [h]) was defined as follows:

$$dAE_i/dt = Q_i*(CEA - CEV_i)$$

where: subscript *i* represents *i*-th compartment; E represents ethane; Q_i represents the blood flow through the *i*-th compartment [L/h]; CEA represents the arterial concentration of ethane [mg/L]; CEV_i represents the venous concentration of ethane [mg/L] leaving the *i*-th compartment ($CEV_i = CE_i/P_i$; where CE_i is a concentration of ethane in the tissue in *i*-th compartment [mg/kg] and P_i is the tissue/blood partition coefficient of ethane for *i*-th compartment; $CE_i = AE_i/V_i$, where V_i represents the volume of the *i*-th compartment [kg]).

Integrating this equation over a given time, one can calculate the amount of ethane present in tissue (AE_i) and, therefore, if the actual volume of tissue (V_i) is known, one can calculate the concentration of ethane in the tissue (CE_i) at any time. Using these simple principles, the model for ethane (schematically shown in Figure 6.1-1) was built by linking these mass transfer equations for each compartment, with the exception of the liver and the lung, which are defined below.

For the liver compartment, a loss term (REAM [mg/h]) was added to the well-stirred compartment description to account for metabolism and an increment term REOX [mg/h] was added to account for ethane production (REAM and REOX are the rates of mass utilization by hepatic metabolism and mass production by lipid peroxidation, respectively). As explained above, rates of mass utilization and mass production followed the Michaelis-Menten kinetic equation and the square root equation, respectively:

$$dAE_L/dt = Q_L*(CEA - CEV_L) - REAM + REOX$$

Because ethane is volatile, for the lung compartment with two theoretically possible mass inputs (mixed venous blood and inhaled air), and two theoretically possible outputs (arterial blood and exhaled air), the amount in alveolar air is in equilibrium with the amount in lung blood at steady state. Therefore:

$$QP^*(CEI - CEX) = QC^*(CEA - CEV)$$
$$CEX = CEA/PEB$$

where: QP is the air flow through the lungs [L/h] (alveolar ventilation rate); CEI is the concentration in inhaled air [mg/L]; CEX is the concentration in alveolar air [mg/L]; CEA is the arterial concentration [mg/L] (leaving the lungs); PEB is blood/air partition coefficient [ratio]; QC is the blood flow through the lungs [L/h] (rate of cardiac output); CEV is the venous concentration [mg/L] (entering the lungs). This equation is solved for CEA.

Numerical Values of PBPD Model Constants Used in Simulations

Physiological parameters used in PBPD model for simulations were adapted from the literature (International Life Sciences Institute IRSI, 1994; and Arms and Travis, 1988). These parameters included alveolar ventilation (QPC [L/h/kg]), cardiac output (QCC [L/h/kg]), tissue blood flows (Q_i [fraction of QCC]), tissue volumes (V_iC [fraction of body weight]) and are listed in Table 6.1-1.

TABLE 6.1-1. PHYSIOLOGICAL PARAMETERS USED FOR PBPD MODEL SIMULATIONS

Parameter	Description	Value	[Unit]
BW	Body weight	Measured	[kg]
QPC	Alveolar ventilation	30.0	[L/h/kg]
QCC	Cardiac output	16.5	[L/h/kg]
QGC	Blood flow to gut	0.175	[ratio]
QLC	Blood flow through hepatic artery	0.24 - QGC	[ratio]
QFC	Blood flow to fat	0.05	[ratio]
QSC	Blood flow to slowly perfused tissues	0.238	[ratio]
QRC	Blood flow to rapidly perfused tissues	0.472	[ratio]
VLC	Volume of liver	0.05	[ratio]
VFC	Volume of fat	0.1	[ratio]
VSC	Volume of slowly perfused tissues	0.558	[ratio]
VRC	Volume of rapidly perfused tissues	0.031	[ratio]
VGC	Volume of gut tissue	0.033	[ratio]

The tissue/air partition coefficients are listed in Table 6.1-2.

TABLE 6.1-2. PHYSICOCHEMICAL PARAMETERS USED FOR PBPD MODEL SIMULATIONS

Parameter	Description	Value	[Unit]
Partition coefficients for ethane*			
PEB	Measured blood/air	1.305	[ratio]
PLA	Measured liver/air	0.828	[ratio]
PFA	Measured fat/air	2.444	[ratio]
PSA	Measured slowly perfused/air	0.979	[ratio]
PRA	Measured rapidly perfused/air	0.996	[ratio]
PGA	Measured gut/air	0.996	[ratio]
	Molecular weight		
MEW	Molecular weight of ethane	30.0	[g/mol]

***The tissue/blood partition coefficients were calculated by dividing the tissue/air partition coefficient by blood/air partition coefficient.**

The metabolic parameters were estimated by fitting and optimizing the PBPD model-simulated curve to our experimental measurements of ethane uptake in a closed chamber, employing the method of Gargas et al. (1986). The final set of metabolic parameters is listed in Table 6.1-3. Pharmacokinetic micro- and macro-constants for TCE and CCl_4 were initially

TABLE 6.1-3. METABOLIC PARAMETERS USED FOR PBPD MODEL SIMULATIONS.

Parameter	Description	Value	[Unit]
KEFC	First order metabolic constant	2.786	[1/h/kg]
KEM	Apparent Michaelis constant	0.510	[mg/L]
VMEXC	Pseudo-V_{max} for ethane metabolism	0.286	[mg/h/kg]
PXFCE	Rate of lipid peroxidation for TCE	9.102E-10	[mg/h/kg]
PXFCE	Rate of lipid peroxidation for CCl_4	3.4132e-9	[mg/h/kg]

estimated from data published in the literature (D'Souza et al., 1985; Gargas et al., 1986; Paustenbach et al., 1986a; 1986b; Gallo et al., 1993; Sanzgiri et al., 1995) and then the estimates were improved by fitting to our experimental results from mice. The final set of pharmacokinetic constants for biexponential function are listed in Table 6.1-4.

TABLE 6.1-4. PHARMACOKINETIC PARAMETERS FOR BIEXPONENTIAL FUNCTION USED FOR PBPD MODEL SIMULATIONS

Parameter	Description	Value		[Unit]
		TCE	CCl_4	
$K_{1,0}$	Transfer micro-constant	0.03	0.15	[1/h/kg]
α	Macro-constant	0.01	1.50	[1/h/kg]
β	Macro-constant	2.00	1.60	[1/h/kg]

Experimental Calibration of PBPD Model

Before the experimental calibration with pro-oxidant chemicals was actually performed in mice, the PBPD model was run with the available literature data (Cojocel et al. 1989) to assess the possible range of concentration of the ethane exhaled due to oxidative stress. It was estimated that under our experimental conditions the concentration of ethane exhaled by mice should not exceed 1 ppm. Therefore, the gas uptake measurement was performed with this initial ethane concentration (1 ppm) in the closed chamber (Figure 6.1-2). Initially, the fitting of the curve to the ethane gas uptake data was performed by

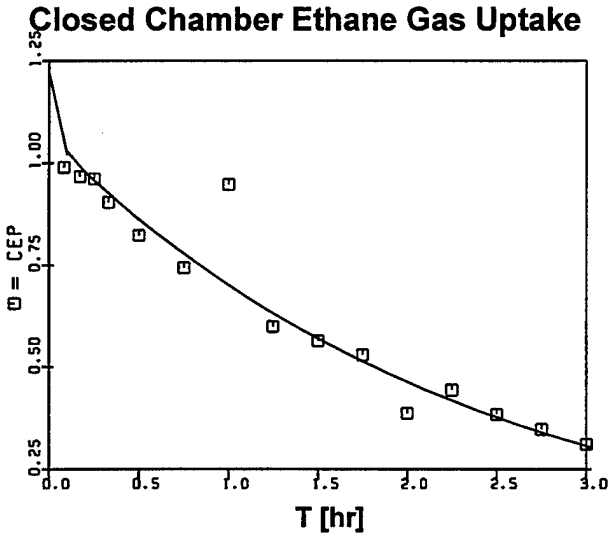

Figure 6.1-2. The results of PBPD model computer simulations of experimental data from our laborat ory from mice inhaling ethane in a closed chamber (initial concentration of ethane 1 ppm). Symbols depict experimental data collected from the closed chamber (four ani mals per time-point); continuous line is the PBPD model simulation). CEP - Ethane concentration [ppm]; T - time [h].

varying the first order metabolism rate constant (KEFC), with the second order metabolism turned off, and visually inspecting the residuals. Then, the Michaelis-Menten-type metabolism was turned on, and the curve was fitted to the ethane gas uptake data by varying the metabolism constants (V_{MEXC} and K_{EM}). Finally, the metabolism constants were optimized with SIMUSOLV. From comparison of the model simulation with the closed chamber ethane gas uptake, shown in Figure 6.1-2, it seems that the combination of the first order and the second order metabolism described the experimental results adequately.

The effect of CCl_4 on ethane exhalation in mice is shown in Figure 6.1-3. The shape of a local CCl_4 concentration in the liver over time predicted by the biexponential function is shown in an inset. At the time of model development, the actual measurements of CCl_4 concentration in the mouse liver were not available. Nevertheless, the experimental data for ethane exhalation evoked by CCl_4 were within the same order of magnitude as our PBPD model predictions.

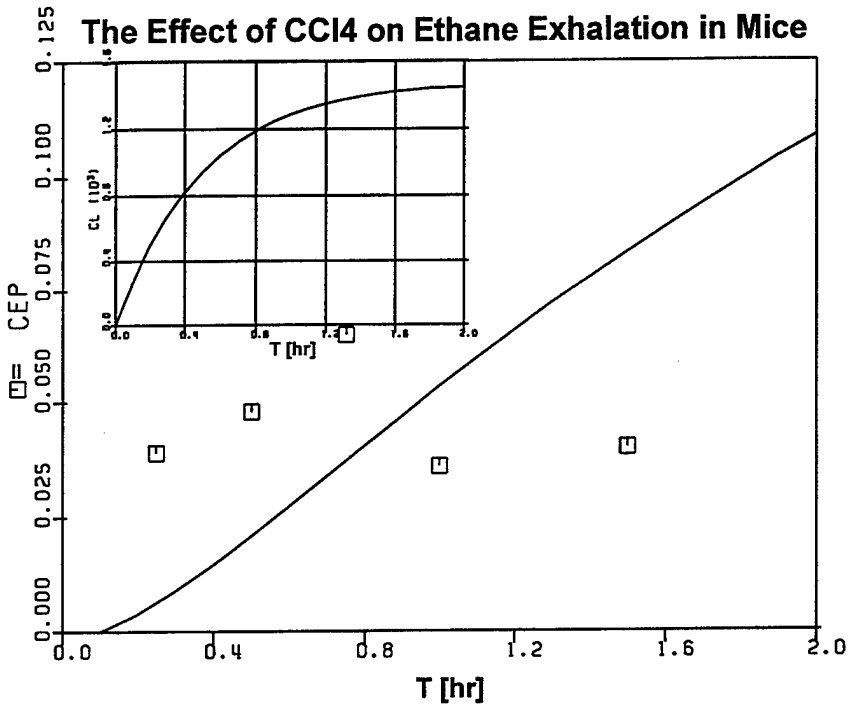

Figure 6.1-3. **The results of PBPD model computer simulations of experimental data from our labora tory from mice exhaling ethane in a closed chamber after treatment with carbon tetra chloride (CCl_4 1.5 g/kg i.p. in mineral oil). Symbols depict experimental data collected from the closed chamber (four animals per time-point); continuous line is the PBPD model simulation. The inset shows simulated concentration of CCl_4 in the liver (CL [mg/kg]). CEP - ethane concentration [ppm]; T - time [h].**

The effect of TCE on ethane exhalation in mice is shown in Figure 6.1- 4. The main plot represents the simulation of ethane exhalation linked with the local TCE concentration in the liver by biexponential function. The inset shows the simulation in

which the PBPD model was linked with a separate PBPK model, calibrated for mice, and estimating the TCE concentration in the liver.

DISCUSSION

Many xenobiotics may be activated by the cytochrome P450 system or other oxidoreductases and generate free radical intermediates directly. For example, tert-butyl hydroperoxide, bromotrichloromethane, CCl_4, and TCE may be split to their respective free radical fragments. Although, these free radicals are

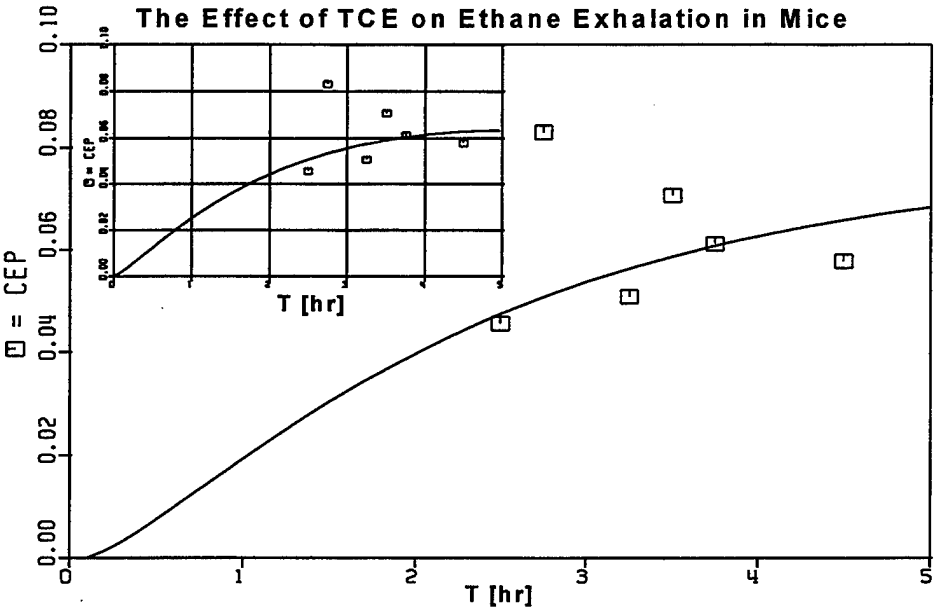

Figure 6.1-4. The results of PBPD model computer simulations of experimental data from our laborat ory from mice exhaling ethane in a closed chamber after the treatment with trichloro ethylene (TCE 2.5 g/kg i.p. in mineral oil). Symbols depict experimental data collected from the closed chamber (four animals per time-point); continuous line is the PBPD model simulation. The inset shows simulation of ethane exhalation by PBPD model interlinked with a PBPK model simulating the TCE disposition and calculating a local concentration in the liver. CEP - ethane concentration [ppm]; T - time [h].

responsible for initiation and propagation of lipid peroxidation, and the extent of the resultant lipid peroxidation can be linked with their local concentration, the quantitative models must also consider the antioxidant defenses (Tappel et al., 1989; Byczkowski et al., 1995). The antioxidants, such as vitamin E, glutathione, etc., are responsible for quenching free radicals and termination of the lipid peroxidation. Some animal species are less protected against free radical attack and lipid peroxidation compared to others. For instance, superoxide dismutase activity, an antioxidant enzyme, is significantly lower in mouse liver than in rat liver (Sohal et al., 1989). Apparently, the same is true for tocopherols and other lipid-soluble

antioxidants whose concentrations are the lowest in mouse liver, higher in rat liver, and the highest in human liver. Therefore, it seems that susceptibility of the mouse to free radical liver damage may be higher than that of the rat (Cutler, 1991) and humans. Thus, the overall process of lipid peroxidation initiated by pro-oxidant chemical depends on the equilibrium between the rates of free radical formation and free radical quenching and termination. In the present PBPD model, this equilibrium has been included in the empirical, lumped factor PXFE [1/h] which must be estimated for different animal species from the experimental data with the respective pro-oxidant chemical (Table 6.1-3). Theoretically, it should be possible to infer the value of PXFE from the *in vitro* experimental measurements of a peroxidative yield of ethane (K_{eff}), the overall rate constant of lipid peroxidation initiation/propagation by the pro-oxidant chemical (k_i), and the overall rate constant of its termination and quenching by the biosystem (k_t). A protocol with precision cut liver slices currently is being developed by our group to address this experimental challenge.

Another model parameter that is animal species-dependent and determined *in vitro* is the partition coefficient of tissues. As the solubility of ethane in tissues was low (the tissue/air partition coefficients within the range of 0.8 to 2.4; Table 6.1-2), even a relatively high variability of the experimental determinations had little effect on the model output (Seckel and Byczkowski, 1996, unpublished data).

As was reported in the available literature (Smith, 1991), the rate of ethane catabolism was low (Table 6.1-3). Accordingly, within about 2 hours under the experimental conditions employed, the concentration of ethane in the closed chamber dropped only by half (Figure 6.1-2). Using the parameters measured experimentally *in vitro* and *in vivo*, the proposed PBPD model described adequately the kinetic behavior of ethane in mice.

Using this calibrated model, the proposed predictions were compared with experimental measurements of ethane exhalation in mice treated with a bolus dose of CCl_4 and TCE (Figure 6.1-3 and 6.1-4). At this preliminary stage of this project, it is difficult to assess how close the PBPD model predicted the experimental data. More experiments are necessary with different doses of CCl_4 and TCE and employing enough animals to allow for a meaningful statistical analysis. Since concentrations of these chemicals in the liver were not measured directly in this project, pharmacokinetic models were used to estimate their local levels. Two possible approaches were explored. One links the ethane exhalation PBPD sub-model with a classic two-compartment open model for CCl_4 or TCE in liver (Figure 6.1-3 and 6.1-4, main plots) and the other was a PBPK model (Figure 6.1-3 and 6.1-4, insets). From comparison of the results of simulation (Figure 6.1-4) it seems that both approaches gave similar fit to the experimental data. In the case when not enough information about the disposition of a chemical is available to construct and validate a separate PBPK model, the multiexponential equation may be used to estimate local concentration in the liver according to the classic multicompartmental pharmacokinetic model. On the other hand, the PBPD model may be interlinked, as a compatible module, with the existing PBPK models for pro-oxidant chemicals.

Although further experiments are necessary to calibrate systematically and validate both delivery and the pro-oxidant effect of the studied chemicals, it seems that this modeling approach is adequate for a quantitative description of ethane exhalation as a valid end point for chemically induced oxidative stress. The described PBPD model was used to simulate the kinetics of ethane exhalation in mice treated with massive doses of either CCl_4 or TCE. It is suggested that the PBPD model, when

systematically calibrated with experimental data for several pro-oxidant chemicals, may be useful for future pharmacodynamic descriptions of oxidative stress and dose-response characterization of these pro-oxidant chemicals.

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6.2 MATHEMATICAL MODELING OF OXIDATIVE STRESS *IN VITRO*

J. Z. Byczkowski and C.D. Flemming

ABSTRACT

A biologically based pharmacodynamic (BBPD) model based on a description by Vroegop et al. (1995) was developed to simulate the dose-dependent biological effects of chemically induced oxidative stress in tissue preparation *in vitro*. The model was written in Advanced Continuous Simulation Language (ACSL) with a FORTRAN sub-routine, and simulations were performed using SIMUSOLV software on a VAX/VMS mainframe computer. The BBPD model simulated formation of free radicals over time as a function of the pro-oxidant chemical concentration, and predicted the dose-dependent response of cellular targets at each time. The BBPD model was calibrated with the literature data for free radical generation in liver slices, and for dose-dependent effects of oxidative stress in cultured neuronal N 18 hybridoma and rat hepatoma cells. The model allowed us to distinguish between the selective "one-hit" targeted mode of action and the "multi-hit" stochastic interaction with multiple nonselective cellular targets. It is suggested that the algorithm developed and calibrated with experimental data *in vitro* may be employed for future dose-response characterization of the action of pro-oxidant chemicals *in vivo* using the physiologically based pharmacokinetic/dynamic models.

INTRODUCTION

Oxidative stress which may be caused by free radicals generated from pro-oxidant chemicals, can be detrimental to biological systems (reviewed by Byczkowski and Gessner, 1988). Unlike most of the stable drugs which selectively interact with more-or-less specific receptors within the biosystem, the free radicals can interact with almost any cellular constituent. Free radicals can irreversibly block many vital cellular targets, and at the molecular level, they can initiate destructive chain reactions (e.g., lipid peroxidation). Figure 6.2-1 shows schematically, as an example, thirteen main cellular targets for free radicals involved in the three most vital functions of the cell: energy production, substrate transport, and signal transduction. Although it is well known that any pro-oxidant chemical may be deleterious to the biosystem, it is often difficult to find its mechanism of action. Thus, depending on the concentration *in vitro*, or its internal dose *in vivo*, the same pro-oxidant chemical can cause cell growth stimulation, apoptosis, or necrosis (Corcoran et al., 1994; Byczkowski and Channel, 1995). Obviously, the risk characterization and/or risk assessment, based on a high internal dose causing necrosis, would be irrelevant to a low internal dose which stimulates cell growth and has a higher probability of occurring in a real-life scenario. This points out the importance of an appropriate dose-dependent modeling of the biological effects of pro-oxidant chemicals in the risk characterization.

Another problem with risk characterization of pro-oxidant chemicals is a species-dependent difference in antioxidant tissue levels (Sohal et al., 1989; Cutler, 1991). Since the concentration of natural antioxidants, e.g., in mouse liver, is about an order of magnitude lower than the concentration in an average, normally fed human, it appears that humans

are much better protected against oxidative stress and lipid peroxidation triggered by free radicals than mice (Cutler, 1991). Therefore, risk characterization based on hepatotoxicity or hepatocarcinogenicity mouse bioassays may be irrelevant to human exposure if free radical quenching by the biosystems is ignored.

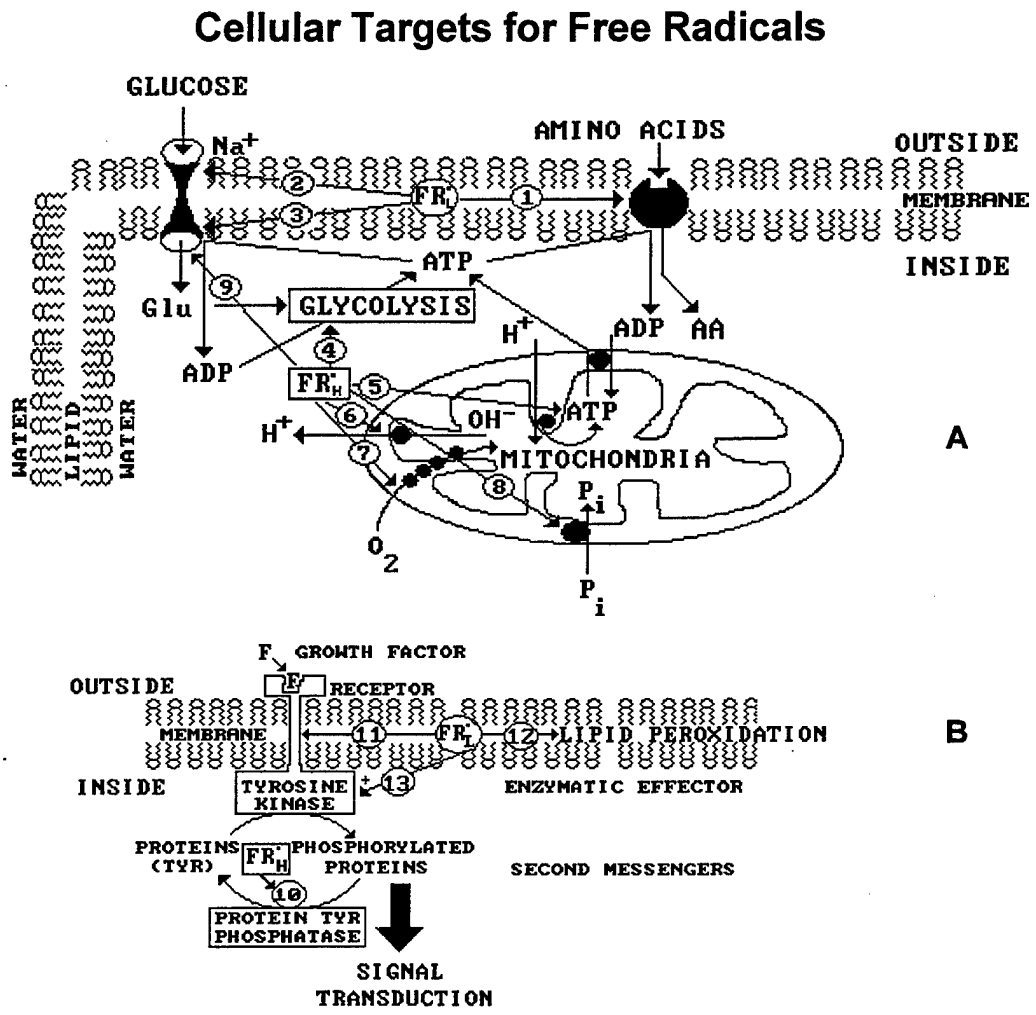

Figure 6.2-1. **Scheme of main cellular targets for lipophilic (FRL) and hydrophilic (FRH) free radicals. A. Energy production and substrate transport; B. Signal transduction. Effects: 1 - interaction with lipophilic domain of amino acid transporter; 2 - inter action with the glucose transporter domain located at the outer face of cellular membrane; 3 - interaction with the glucose transporter domain located at the inner face of cellular membrane; 4 - interaction with glycolytic enzymes; 5 - interaction with vectorial ATPase; 6 -interaction with mitochondrial inner membrane perme ability; 7 - interaction with mitochondrial respiratory chain; 8 - interaction with mitochondrial membrane transporters; 9 - interaction with hydrophilic domain of glucose transporter; 10 - interaction with protein tyrosine phosphatase; 11 -inter- action with lipophilic domain of growth factor receptors; 12 - initiation of lipid peroxidation; 13 - interaction with activators of protein tyrosine kinase.**

A widespread usage of pro-oxidant chemicals in the Department of Defense operations, and a scientific interest in the causative role of oxidative stress in toxicity were important considerations in exploring the feasibility of developing a quantitative, dose-dependent mode of this process in tissue preparations *in vitro*. A biologically based pharmacodynamic (BBPD) model has been developed using data available in the literature (Byczkowski and Flemming, 1996). This model, once adequately validated, should aid the risk characterization by addressing quantitatively the above mentioned problems. The other existing mathematical models of biologically relevant free radical reactions (Suzuki and Ford, 1994) and lipid peroxidation (Tappel et al., 1989; Babbs and Steiner, 1990; McKenna et al., 1991) were unsuitable for interlinking with physiologically based pharmacokinetic (PBPK) models which are used to simulate tissue distribution of toxicants (Yang and Andersen, 1994). This newly developed BBPD model was compatible with PBPK models, simulated the formation of free radicals from pro-oxidant chemicals, and predicted the dose-dependent inhibition of cellular targets caused by free radicals.

MATERIALS AND METHODS

The BBPD model was written in Advanced Continuous Simulation Language (ACSL; Mitchell and Gauthier Associates, Inc. 1993) with a sub-routine written in FORTRAN. The simulations were performed using SIMUSOLV software with optimization capabilities (DOW Chemical Co., Midland, MI) on a VAX/VMS mainframe computer. Parameters were optimized by SIMUSOLV which uses the log likelihood function as the criterion. Either the generalized reduced gradient method for single parameter optimization or the Nelder-Mead search method for multiple parameters optimization was used to adjust the values (Steiner et al., 1990).

RESULTS AND DISCUSSION

BBPD Model Structure

A general simplified scheme of the BBPD model is shown in Figure 6.2-2. The first part of the model describes a metabolic reaction. A molecule of xenobiotic is split to produce two free radicals that further recombine or interact with the biosystem to yield nonradical products. There were two basic model assumptions: first, the current concentration of xenobiotic (PCONC [μM]) is not changing significantly from the initial concentration (C_0 [μM]) at each time of preincubation with free radicals; second, free radicals (FR [μM]) are produced at a constant rate:

$$\begin{array}{ccc} & k_i\rightarrow & k_t\rightarrow \\ & \downarrow & \downarrow \\ \text{XENOBIOTIC} & \text{----> FR} & + \text{ FR ----> Nonradical products} \end{array}$$

$$\text{assumptions: XENOBIOTIC concentration PCONC} \approx C_0$$

$$\text{and } dFR/dt = k_i * C_0 - k_t * FR * FR = 0$$

where: k_i is a rate constant of free radical formation from the pro-oxidant xenobiotic [1/μM/h]; k_t is the lumped rate constant of free radical recombination and quenching by the biosystem [1/μM/h].

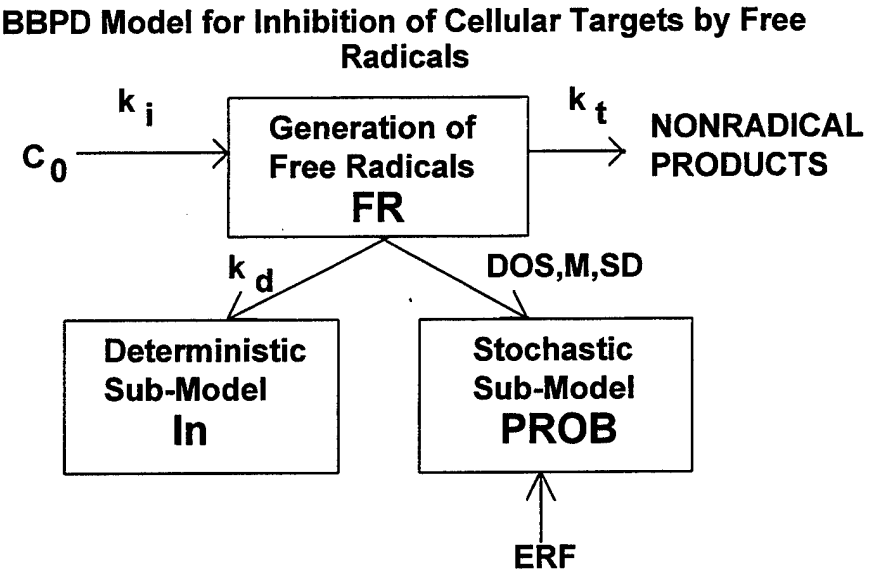

Figure 6.2-2. **A schematic diagram of the biologically based pharmacodynamic model (PBPD) describing the inhibition of cellular targets by chemically generated free radicals. Symbols: C_0 - initial pro-oxidant xenobiotic concentration; k_i - rate constant of free radical formation from the pro-oxidant xenobiotic [1/μM/h]; FR - concentration of free radicals [μM]; k_t - lumped rate constant of free radical recombination and quenching by the biosystem [1/μM/h]; k_d - rate constant of cellular target inactiva tion by free radicals [100%/μM/h]; In - fraction of remaining active cellular targets, relative to the amount before inhibition [ratio]; DOS - array of free radical concen tration values [μM]; M - mean of the cumulative Gaussian distribution of free radical concentration values [μM]; SD - standard deviation of the cumulative Gaussian distribution of free radical concentration values [μM]; PROB - probabil ity of cellular targets to remain active, relative to the amount before inhibition [ratio]; ERF - error function.**

Under these steady state conditions, free radical concentration (FR [μM]) is proportional to the square root of the initial concentration of the pro-oxidant xenobiotic (C_0 [μM]):

$$FR = \sqrt{k_i * C_0 / k_t}$$

From each split pair of electrons, eventually two unpaired electrons with opposite spins must be formed, which then produce two free radicals. The free radicals further can interact with each other, with cellular macromolecules, antioxi-dants, and/or cellular targets. Thus, the above equation was used to estimate concentrations of free radicals at each time of preincubation, and to generate an array of free radical concentrations (DOS [μM]) over all initial concentrations of xenobiotic simulated (ND [integer] = number of simulated concentrations).

The second and third parts of the model described the interactions of free radicals with cellular targets. Two different modes of action of free radicals on cellular targets were considered: 1) one-hit, targeted, or selective interaction of free radicals with a homogenous population of cellular targets, and 2) multi-hit stochastic interaction with heterogenous nonselective cellular targets.

Mode of Action 1

Assuming that all cellular targets react with all radicals with the same rate constant (k_d [1/μM/h]) and that the target inactivation is directly proportional to the number of free radicals, and assuming the same time of preincubation at different concentrations of free radicals (t_p [h]), the probability (In) that 100% of cellular targets initially present at time = 0 will remain active, decays according to this simple exponential equation:

$$k_d$$

$$\text{FR} + \text{CELLULAR TARGET} \text{----->} \text{INACTIVE TARGET}$$

$$\text{assumptions: uniform sensitivity of CELLULAR TARGETS to FR}$$

$$In = I_0 * \exp(-k_d * FR * t_p)$$

where: I_0 is the initial concentration of active cellular targets, assumed to be 100% (I_0 = 1.[%/100]); k_d is the rate constant of cellular target inactivation by free radicals [100%/μM/h]; In is a probability, measured as a fraction of remaining active cellular targets, relative to the amount before inhibition [ratio]; t_p is time of preincubation with free radicals [h]; FR is the concentration of free radicals [μM].

Mode of Action 2

The heterogenous nonselective cellular targets, with different rate constants and different sensitivities, give a range of inhibitory effects in response to free radicals and, eventually, the probabilities of inactivation of the overall process at a given time of preincubation should have normal Gaussian distributions at each free radical dose level. Therefore, activity of cellular targets surviving this stochastic process resulting from "indiscriminate shooting" at differently sensitive targets, may be described as 1 minus cumulative Gaussian distribution, as if the probability of a target being hit or not depends on pure chance.

$$\text{FR} + \text{MULTIPLE TARGETS} \text{----->} \text{RANGE OF INHIBITORY RESPONSES}$$

$$\text{assumption: normal distribution of the INHIBITORY RESPONSES}$$

$$PROB = 1 - \int_{-\infty}^{x} 1/\sqrt{2\pi} * \exp(-z^2/2) * dz$$

where:

$$x = (DOS - M)/SD;$$

PROB is probability of cellular targets to remain active, relative to the amount before inhibition [ratio]; DOS is array of free radical concentration values [μM]; M is mean of the cumulative Gaussian distribution of free radical concentration values [μM]; SD is standard deviation of the cumulative Gaussian distribution of free radical concentration values [μM]; z is a variable of integration. Of course, values of the free radical concentration in the array DOS [μM] used in this error function were proportional to the square root of the xenobiotic concentration (C_0 [μM]), as described above.

Numerical Values of BBPD Model Parameters Used in Simulations

The suitable experimental data-set by Vroegop et al. (1995) in cultured neuronal N 18 hybridoma cells was adopted in the model and used for the calibrations. The assumption of a steady-state level of free radicals in tissue preparations *in vitro* (McKenna et al., 1991) was confirmed with experimental data using free radical generation from trichloroethylene (TCE) in liver slices (Steel-Goodwin et al., 1995). Parameters used in a BBPD model for simulation of the inhibition of cellular targets by chemically generated free radicals are listed in Table 6.2-1. The initial parameter estimates were taken from the literature (Vroegop et al., 1995; and Steel-Goodwin et al., 1995), then the model was fitted to their respective experimental data using the optimization with SIMUSOLV software.

TABLE 6.2-1. PARAMETERS FOR THE BIOLOGICALLY BASED PHARMACODYNAMIC MODEL DESCRIBING THE INHIBITION OF CELLULAR TARGETS BY CHEMICALLY GENERATED FREE RADICALS.

Symbol	Numerical value	Description [Units]	Data source
Rate constants of FR formation			
ki	18.0 from H_2O_2	[1/μM/h]	fitted to Vroegop et al. (1995)
ki	900.0 from TCE	[1/μM/h]	fitted to Steel-Goodwin et al. (1995)
Rate constant of FR quenching			
kt	200.0	[1/μM/h]	fixed, est. from Vroegop et al. (1995)
Rate constants of cellular target inactivation			
kd	0.075 AA transport	[100%/μM/h]	fitted to Vroegop et al. (1995)
kd	0.05 mitochondria	[100%/μM/h]	fitted to Vroegop et al. (1995)
kd	0.1GLU transport	[100%/μM/h]	fitted to Vroegop et al. (1995)
kd	1.0 PTPase	[100%/μM/h]	fitted to Hecht and Zick (1992)
Initial concentration of cellular targets			
I0	1.0	[%/100]	assumed
m			
Time of preincubation with FR			
tp	1.0	[hr]	experimental from Vroegop et al. (1995)
tp	0.5	[hr]	experimental from Hecht and Zick (1992)
tp	0.333	[hr]	experimental from Steel-Goodwin et al. (1995)
Length of experiment			
TSTOP	2.0	[hr]	experimental from Vroegop et al. (1995)
TSTOP	0.75	[hr]	experimental from Hecht and Zick (1992)
TSTOP	0.333	[hr]	experimental from Steel-Goodwin et al. (1995)

Abbreviations: TCE - trichloroethylene; FR - free radicals; AA - amino acids; GLU - glucose; PTPase -protein tyrosine phosphatase.

BBPD Model Simulations

After calibration of the algorithm for concentrations of free radicals at each time of preincubation with data from Steel-Goodwin et al. (1995), it was possible to verify the dose-dependence of free radical interactions with cellular targets using the second, deterministic sub-model. Assumption that all targets react with all radicals with the same rate constant (k_d), and the algorithm predicting exponential decay of the probabilities that 100% of cellular targets initially present at the time = 0 will remain active, were tested against the data of Vroegop et al. (1995). Figure 6.2-3 A shows effects of different concentrations of hydrogen peroxide (H_2O_2) on the glucose transporter (GLU) in the cells in culture (Vroegop et al., 1995).

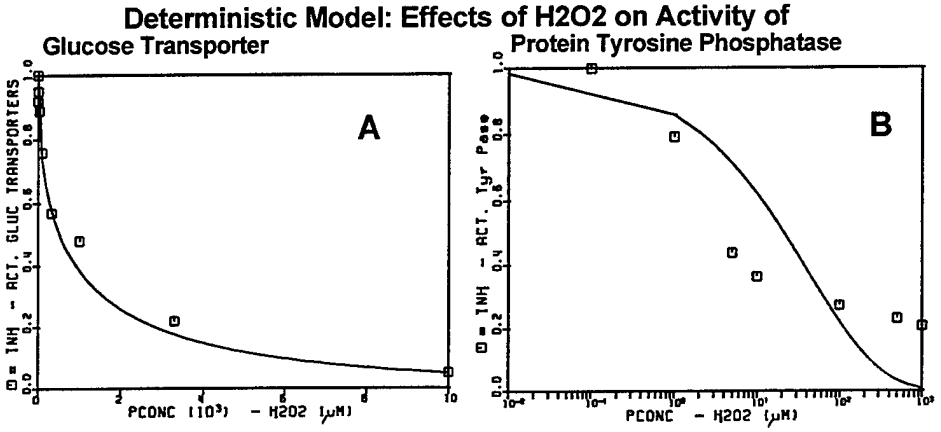

Figure 6.2-3. Calibration of the deterministic BBPD sub-model (A) and simulation of biological effects (B) of different concentrations of hydrogen peroxide (PCONC [µM H_2O_2]). A. Inhibition of the activity of glucose transporter (GLU) in N 18 neuronal hybri doma cell line in culture (INH [ratio]), after 1 hour (*plus* t_p= 1 h preincubation with H_2O_2), data from Vroegop et al. (1995). B. Inhibition of the activity of protein tyrosine phosphatase (PTPase) in rat hepatoma cells in culture (INH [ratio]), after 15 min (*plus* t_p= 0.5 h preincubation with H_2O_2), data from Hecht and Zick (1992). The continuous line is a computer-generated simulation, whereas the small squares represent actual experimental data points.

For these simulations, the values of rate constant k_i (for free radical formation from the pro-oxidant xenobiotics) were estimated by fitting the deterministic sub-model predictions to the respective data. Constant k_t (for free radical recombination and quenching by the biosystem) was fixed at the overall average value for primary, secondary, and tertiary free-radical interactions with molecules in this biosystem (Babbs and Steiner, 1990; Vroegop et al., 1995). The values of rate constant k_d (for cellular target inactivation by free radicals) were optimized with the deterministic sub-model, assuming that the same cellular targets have the same sensitivity towards free radicals initiated by different xenobiotics. The numerical values of rate constants are listed in Table 6.2-1. The same rate constants for free radical formation or quenching were used to simulate the dose-dependent experiments published by Hecht and Zick (1992). The inhibition of protein tyrosine phosphatase activity in rat hepatoma cells in culture by H_2O_2, followed a familiar exponential decay function (Figure 6.2-3 B).

Using these rate constants, estimated with the deterministic sub-model, it was possible to verify the third, stochastic sub-model. Figure 6.2-4 A shows effects of different concentrations of H_2O_2 on the activity of amino acid transporter (AA) in the N 18 neuronal hybridoma cell line in culture (Vroegop et al., 1995). The small circles are the computer-generated simulations, whereas the small squares represent actual experimental data points. For comparison, the inset shows predictions of the second, deterministic sub-model (line) *versus* the same data points (depicted by squares). Figure 6.2-4 B shows effects of different concentrations of H_2O_2 on mitochondrial reduction of MTT (Mito) in the same cells in culture (after 1 h, *plus* 1 h preincubation with H_2O_2; Vroegop et al., 1995). The small circles are the computer-generated simulations, whereas the small squares represent actual experimental data points. The inset shows predictions of the second, deterministic sub-model (line) *versus* the same data points (depicted by squares).

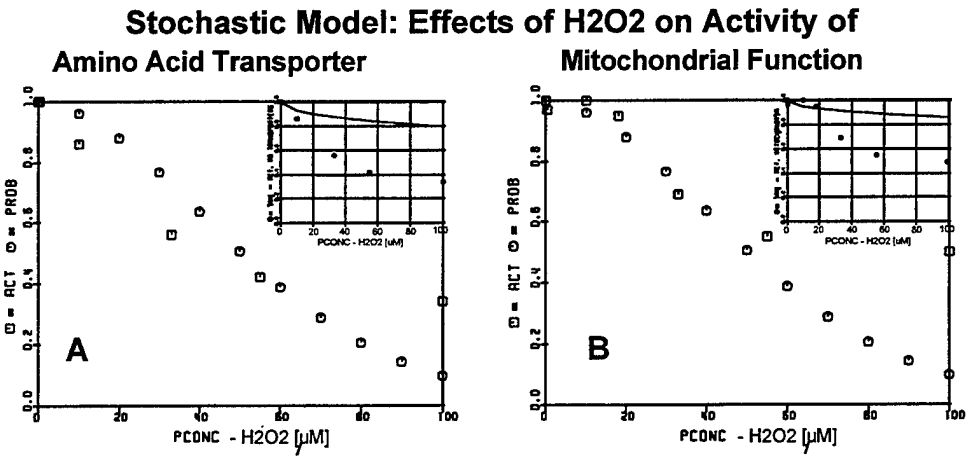

Figure 6.2-4. Simulations with the stochastic BBPD sub-model of the biological effects of different concentrations of hydrogen peroxide (PCONC [µM H_2O_2]) on (A) activity of amino acid transporter (AA) and (B) on mitochondrial reduction of MTT (Mito) in N 18 neuronal hybridoma cell line in culture (PROB [ratio]), after 1 hour (*plus* 1 hr preincubation with H_2O_2). The small circles are computer-generated simulations (PROB), whereas the small squares (ACT [ratio]) represent actual experimental data points from Vroegop et al. (1995). For comparison, the insets show predictions of the deterministic BBPD sub-model (INH [ratio], continuous line) *versus* the same data points.

To avoid modeling of hundreds of possible free radical reactions with their bimolecular rate constants (Babbs and Steiner, 1990), the rate constant in denominator (k_t) was fixed at the "average" lumped value for the biosystem employed, and the rate constant in numerator (k_i) was fitted to the experimental data obtained with a specific xenobiotic (Table 6.2-1). Therefore, the numerical values for k_i reported in Table 6.2-1 have only a relative meaning and correspond to the ratio of free radical formation *versus* free radical quenching. This practical approach, although not very exact from a theoretical point of view, was advantageous and probably the only realistic possibility at the present stage of knowledge of molecular events in cellular oxidative stress. Since the measured bimolecular rate constant values reported in the literature for the same free radical reaction varied several fold (sometimes even several orders of magnitude; Babbs and Steiner, 1990), they were not used for modeling purposes. Therefore, given the difficulties in determination of k_i, the lumped rate constants k_t should be measured experimentally for a realistic description of the

amount of free radicals available in different tissues. Clearly, more experimental data are needed for adequate description of antioxidant and free radical quenching capacity of different tissues.

Once the concentration of free radicals was adequately estimated at each time of preincubation over a wide range of xenobiotic concentrations, it became possible to calibrate the deterministic sub-model for interaction of free radicals with cellular targets (Figure 6.2-3). Assuming that all radicals react with all targets with the same rate constant (k_d), that one free radical can knock down only one target, and assuming the same time of preincubation with different concentrations of free radicals (t_p), the probability that 100% of cellular targets initially present at the time = 0 remain active (In) decayed according to the simple exponential equation (Figures 6.2-3 A and 6.2-3B). These assumptions, and therefore the exponential equation, held when either lipophilic free radicals (e.g., generated by cumyl hydroperoxide) interacted in the membrane lipid phase with the phospholipid-immersed amino acid transporter (Figure 6.2-1, effect 1; Byczkowski and Flemming, 1996), or when hydrophilic free radicals (e.g., generated by hydrogen peroxide) interacted with hydrophilic domain of glucose transporter (Figure 1, effect 9; Figure 6.2-3 A). When both the target and radical stayed in the same phase, it was possible to describe adequately the inactivation of each type of cellular target with different free radicals using the same rate constant (k_d; Table 6.2-1). The targets for these homogenous interactions are shown schematically in Figure 6.2-1 (effects 1, 9, 10 and 11). The deterministic sub-model failed when confronted with quantitative data on hydrophilic free radicals (e.g., generated by hydrogen peroxide) affecting the phospholipid-immersed amino acid transporter (Byczkowski and Flemming, 1996). In this case, the hydrophilic free radicals affected the amino acid transporter indirectly by interfering with energy production (ATP) by glycolysis (Figure 1, effect 4) and mitochondria (effects 5,6,7,8), as well as interfering with the substrate supply to the cell (effect 9). These types of multiple points of action or heterogenous targets have different sensitivities toward free radicals (Figure 6.2-1).

The heterogenous targets gave a range of inhibitory effects in response to free radicals and, eventually, the probabilities of inactivation of the overall process after a given time of preincubation had normal distributions at each free radical dose level. Therefore, activity of targets surviving this stochastic process resulting from "indiscriminate shooting" at differently sensitive targets, was adequately described by a stochastic sub-model (PROB; Figure 6.2-4), as if the probability of a target being hit or not depended on pure chance. On the other hand, this stochastic sub-model failed to describe the "one-hit" interaction; for instance, of lipophilic free radicals generated by cumene hydroperoxide with amino acid transporter (results not shown here). This process was adequately described by the deterministic sub-model (Byczkowski and Flemming, 1996).

The stochastic sub-model was successfully tested *versus* experimental data from Vroegop et al. (1995) for inhibition of amino acid transporter and mitochondrial function by free radicals generated by hydrogen peroxide (Figure 6.2-4). However, in these cases, the worse fit was obtained at very high concentrations as if the number of "miraculous survivors" at an extreme condition was higher than the normal distribution predicted. Another explanation for this discrepancy is that among the experimentally measured biological functions, some percentage of activity (background) did not depend on free radical-inhibitable targets. It seems that in the case of heterogenous, multiple cellular targets interacting with free radicals, the stochastic modeling may give good predictions in low- and mid-range concentrations, the most important range for a risk characterization.

It is postulated that using the interlinked deterministic and stochastic sub-mathematical models with the aid of computer simulation one may distinguish and differentiate a so-called "nonselective" multiple-hit cellular process from a targeted selective "one-hit" mechanism of interaction between free radicals and cellular targets. Thus, it seems feasible that different discontinuous cellular events, such as cell growth stimulation, apoptosis, or necrosis may be described by such an interlinked BBPD model. Combining the BBPD modeling approach of the type presented here with PBPK models may provide insight concerning the dose-response characteristics and may be very useful for mechanistically based risk characterization.

CONCLUSIONS

Even at the present state of incomplete knowledge of cellular and molecular mechanisms involved in oxidative stress, the use of stochastic computer modeling can provide insight concerning nonselective interaction with multiple cellular targets. At the present stage of knowledge, a model could contain only enough detail to capture the essence of the process. As proven by the results presented in this report, this simplified approach provided a useful tool to distinguish between the one-hit targeted mode of action of free radicals from multi-hit heterogenous stochastic interactions.

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SECTION 7

TRIMETHYLOPROPANE PHOSPHATE AND 2,6-DI-tert-BUTYL-4-NITROPHENOL TOXICITY PROJECTS

7.1 INVESTIGATION OF TRIMETHYLOLPROPANE PHOSPHATE INDUCED CENTRAL NERVOUS SYSTEM SENSITIZATION OF THE MESOCORTICO-LIMBIC PATHWAY

J. Lin, J. Cassel[1], J. Rossi III[1]

ABSTRACT

Brain electrical activities were recorded from the nucleus accumbens and ventral tegmental area of freely moving rats. Trimethylolpropane phosphate (TMPP) (0.275 mg/kg), pentylenetetrazol (PTZ) (20 mg/kg), and N-methyl-β-carboline-3-carboxamide (FG7142) (7.5 mg/kg) induced both spontaneous behavioral jerking, seizure activities, and epileptic electrical activities in both the nucleus accumbens and ventral tegmental area 20 min following chemical administration. The TMPP, PTZ and FG 7142 treatments were three, seven, and eight weeks duration, respectively. Twenty-four hours after chemical administration, spontaneous behavioral and electroencephalograms (EEG) change were not observed. Electrical stimulation (0.1 Hz, 0.1 ms duration, 6-12 V) of the ventral tegmental area, evoked both behavioral jerking and nucleus accumbens EEG paroxysms (after discharge) in 100% of TMPP (0.275 mg/kg, after 16th dose), 80% of PTZ (20 mg/kg, after 21st dose) and 20% of FG7142 (7.5 mg/kg, 21st dose) treated rats. The animals treated with vehicle solution showed neither immediate nor prolonged responses to vehicle injection and electrical stimulation of the ventral tegmental area. The present study indicates that long-term treatment of rats with low doses of TMPP, PTZ, and FG7142 induces sensitization of the mesocortico-limbic pathway.

INTRODUCTION

Highly neurotoxic trimethylolpropane phosphate (TMPP), generated from the pyrolysis of phosphate ester-based lubricants, is a concern to the military. Completed and ongoing work at the Naval Medical Research Institute/ Toxicology Detachment indicates that TMPP induces EEG paroxysms, sub-clinical seizures, clinical seizures and lethality, as well as long-term central nervous system (CNS) sensitization/kindling effects (i.e., progressive increases in EEG paroxysms, audiogenic seizure susceptibility, and hyperreactivity to amphetamine challenge) in rats. It is hypothesized that TMPP-induced effects reflect chemical modulation of specific neural pathways. The mesocortico-limbic pathway (A_{10}), with dopamine neuron cell bodies located in the midline of the ventral tegmental area of Tsai innervating forebrain areas including nucleus accumbens, olfactory tubercle, interstitial nucleus of the stria terminalis, septum and nucleus of the diagonal band of Broca, has been shown to involve time-dependent sensitization to emotional stressors (Antelman et al., 1991), and the processes underlying the kindling of epileptogenic events and neural sensitization, in general (Sato et al., 1976). Preliminary data from Dr. Lindsey's work (Lindsey et al., 1995) have shown that perfusion of TMPP directly into the nucleus accumbens *via* the microdialysis probes resulted in an increase in forequarter-jerking, hyperlocomotor activity, and stereotyped behaviors such as activated sniffing and defensive burrowing in rats. The data provided a direct evidence of the involvement of brain A_{10} dopaminergic pathway in TMPP-induced behavioral change. Recent work of Narayanan et al. has also shown that TMPP binds to benzodiazepine-$GABA_A$ receptor complex (Narayanan et al., 1995). The purpose of the present experiments was to determine if and how the mesolim-

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bocortical pathway is involved in long-term TMPP treatment-induced behavior change and sensitization by using electrophysiology techniques in awake, freely moving rats. The present study was also designed to investigate the mechanism of TMPP-induced sensitization on mesolimbocortical pathway by comparison of effects induced in the same pathway by similar chemical convulsants, pentylenetetrazol (PTZ), a known GABA-benzodiazepine receptor antagonist and N-methyl-β-carboline-3-carboxamide (FG7142), a known GABA-benzodiazepam receptor inverse agonist.

MATERIALS AND METHODS

Subjects and Surgical Procedures

Twenty male Sprague-Dawley rats weighing 150-250 g upon receipt were anesthetized with ketamine HCl (70 mg/kg) and xylazine (6 mg/kg) for stereotaxic surgery. Bipolar electrodes, made of twisted stainless steel insulated wire (0.2 mm in diameter; Plastic One, Roanoke, VA), were implanted unilaterally in the ventral tegmental area at the following coordinates: -4.9 AP; 0.8 ML; 7.9 DV, and in nucleus accumbens at the following coordinates: +1.7 AP; 1.2 ML; 6.7 DV. Three screw electrodes were placed on the skull over frontal and occipital cortices. An amphenol connector was attached to one of the three steel skull screws to serve as ground wire. The electrodes and amphenol connector were affixed to the skull with the aid of steel skull screws and dental acrylic cement. Animals were then allowed at least two weeks to recover before being subjected to drug treatment and electrophysiological assessments.

Drug Studies

Animals were divided into four groups (n=5 each) and were intraperitoneally injected with vehicle (0.5 mL/kg), TMPP (0.275 mg/kg) PTZ (20 mg/kg), or FG7142 (7.5 mg/kg), three times per week for ten weeks (a total of 30 administrations). Rats received the same dose at the same time of day throughout the 10-week period. Electroencephalograms were recorded from the ventral tegmental area and nucleus accumbens 5 min, 10 min, 15 min, 20 min, 30 min, 45 min, 60 min and 24 h following dosing on preselected days throughout the 10-week period. Behavior was observed and categorized at 0 (absence of behavioral change), 1 (active sniffing, jaw clonus, extended periods of immobility, staring), 2 (myoclonic jerk-incidences of abrupt flexion and extension of the forelimb, head, and neck musculature), or 3 (clonic motor seizures-unilateral or bilateral forelimb clonus that lasted for more than 10 seconds, rearing, then loss of righting).

Electrical Stimulation

Rats in all groups were electrically stimulated. Electrical stimulation was delivered through a bipolar stimulating electrode to the ventral tegmental area or nucleus accumbens (monophasic square wave, 0.1 ms pulse duration, 0.1 Hz at 6-12 V) by Grass stimulator-isolator. Twenty pulses were presented to rats 25 min and 24 h following the test chemical or vehicle administration. Electrical signals from the nucleus accumbens and ventral tegmental area were directly recorded on a computer and continuously displayed on an oscilloscope and a thermal chart recorder.

Chemicals and Drugs

The vehicle contained Dimethyl Sulfoxide: Propylene Glycol: Saline=(25%/25%/50%). PTZ (RBI), TMPP (Department of Chemistry, Wright State University) and FG7142 (RBI) were dissolved in the vehicle solution.

Data Acquisition and Data Analysis

An Assyst4 program written by Dr. J. Lindsey was used for data acquisition.

RESULTS

Drug-Induced Spontaneous Behavioral and EEG Change

Following long-term TMPP (0.275 mg/kg) treatment, rats developed spontaneous head jerking (3 week of treatment), myoclonic jerk (4 weeks of treatment), and clonic motor seizure (8 weeks of treatment) 10-20 min after intraperitoneal injection of TMPP (0.275 mg/kg) (Figure 7.1-1a). Spontaneous epileptic after discharge electrical activities were also recorded from nucleus accumbens (Figure 7.1-2) and the ventral tegmental area. Both behavioral and EEG changes lasted for 2-4 h. In contrast, the onset of the behavior and EEG change from PTZ or FG7142 were much delayed. The observable behavioral change and epileptic EEG can be recorded after seven and eight weeks of PTZ (Figure7.1-1b) and FG7142 (Figure 7.1-1c) treatment, respectively. The latencies for drug-induced responses are 2-10 min for PTZ and 15-25 min for FG7142. All rats treated with PTZ exhibited tonic clonic motor seizure once or more, while 2 of 5 rats treated with TMPP and none from the FG7142-treated group, exhibited tonic clonic motor seizures (Figures 7.1-1a, b, c). After 24 h of chemical administration, no spontaneous behavioral jerking, seizure, or EEG paroxysms were observed. Vehicle-treated rats did not show any observable behavioral or EEG change upon administration of the vehicle solution.

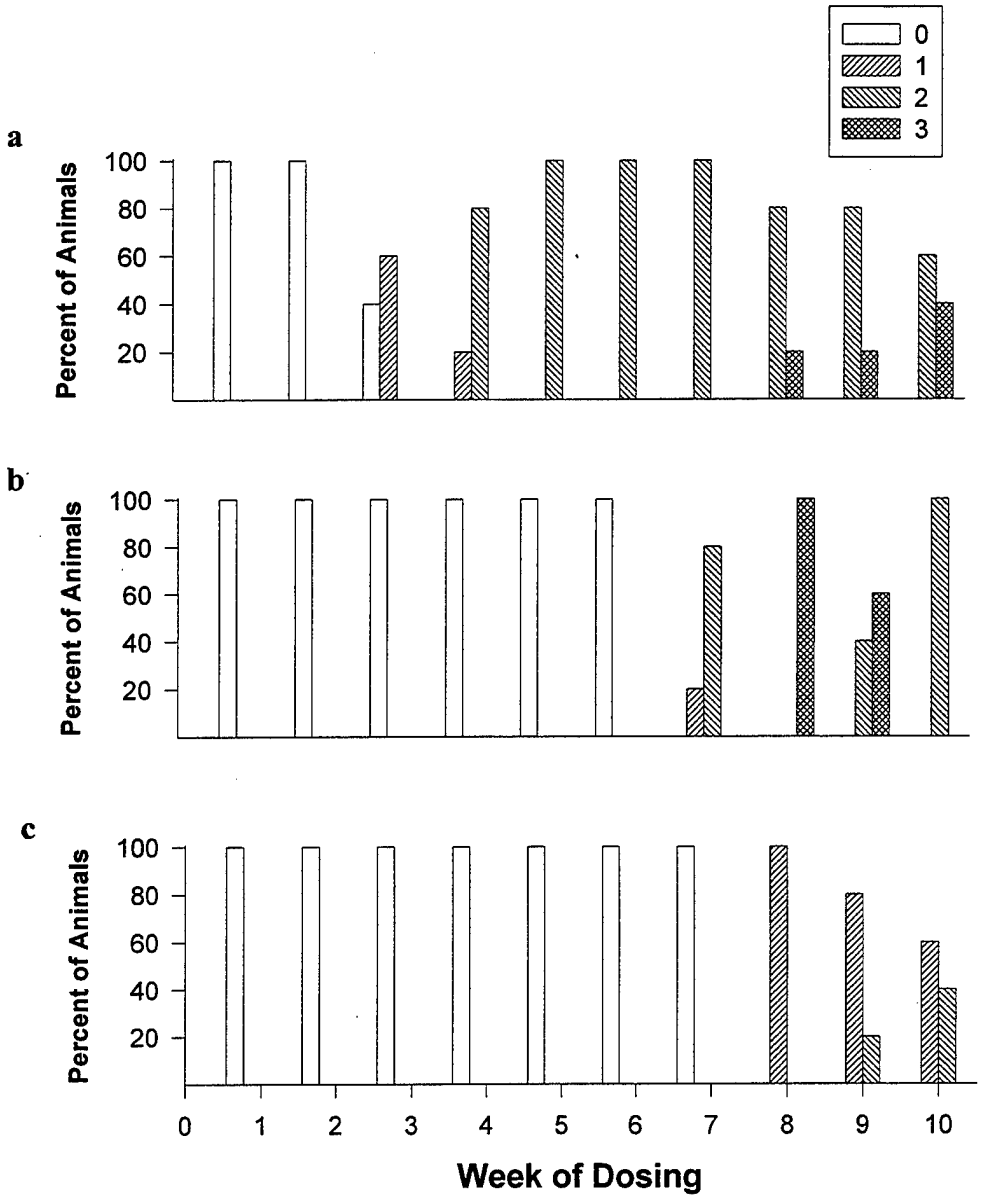

Figure 7.1-1a,b,c. Immediate spontaneous behavioral response following (a) TMPP (0.275 mg/kg), (b) PTZ (20 mg/kg) and (c) FG7142 (7.5 mg/kg) intraperitoneal injection. Ordi nate, percent of animals. Abscissa, time period of treatment in weeks. 0, absence of behavioral change; 1, active sniffing, jaw clonus, extended periods of immobility, staring; 2, myoclonic jerk-incidences of abrupt flexion and extension of the forelimb, head and neck musculature and;3 clonic motor seizures-unilateral or bilateral forelimb clonus that endures for more than 10 seconds, rearing the loss of righting.

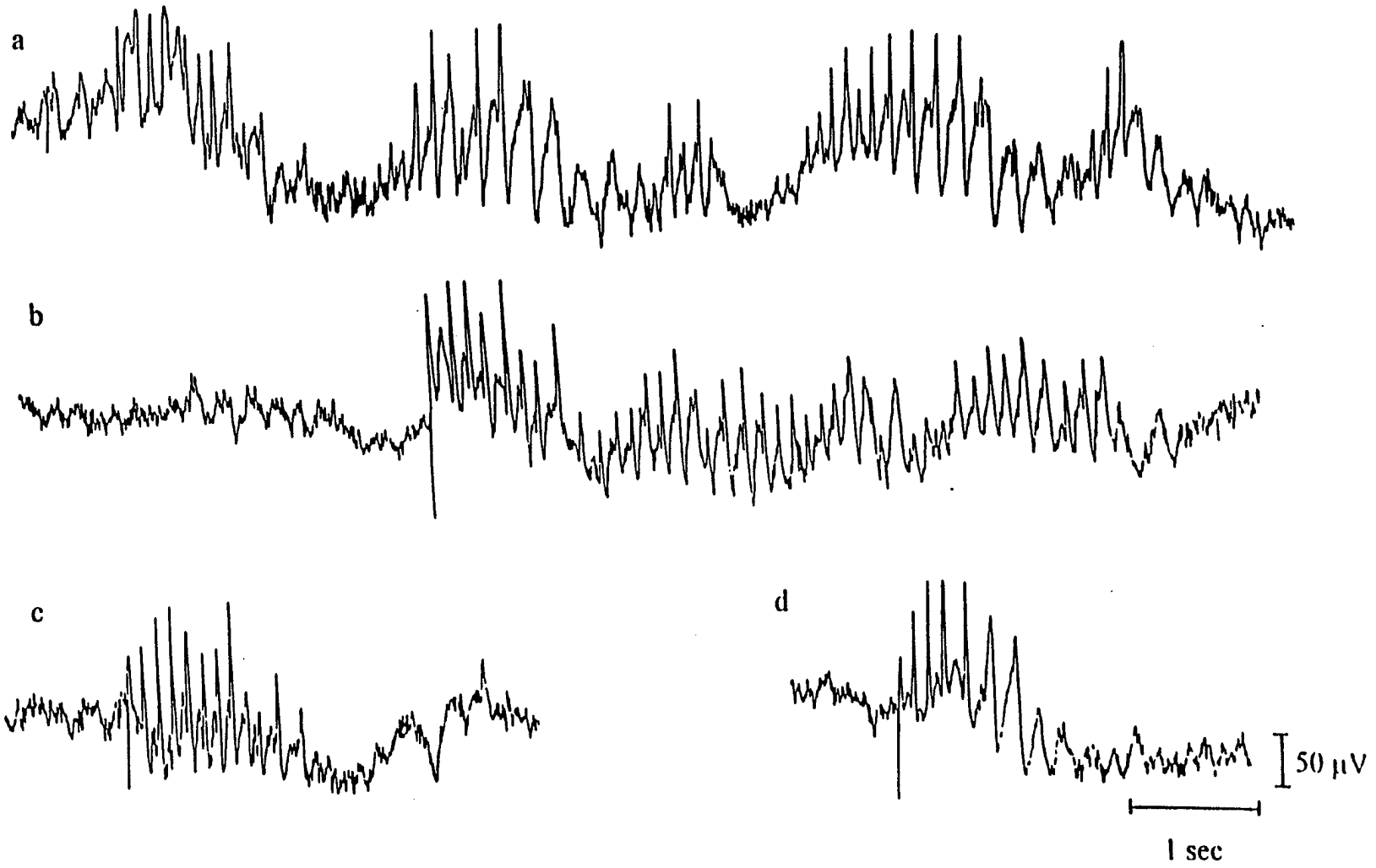

Figure 7.1-2. **Continuous recording of EEG from nucleus accumbens of one rat 25 min following TMPP (0.275 mg/kg) intraperitoneal injection.**

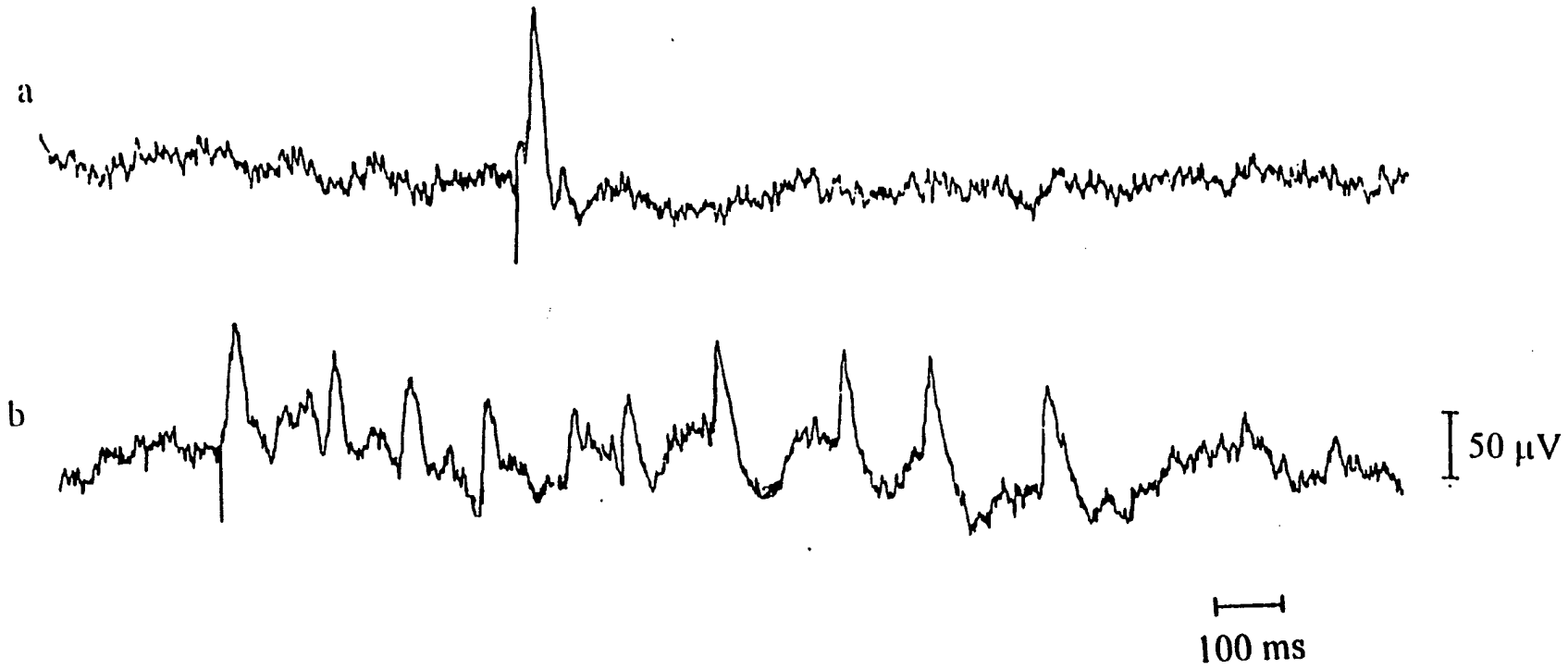

Figure 7.1-3 **Continuous recording of evoked potentials from nucleus accumbens of three rats (a,b,c) during electrical stimulation of ventral tegmental area (single pulse, 6V, 0.1 ms duration), 25 min following TMPP (0.275 mg/kg) (a), PTZ 920 mg.kg) (b) or FG7142 (7.5 mg/kg) (c) intraperitoneal injection. Downward deflections, stimulating artifact.**

Electrical Stimulation-Evoked Responses In Drug Treated Animals

Electrical stimulation (0.1 Hz, 0.1 ms duration, 6-10 V, 20 pulses) of the ventral tegmental area of vehicle and naive rats evoked single field potential (Figure 7.1-4). No observable behavioral change could be obtained from these rats upon electrical stimulation of the ventral tegmental area or nucleus accumbens (0.1 Hz, 0.1 ms duration, 6-10 V, 20 pulses). However, after 3 weeks of TMPP (0.275 mg/kg), 7 weeks of PTZ (20 mg/kg), or 7 weeks of FG7142 (7.5 mg/kg) treatment, 20 min following intraperitoneal injection of TMPP (0.275 mg/kg) (Figure 7.1-3a, 7.1-4b), PTZ (20 mg/kg) (Figure 7.1-3b) or FG7142 (7.5 mg/kg) (Figure 7.1-3c), electrical stimulation (0.1 Hz, 0.1 ms duration, 6-10 V, 20 pulses) of the ventral tegmental area induced EEG after discharges in nucleus accumbens that were associated with behavioral jerking. After 6 weeks of TMPP treatment, 24 h following TMPP injection (0.275 mg/kg), electrical stimulation of the ventral tegmental area (0.1 Hz, 0.1 ms duration, 6-10 V, 20 pulses) caused behavioral jerking in all TMPP-treated rats (Figure 7.1-5a) and induced EEG after discharges in nucleus accumbens (Figure 7.1-6a). In contrast, after 6 weeks of PTZ or FG7142 treatment, 24 h following PTZ (20 mg/kg) or FG7142 (7.5 mg/kg) injection, electrical stimulation of the ventral tegmental area (0.1 Hz, 0.1 ms duration, 6-10 V, 20 pulses) induced EEG after discharge and behavioral jerking in 80% and 20% of PTZ-treated (Figure 7.1 5b) and FG7142-treated (Figure 7.1-5c) rats, respectively. However, electrical stimulation of the nucleus accumbens failed to induced any epileptic EEG in the ventral tegmental area for the vehicle and all chemical-treated rats (Figure 7.1-6b) after 10 weeks of the experimental period. Electrical stimulation of the ventral tegmental area-evoked epileptic after discharge of nucleus accumbens and behavioral jerking, declined after eight weeks of treatment (Figure 7.1-5a, b, c), indicating the tolerance to electrical stimulation also occurred.

DISCUSSION

A thorough discussion will not be presented at this time because the study is in progress. However, based upon the available data, the following conclusions can be stated.

1. Long-term treatment (10 weeks, 3 doses/week) of rats with PTZ (20 mg/kg) induced kindling. However, long-term treatment (10 weeks, 3 doses/week) of rats with TMPP (0.275 mg/kg) and FG7142 (7.5 mg/kg) failed to induce kindling in rats.

2. Electrical stimulation of the ventral tegmental area evoked epileptic after discharge in rats exposed to long-term treatment of low doses of TMPP (0.275 mg/kg), PTZ (20 mg/kg), and FG7142 (7.5 mg/kg). The same stimulation failed to induce any behavioral or EEG changes in rats treated with vehicle for the same period of time. This indicates that TMPP, PTZ, and FG7142 sensitized the A_{10} dopaminergic pathway.

FUTURE EXPERIMENTS

To complete the study, the following experiments are planned.

Kindling

Following the completion of treatment and neural sensitization testing, all rats will have a recovery period of 3-4 weeks and then be subjected to electrical kindling and audiogenic seizure experiments.

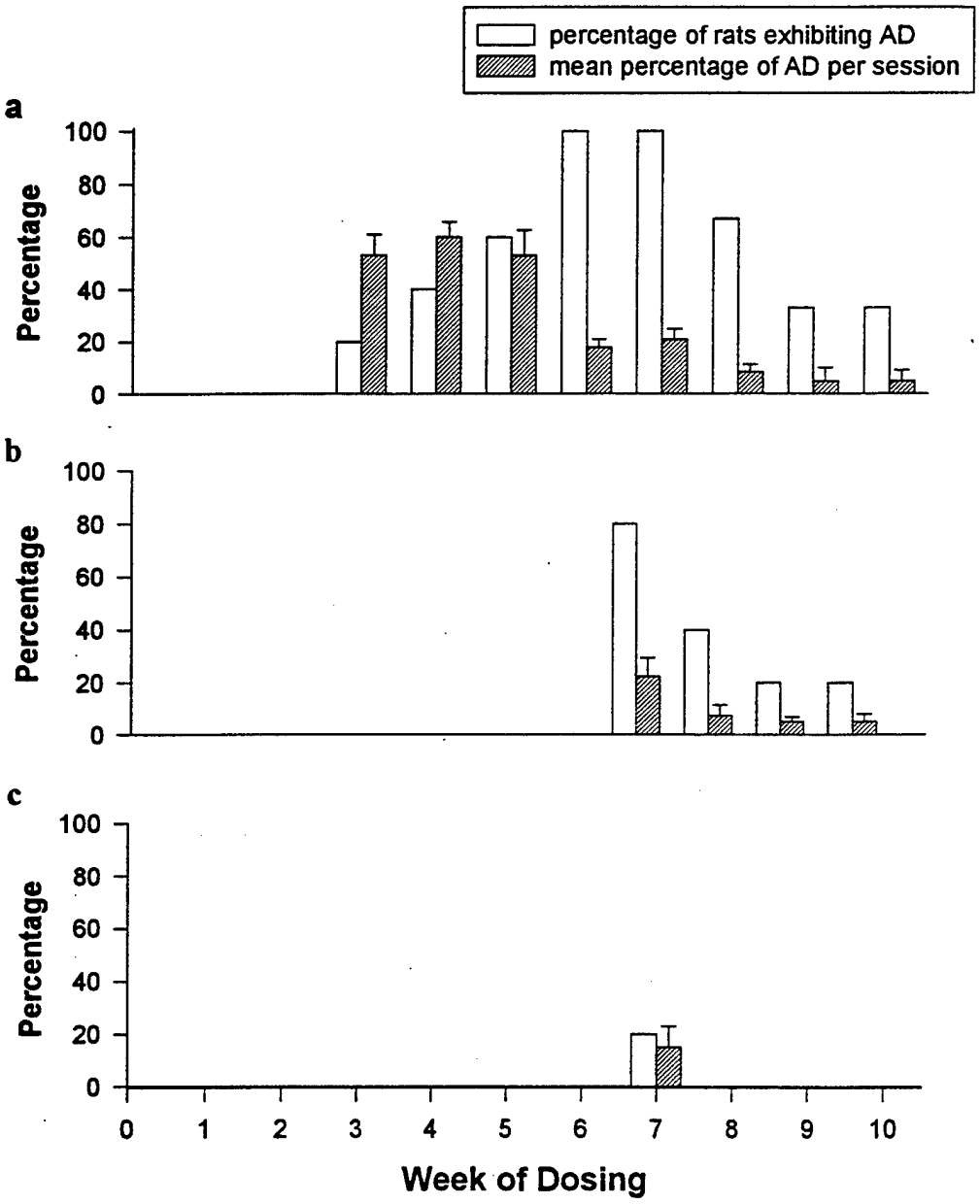

Figure 7.1-4. **Electrical stimulation of ventral tegmental area (single pulse, 6V, 0.1 ms duration) evoked responses recorded from nucleus accumbens of one rat before (a) and 25 min after (b) 7th dose of TMPP (0.275 mg/kg) intraperitoneal injection. Downward deflections, stimulating artifact.**

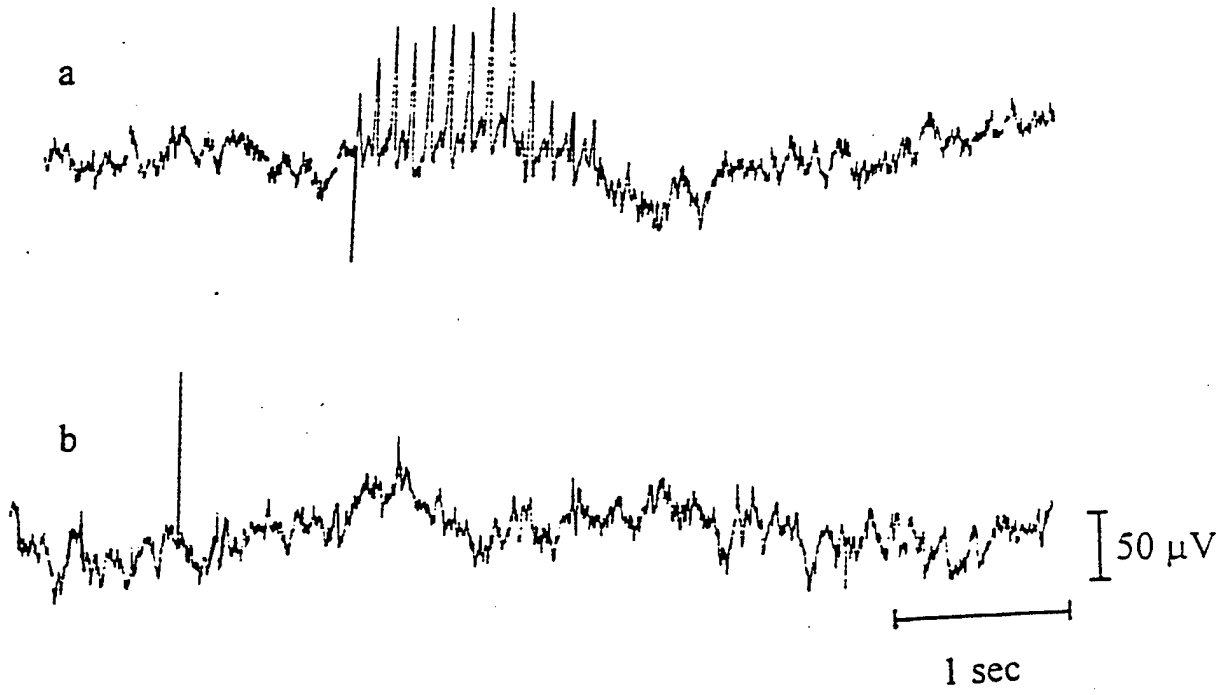

Figure 7.1- 5. **Electrical stimulation of ventral tegmental area (single pulse, 6V, 0.1 ms duration) induced nucleus accumbens EEG paroxysm and myoclonic jerk behavior following 24 hr of (a) TMPP (0.275 mg/kg), (b) PTZ (20 mg/kg) or (c) FG7142 (7.5 mg/kg) treatment. Ordinate, percentage. Abscissa, time period of treatment in weeks. AD, after discharge. Each session contains 20 pulses of electrical stimulation (0.5 Hz, 05 ms duration at threshold intensities)..**

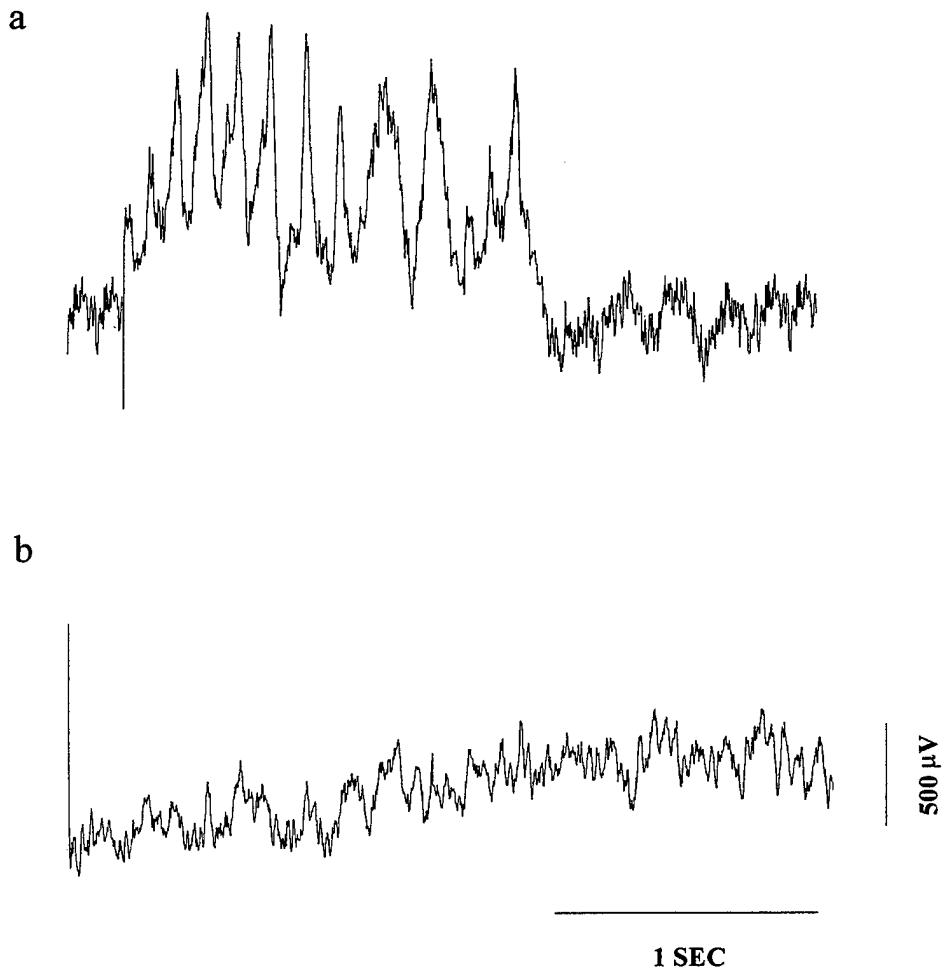

Figure 7.1-6 **Continuous recording of electrical activities from nucleus accumbens (a) and ventral tegmental area (b) of one rat upon stimulation of ventral tegmental area (single pulse, 6V, 0.1 ms duration) and nucleus accumbens (single pulse, 6V, 0.1) ms duration), respectively. Downward and upward deflections in (a) and (b), stimulating artifact.**

Electrical Kindling

The after discharge threshold (ADT) of the stimulation (ventral tegmental area and nucleus accumbens) will be determined by a 2 sec, 60 Hz stimulation beginning at 2V with subsequent increases of double intensity at 2-min intervals until ADTs are observed. The first stimulus eliciting an after discharge will be designed as the ADT and this current level will be used for the corresponding rat once per day until five consecutive generalized tonic-clonic seizures are observed. Sub-clinical seizure activity will be rated daily using the Racine Seizure Identification Scale (Racine, 1972). Dependent measures include ADT, after discharge duration, epileptogenesis amplitude, and number of days of kindling to observe generalized motor seizures. All the parameters obtained will be compared among four groups by using two-way ANOVA. The comparison of drug-treated group with vehicle group will determine the drug-induced potentiation.

Audiogenic Seizure

All rats will be subjected to a single presentation of a 110 dB audiogenic stimulus. Seizure response (wild running, tonic-clonic seizures, etc.) will be rated using the Racine Seizure Identification Scale (Racine, 1972). Seizure incidence, time to seizure induction, seizure duration, and highest seizure grade observed in drug-treated groups that statistically exceed comparable measures from vehicle group will be considered evidence of drug-induced potentiation.

Histology

After completion of sensitization, audiogenic seizure susceptibility, and electrical kindling testing, animals will be anesthetized with an overdose of sodium pentobarbital and perfused intracardially with 4% formalin in 165 mM NaCl. The brains will be removed, sliced in 40 μm coronal sections and stained with thionine for varification of electrode tracts. The data from animals in which electrode placements were located outside the regions of the ventral tegmental area and nucleus accumbens will be excluded from statistical analyses.

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7.2 COMPARATIVE TOXICITY OF 2,6-DI-*TERT*-BUTYL-4-NITROPHENOL AND OTHER NITROPHENOLS IN HUMAN AND RAT HEPATIC TISSUE SLICES

J. F. Wyman, R. Fisher[1], S. Prues[2], C.D. Flemming, J. Rivera[3], and K. Brendel

ABSTRACT

The toxicity of nitrophenols to human and rat liver tissue slices was evaluated during a 4-h exposure period. The specific compounds studied were 2,6-di-*t*-butyl-4-nitrophenol (DBNP), 2,4-dinitro-6-*t*-butylphenol (DN*t*BP), 2,4-dinitrophenol (DNP), and 4-nitrophenol (4-NP) at concentrations of 25, 50, 100, and 200 μM. Indices of toxicity to liver slices were changes in K^+ content of slices, protein synthesis, adenosine 5'triphosphate (ATP) content, and lactate dehydrogenase (LDH) release relative to untreated slices. Indices most sensitive to treatment with the nitrated phenols were ATP content and protein synthesis, followed by potassium content, and then LDH release. All indices were altered by DN*t*BP and DBNP at high concentrations. The onset of toxicity for nitrophenols was concentration dependent, except for 4-nitrophenol, which was essentially non-toxic at all concentrations tested. Comparison of changes in indices at different concentrations and times to onset demonstrated that the order of toxicity (highest to lowest) was DNtBP > DBNP > 2,4-DNP >> 4-NP. Human liver slices were more resistant to insult from nitrophenols than rat liver slices. The authors conclude that hepatotoxicity of nitrophenols *in vitro* is a function of bioavailability and disruption of ATP synthesis.

INTRODUCTION

On 20 March 1992, the Navy Environmental Health Center, Norfolk, VA, was contacted by Submarine Squadron Two, Groton, CT, regarding a yellow discoloration of bulkheads aboard Navy submarines which occurred while the submarines were underway (Huber, P.M. 1992, Department of the Navy, Naval Sea Systems Command ltr 9190 OPR:PMS393TR, Ser 393T/2367 of 22 May 1992, Washington, DC 20362-5101). The contaminant which caused the discoloration has since been identified by the Naval Research Laboratory, Washington, DC as 2,6-di-*t*-butyl-4-nitrophenol (DBNP). The yellowing process occurred on painted surfaces and on bedsheets. Crew members had potentially been exposed to DBNP for extended periods in the closed atmosphere of submarines.

The source of DBNP contamination is believed to be from the reaction of 2,6-di-tert-butylphenol, an antioxidant additive for lubricant 2190 TEP, with nitrogen oxides (dioxide) from the air. Nitrogen oxides are thought to be produced from atmospheric nitrogen by electrostatic precipitators aboard the submarines. Under the conditions in which this contaminant was produced, the most probable routes of exposure of submarine personnel included dermal, inhalation, and oral routes.

DBNP was originally evaluated for use as a mitocide, but was found to have substantial toxicity to mammals (Vesselinovitch et al., 1961). The acute toxicity for three species by oral and i.p. routes of exposure was evaluated; oral exposure proved less

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toxic, as would be expected. The LD_{50} values for male rats were 270 mg/kg and 450 mg/kg for i.p. and oral exposures, respectively. Additionally, subacute (60-day i.p. treatment) and chronic (16-week feeding) studies were performed. The toxicity of DBNP in rats was found to be cumulative with the liver, kidney, lung, heart, and spleen being target tissues. Specific injuries observed were periportal fatty infiltration of the liver, degeneration of the tubular epithelium and loss of renal function, degeneration of the myocardium and lymphorrhexis, and aborted cell division in the spleen. In another investigation, DBNP was found to produce elevated liver weights and induce both Phase I and Phase II metabolic pathways (Gilbert et al., 1969). In a recent study, DBNP was shown to be an uncoupler of oxidative phosphorylation (Yoshikawa et al.,1980). No other reports of DBNP toxicity were available from the literature.

Evaluation of DBNP toxicity has been very limited and the relative toxicity of DBNP compared to other nitrophenols has not been studied. The study described here is an evaluation of the hepatotoxicity of DBNP, compared to other nitrophenols, in rat and human liver slices. Structural comparison of the nitrophenols studied is depicted in Figure72-1.

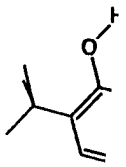

Figure 7.2-1. Nitrophenol Structures.

MATERIALS AND METHODS

Materials

Chemicals. DBNP was synthesized from 2,6-di-*t*-butylphenol (Sigma Chemical Co., St. Louis, MO) and NO_2 gas as previously described (Rivera et al., 1995). 2,4-Dinitrophenol and 4-nitrophenol were purchased from Sigma Chemical Co. 2-*t*-Butyl-4,6-dinitrophenol was synthesized as previously described (Hart and Cassis, 1951) with structural confirmation by mass spectral and elemental analyses (Galbraith Laboratories, Inc., Knoxville, TN) as described in (Rivera et al., 1995).

The liver slice incubation medium was prepared with Waymouth's MB752/1 powdered medium (without phenol red), purchased from Gibco Laboratories (Grand Island, NY). The basic Waymouth's medium contained the following additives: fetal calf serum, purchased from Hyclone Laboratories (Logan, UT); gentamicin sulfate and L-glutamine were obtained from Sigma Chemical Co. (St. Louis, MO); Fungi-Bact® was obtained from Irvine Scientific (Irvine, CA). This incubation medium was used for both an initial acclimation period as well as for nitrophenol exposure. Nitrophenols, in an amount required for a specific concentration, were dissolved in 100 μL dimethylsulfoxide and added to the incubation medium. Control exposures contained 100 μL dimethylsulfoxide without a nitrophenol toxicant. Viaspan® cold preservation solution was obtained from DuPont Pharmaceuticals (Wilmington, DE) and V-7® preservation buffer was obtained from Vitron, Inc. (Tucson, AZ).

Toxicity assays required the following: Lactate dehydrogenase kit (Sigma Chemical); ^{3}H-leucine purchased from ICN Radiochemicals (Irvine, CA); Biosafe II® scintillation cocktail was purchased from Research Products International (Mt. Prospect, IL).

All other chemicals used in this study were reagent grade quality or higher. Tissue slice incubations were carried out using teflon/viton/titanium rollers, a dynamic organ culture incubator, and a Brendel/Vitron® tissue slicer, all obtained from Vitron, Inc. (Tucson, AZ).

Animals. Adult male Sprague-Dawley rats (200-250 g) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The animals were fed Tek-lad® (4%, mouse/rat, 7001), housed on Sani-chips®, maintained on a 12-h light cycle, and allowed free access to drinking water. Animals were killed by cervical dislocation, the livers removed through a midventral incision and immediately placed in ice-cold V-7 preservation solution.

Human Liver Tissue Human liver tissue was obtained from the Association of Human Tissue Users (Tucson, AZ). The liver was procured for transplantation by organ banks but was not transplanted for various medical reasons. Once it was decided that the tissue would not be transplanted, it was immediately placed in an ice-cold solution of Viaspan® for transportation to the laboratory. The age, sex, race, height, weight, serologies, blood group, medications, cause of death, medical history, warm ischemia time, and cold ischemia time were recorded for each human liver (Table 7.2- 1)

TABLE 7.2-1. HISTORY OF HUMAN LIVER TISSUE[1]

	#1	# 2	# 3
AGE:	41	42	43
SEX:	male	female	female
RACE:	caucasian	caucasian	caucasian
HEIGHT:	180 cm	163 cm	170 cm
WEIGHT:	80 kg	86 kg	85 kg
SEROLOGIES:	negative	negative	negative
BLOOD GROUP:	A +	B +	A +
CAUSE OF DEATH:	CI[2]	CA[2]	ICB[2]
MEDICAL HISTORY:	healthy	TB,AL[3]	NIDDM[4]
MEDICATIONS:	NL[5]	none	none
WARM ISCHEMIA TIME:	0 h	0 h	0 h
COLD ISCHEMIA TIME:	12 h	25 h	13 h

1) **All livers were procured for transplantation but were not transplanted into a recipient be cause of various medical reasons. The Association of Human Tissue Users received the tissue for research purposes.**
2) **CI = cranial injury, CA = cardiac arrest, and ICB = intracranial bleed.**
3) **The donor smoked tobacco (TB) and drank alcohol occasionally (AL).**
4) **The donor had gestational Non-Insulin-Dependent Diabetes Mellitus (NIDDM) with last pregnancy at age 37 and borderline hypertension. Had regular medical attention and was taking oral agents[5] for this condition.**
5) **The types of oral agents taken were not listed (NL).**

Incubation Procedure

Tissue Slice Preparation. Tissue cylinders were prepared from both rat and human livers with a sharpened, stainless steel tube (8 mm) by slowly turning and advancing the metal tube into the liver, which was suspended on a wax support. Using a Brendel/Vitron® tissue slicer, tissue slices (200 µM) were prepared from the cylinders in oxygenated and ice-cold V-7® preservation solution.

Slice Organ Culture. The slices in V-7® preservation solution were floated onto teflon/viton/titanium rollers. The rollers were then carefully blotted and loaded horizontally into glass scintillation vials containing 1.7 mL of Waymouth's culture medium which had been supplemented with 10% fetal calf serum, 10 mL/L Fungi-Bact®, 84 µg/mL gentamicin, 350 µg/mL L-glutamine, and 2.24 g/L sodium bicarbonate. Vials were closed with a cap which had a central hole of approximately 2 mm, placed in the dynamic organ culture incubator, and gassed with 95% O_2/5% CO_2.

Liver slices were acclimated for a period of 1 h prior to exposure to individual nitrophenols. The exposure procedure involved removing the roller and slice from the acclimation medium, blotting dry, and placing the roller and slice in a new vial containing the nitrophenol. Nitrophenol solutions were prepared as described above at concentrations of 25, 50, 100, and 200 µM.

Indices of Toxicity

Intracellular K+ Content. Individual tissue slices were removed from incubation, blotted, weighed, and put in 1 mL of distilled water. The tissue slice was then homogenized by sonication using a cell disrupter at an intermediate setting (Model 350, Branson Sonic Power Co., Danbury, CT). After the addition of 20 μL of 70% perchloric acid, the homogenate was centrifuged. The supernatant was then assayed for K+ content using a flame photometer (Model CA-51 Perkin-Elmer, Danbury CT). Results are expressed as μmol K+/g slice wet weight.

Protein Synthesis. Slices were exposed to ^{3}H-leucine, incubated for a specified time, were immediately washed twice in buffer, and homogenized in 1 mL of 1 M KOH. Following the addition of an equal volume of 1.5 M acetic acid, the samples were centrifuged (3000g, 20 min) to pellet the protein precipitate. The pellets were washed twice in 2 M acetic acid and dissolved in 0.5 M NaOH (0.5 mL). The incorporation of ^{3}H-leucine into acid precipitable protein was determined by counting a 0.4 mL aliquot of the dissolved pellets after neutralization with 125 μL of 2 M HCL. Results are expressed in dpm ^{3}H- leucine/mg wet weight.

LDH Leakage. Lactate dehydrogenase (LDH) was measured by using a Sigma kit. Aliquots of the culture medium and slice homogenate were taken and analyzed in a micro assay using 96 well plate technology. Results are expressed as percent of total LDH released.

ATP Content. ATP content was determined using a luciferin-luciferase bioluminescent assay (Kricka, 1988). Upon harvesting, slices were homogenized in a 5% trichloroacetic acid solution and the homogenate frozen in liquid nitrogen and stored in a -80 °C freezer until the analysis. Results are expressed as nmoles ATP/mg wet weight of tissue.

Statistical Analyses

Data were examined to determine which index of toxicity was the most sensitive measurement of change for both rat and human slices for each nitrophenol examined. Sensitivity was defined as a measure of the greatest change in a specific index from the control value over time. Additionally, whether a correlation existed between individual indices of toxicity, and which nitrophenol produced the greatest change for a specific index of toxicity, in a specific species, was examined.

A two factorial multivariate analysis of variance was performed, with the two factors being dose (at 5 levels) and time (at 4 levels). Levine's test for homogeneity of variance was applied, using a two factorial multivariate analysis of variance. The Wilk-Shapiro test was used to test for normality. If either normality or homogeneity were violated, an appropriate transformation such as log or rank was used.

RESULTS

Incubation of liver slices with DBNP produced a significant decrease in ATP content. The effects of dose and length of incubation for both rat and human liver slices is shown in Figure 7.2-2. Rat liver slices were significantly different from the control values at DBNP concentrations ≥100 μM, while ATP content of human slices were significantly changed at concentrations of 200 μM. No other viability indices were changed after 2 h. Figures 7.2-2 and 7.2-3 depict the effect of increasing concentration of DBNP on slice viability for rat and human liver, respectively, after a 2 h incubation time. The response

of rat liver slices to DBNP was greater than that of human liver slices, with all indices of toxicity being significantly changed at the highest concentration (200 μM).

Human liver slices were altered to the same extent, but only after a 4-h incubation period at 200 μM (data not shown). For both rat and human liver slices, the most sensitive index of toxicity was ATP content (based on significant changes observed at the earliest time points and at high concentrations of DBNP). Of the indices of toxicity monitored, the order of susceptibility for both species was ATP content > protein synthesis > LDH release > K^+ content.

The sensitivity of protein synthesis as an index of toxicity was greater than that of ATP content if DBNP concentrations were low and the incubation time was extended. The effect of increasing incubation time for DBNP concentrations of 0, 25, and 50 μM on rat and human liver slices is shown in Figures 7.2-4 and 7.2-5, respectively. At the lowest concentration of 25 μM, protein synthesis in rat liver slices was significantly decreased after 2 h; whereas, at the same concentration,a significant decrease in human liver slices did not occur until after 4 h. No other indices were altered at this low concentration for either rat or human slices. Rat liver slices exposed to DBNP for 4 h at 50 μM showed changes in all of the viability parameters which were monitored. Only ATP content and protein synthesis were affected in human slices under the same conditions.

Based on the findings shown in Figures 7.2-2 through 7.2-5, the most sensitive indices of insult were ATP content and protein synthesis. Potassium content and release of LDH were affected only after extended incubation or at higher concentrations. Evaluation of other nitrophenols (DN*t*BP, DNP, and 4-NP) under the same conditions revealed the same response as that of DBNP, with ATP content and protein synthesis being most strongly affected in rat and human liver slices. The relative toxicity of nitrophenols, at an equivalent concentration of 50 μM and 2-h incubation time, for both rat and human liver slices is shown in Table 7.2-2. Compared to control values, only DN*t*BP produced a significant change in both rat (ATP content and protein synthesis) and human slices (ATP content only). DBNP significantly changed ATP content and protein synthesis in rat slices, but had no significant effect on human slices at the 50 μM 2-h incubation conditions. At higher concentrations and/or longer incubation times, the order of decreasing toxicity was found to be DN*t*BP ≥ DBNP >DNP >> 4-NP in rat liver. The order of decreasing toxicity in human liver was essentially the same with DN*t*BP > DBNP >DNP >>4-NP. Based on the relative decrease in the level of protein synthesis and ATP content, rat liver was more susceptible to nitrophenol toxicity than was human liver.

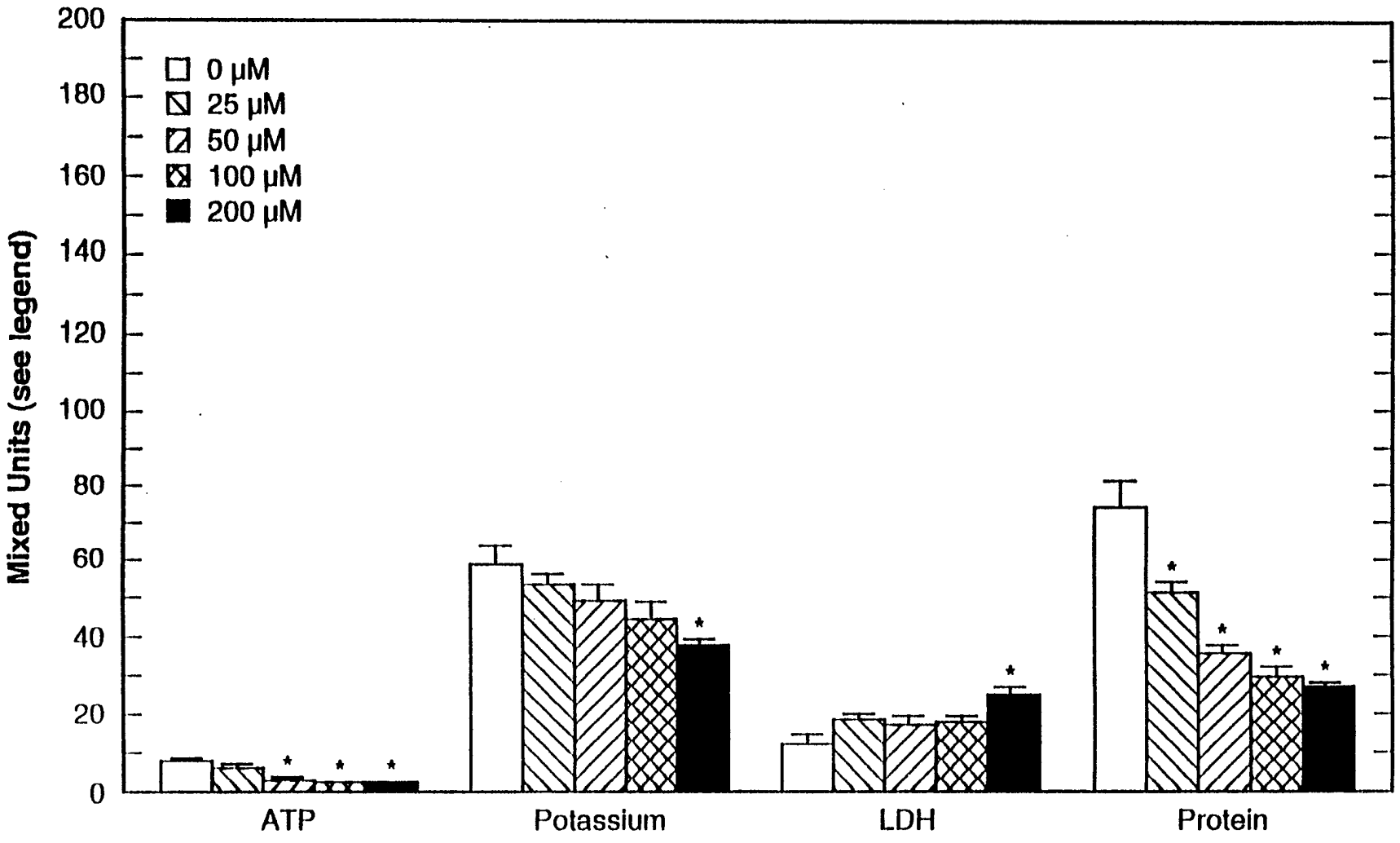

Figure 7.2- 2. **Toxicity of Varied Concentrations of DBNP to Rat Liver Slices After 2 Hrs Incubation**
Values represent the mean ± S.D. for three determinations.
Significantly different from control (0 µM) values.
Mixed Units: ATP = µmol/g wet weight; potassium = µmol/g wet weight; LDH = % of total LDH released; protein = DPM/mg protein

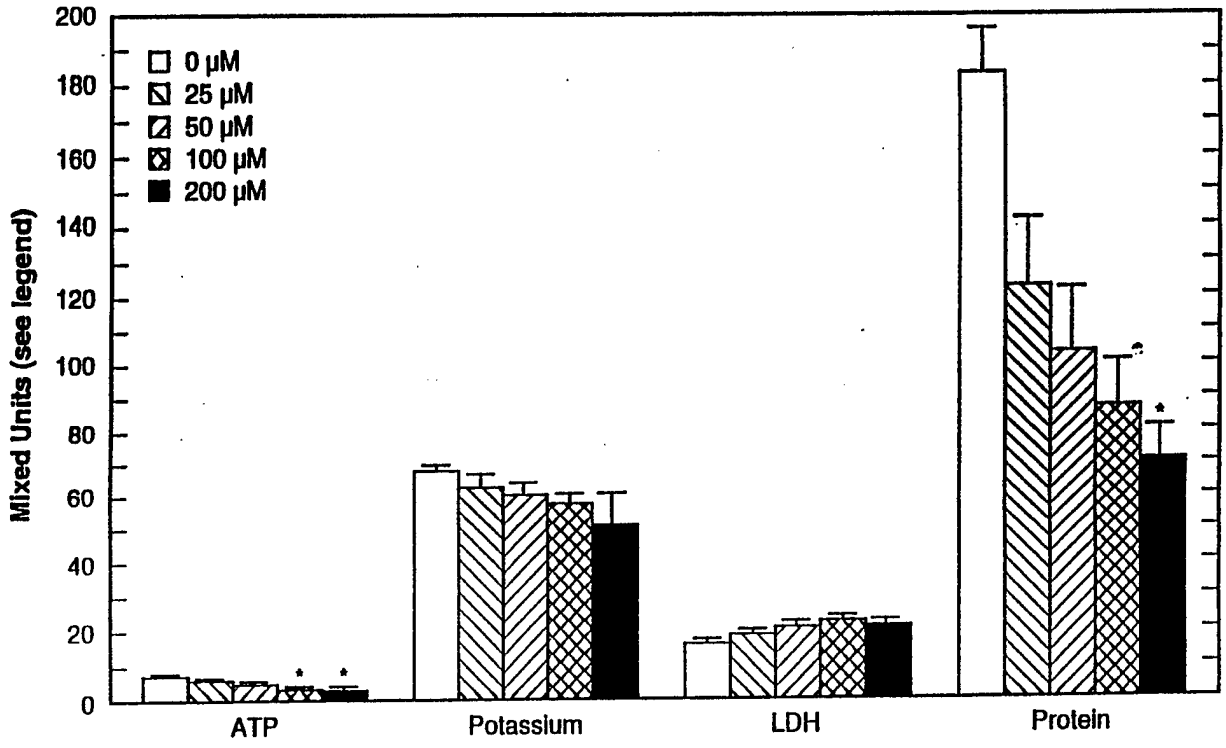

Figure 7.2-3. **Toxicity of Varied Concentrations of DBNP to Human Liver Slices After 2 Hrs Incubation.**
Values represent the mean ± S.D. for three determinations.
***Significantly different from control (0 µM) values.**
Mixed Units: ATP = µmol/g wet weight; potassium = µmol/g wet weight; LDH = % of total LDH released; protein = DPM/mg protein

TABLE 7.2-2. COMPARISON OF NITROPHENOL TOXICITY IN RAT AND HUMAN LIVER SLICES.

	RAT		HUMAN	
Nitrophenol (50 μM)	ATP Content[a]	Protein Synthesis[b]	ATP Content[a]	Protein Synthesis[b]
Control	5.9 ⇧ 0.7[c]	112.7 ⇧ 23.2[c]	7.2 ⇧ 0.4	177.3 ⇧ 21.1
DN-*t*-BP	2.2 ⇧ 0.0*	57.0 ⇧ 19.3*	3.8 ⇧ 1.7*	77.0 ⇧ 12.2
Control	7.0 ⇧ 0.7	73.0 ⇧ 9.6	----------	----------
DBNP	2.2 ⇧ 0.7*	34.3 ⇧ 3.5*	5.1 ⇧ 0.4	99.0 ⇧ 32.7
2,4-DNP	6.1 ⇧ 0.2	57.3 ⇧ 28.0	6.5 ⇧ 0.8	156.3 ⇧ 26.8
4-NP	7.0 ⇧ 0.6	79.0 ⇧ 16.5	7.3 ⇧ 1.2	142.7 ⇧ 19.4

Values represent the mean ⇧ S.D. of three determinations. The concentration and exposure time selected for comparison were 50 μM and 2 hr, respectively.
a) nmo/mg wet wt.
b) DPM/mg wet wt.
c) Control values for DN-*t*-BP exposures were different from the control used for the other nitrophenols evaluated because these studies were conducted at a different time.
*** Statistically different from control values; $p \geq 0.05$.**

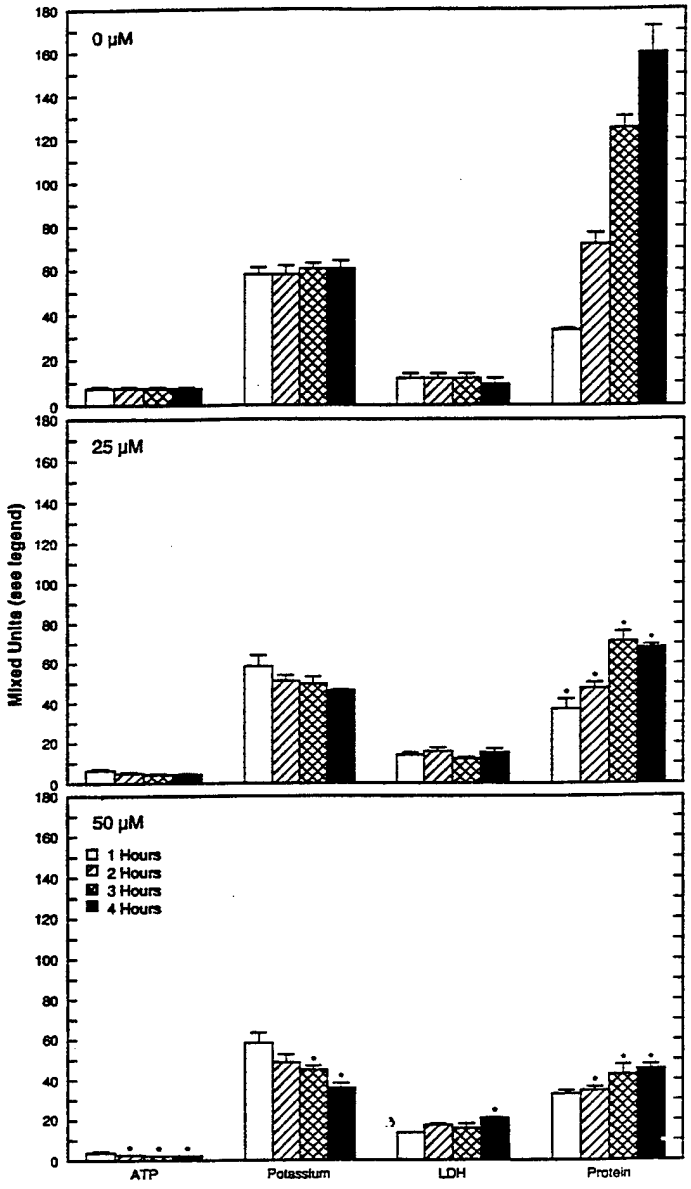

Figure 7.2-4. **Temporal Effect of DBNP on Rat Liver Slices at Varied Concentrations. Values represent the mean ± S.D. for three determinations. *Significantly different from control (0 µM) values. Mixed Units: ATP = µmol/g wet weight; potassium = µmol/g wet weight; LDH = % of total LDH released; protein = DPM/mg protein**

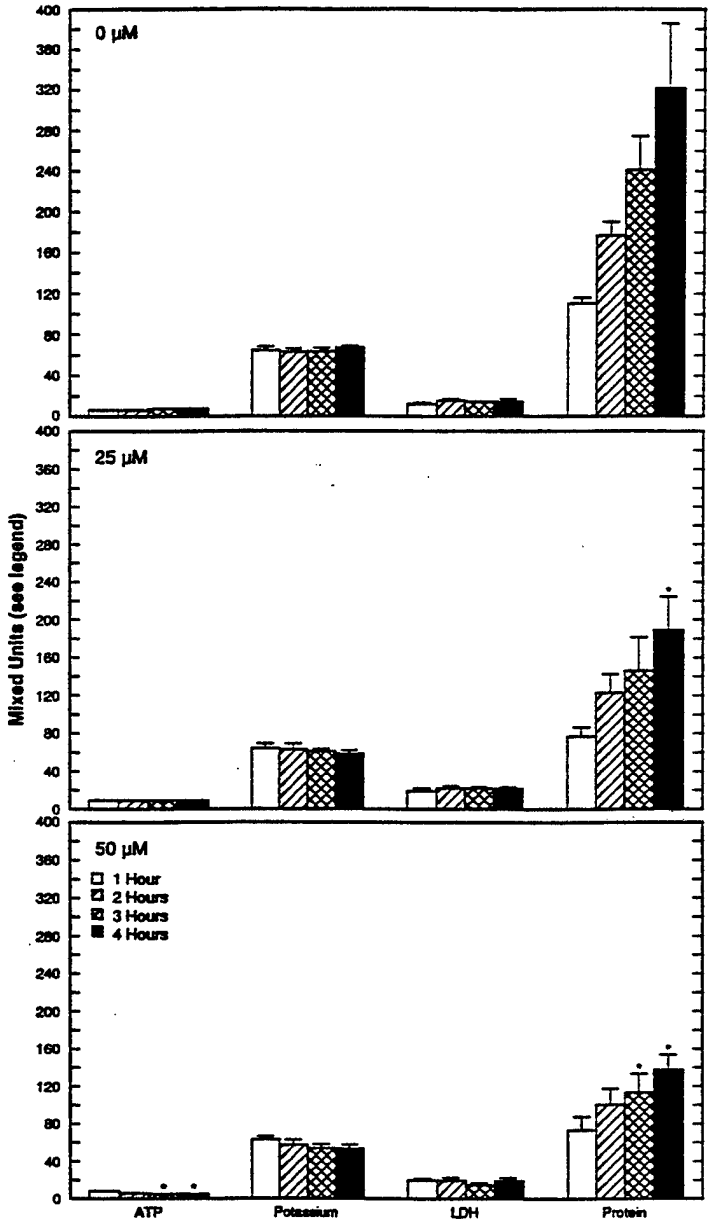

Figure 7.2-5 **Temporal Effect of DBNP on Human Liver Slices at Varied Concentrations.**
Values represent the mean ± S.D. for three determinations.
***Significantly different from control (0 µM) values.**
Mixed Units: ATP = µmol/g wet weight; potassium = µmol/g wet weight; LDH = % of total LDH released; protein = DPM/mg protein

DISCUSSION

Hepatotoxicity has been observed following exposure to some, but not all nitrophenol compounds. Repeated exposure of rats to DBNP produced a dose dependent fatty degeneration of the liver of rats (Vesselinovitch et al., 1961). Acute oral exposure of humans to DNP produced severe fatty changes in the liver (Goldman and Haber, 1936), although these findings were not consistent in other DNP fatalities ("Toxicological Profile for Dinitrophenols: Draft for Public Comment", 1993). No reports of hepatotoxicity following 4-NP exposure are available in the literature (Toxicolgical Profile for Nitrophenol: 2-Nitrophenol, 4-Nitrophenol", 1992).

The order of toxicity of nitrophenols evaluated in both rat and human liver slices was DN*t*BP > DBNP > 2,4-DNP >> 4-NP. A different ordering of toxicity was determined for nitrophenols *in vivo* based on LD_{50} values from the literature. Reported oral LD_{50} values for nitrophenols are DNP (30 mg/kg ["Toxicological Profile for Dinitrophenols: Draft for Public Comment", 1993]) > DBNP (450 mg/kg; [Vesselinovitch et al., 1961]) > 4-NP (620 mg/kg; [Vernot et al., 1977]) > 2-NP (2830 mg/kg; [Vernot et al., 1977]). No data were available for the oral LD_{50} of DN*t*BP. Based on NP toxicities, placement of the nitro group at the 4 position increases the acute toxicity of the phenol; 2-NP was not evaluated in this study.

A structure-activity comparison of nitrophenol-induced hepatotoxicity indicates butylated nitrophenols have a significantly higher toxicity than non-butylated nitrophenols. However, toxicity requires a nitro-function on the molecule because the parent molecule, 2,6-di-*t*-butylphenol, produced no change in viability indices when evaluated under identical conditions (data not shown). The presence of two nitro-functions (2 and 4 positions) increases the toxicity of the molecule, as demonstrated by DN*t*BP being more toxic than DBNP, and 2,4-DNP being more toxic than 4-NP. The observation that DN*t*BP and DBNP were both more toxic than 2,4-DNP suggests that increased hepatotoxicity may be a function of bioavailability. The presence of a *t*-butyl group(s) increases the lipophilic character of the molecules and may facilitate passage through hepatocyte membranes to a greater extent than more polar molecules, such as 2,4-DNP.

Typical toxic effects of dinitrophenols are best exemplified by 2,4-dinitrophenol (DNP), which has undergone the most extensive study. A thorough review has recently been provided ("Toxicological Profile for Dinitrophenols: Draft for Public Comment", 1993). DNP toxicities, including fatalities, in humans have been well characterized because DNP was previously used as an aid for weight loss. Use of DNP as a diet pill occurred from 1932 to 1938, after which time it was discontinued because of adverse health effects. Toxicities included an increase in basal metabolic rate with profuse sweating, elevated temperature, rapid onset of fatigue, labored respiration, dehydration, and weight loss. Similarly, increased basal metabolic rate, elevated temperature, increased oxygen consumption, and weight loss have been observed in numerous animal species including dogs, rats, mice, guinea pigs, and pigeons ("Toxicological Profile for Dinitrophenols: Draft for Public Comment", 1993). All of the metabolic effects caused by DNP have been attributed to an uncoupling of oxidative phosphorylation from the electron transport chain in mitochondria with a resultant decrease in synthesis of ATP.

The indices of toxicity selected for assay in this investigation would be directly or indirectly affected by a perturbation of ATP synthesis. Protein synthesis, maintenance of the Na+/K+ ionic gradient, and the integrity of the cell membrane (LDH release) all are energy-dependent processes. Because 2,4-DNP and DBNP are known uncouplers of oxidative phosphory-

lation, it is a reasonable speculation that DN*t*BP will also disrupt ATP synthesis. 4-NP, which was essentially non-toxic under the conditions of this study, does not uncouple oxidative phosphorylation. The authors postulate that nitrophenol hepatotoxicity is a function of bioavailability and disruption of ATP synthesis.

Human liver slices were less susceptible to injury from nitrophenols than rat liver slices. No explanation for this finding is yet available.

SUMMARY

An overall summary of statistically significant changes in viability indices for both rat and human liver slices is shown in Table 7.2-3. The order of toxicity was DN*t*BP > DBNP > 2,4-DNP >> 4-NP. Differences in toxicity are believed to be due to greater cellular uptake of the more lipophIlic, butylated nitrophenols. Human liver slices were consistently more resistant to insult from nitrophenols than were rat liver slices. The sensitivity of the indices of toxicity monitored was ATP content $\geq$ protein synthesis > K+ content > LDH release.

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TABLE 7.2-3 SUMMARY OF SIGNIFICANT CHANGES IN INDICES OF NITROPHENOL TOXICITY IN LIVER SLICES AT VARIABLE CONCENTRATIONS AND EXPOSURE TIMES

RAT LIVER SLICES

NITRO-PHENOL	1 Hr				2 Hr				3 Hr				4 Hr			
	25µM	50µM	100µM	200µM	25µM	50 µM	100µM	200µM	25µM	50µM	100µM	200µM	25µM	50µM	100µM	200µM
DBNP			A	A	P	A,P	A,P	AKLP	P	A,K,P	A,K,P	AKLP	P	AKLP	AKLP	AKLP
DNBP	A	A	A,K	A,K,P	A	A,P	A,K,P	AKLP	A	A,P	A,K,P	AKLP	A,P	A,P	A,K,P	AKLP
2,4-DNP							A	A			A	A,P	A	A	A	A,P
p-NP															K	

A = ATP Content; K = Potassium Content; L = LDH Activity; P = Protein Synthesis

HUMAN LIVER SLICES

NITRO-PHENOL	1 Hr				2 Hr				3 Hr				4 Hr			
	25µM	50µM	100µM	200µM	25µM	50µM	100µM	200µM	25µM	50µM	100µM	200µM	25µM	50µM	100µM	200µM
DBNP				A			A	A,P		A,P	A,P	A,P	P	A,P	A,P	A,K,P
DNBP	A		A	A		A	A,P	A,P	P	A,P	A,P	A,K,P	P	A,P	AKLP	AKLP
2,4-DNP							P	P			A,P	A,P		A	A,P	A,P
p-NP																

A = ATP Content; K = Potassium Content; L = LDH Activity; P = Protein Synthesis

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SECTION 8

APPLICATION OF STATISTICS AND METHODS DEVELOPMENT

8.1 STATISTICAL DESCRIPTION OF PHYSIOLOGICAL VARIABLES FOR SEVEN NAVAL POPULATIONS

C.D. Flemming, O.M Little[1], and R.L. Carpenter[2]

INTRODUCTION

It has long been recognized that human health risk assessment must be done on the basis of extrapolation from animal experimentation (Casarrett and Doull, 1986), whether the ultimate purpose is to demonstrate a beneficial effect (pharmacology), or protect against a harmful effect (toxicology). To execute such a program, it is necessary to have some basis for extrapolation from laboratory animal experiments to the human population (NAS/NRC, 1983). Physiological variations are a major factor determining sensitivity to chemical exposure. These variations may affect either the absorption, distribution, metabolism, or elimination as determined by pharmacokinetics or the mechanism of action as determined by pharmacodynamics of a compound. These differences can be evaluated by using pharmacokinetic modeling. Of particular interest to toxicologists is a extrapolation modeling technique that relies on the known physiological differences between species (physiologically based pharmacokinetic, or PBPK modeling) (Andersen, 1991).

In general, exposed populations may vary due to differences in their exposure characteristics. For example, ingestion of contaminated drinking water may lead to an exposed population with quite varied physiological properties. Conversely, a specific group having narrowly defined properties may be exposed through occupational chemical use or contact. Civilian or military workers doing a specific job would be an example of this latter case. Thus, exposed population heterogeneity can be affected by circumstances other than the inherent properties of the population itself. These variations may affect either the absorption, distribution, metabolism, or elimination (pharmacokinetics), or the mechanism of toxicity(pharmacodynamics) of a chemical. Evaluation of these differences in pharmacokinetics using physiologically based pharmacokinetic models (PBPK) is the focus here. Consideration of these factors affecting an exposed population is addressable through the application of Monte Carlo methods to PBPK model parameters for estimation of the model prediction population distributions. However, the data necessary to conduct such simulations is sparse and many approximations currently need to be made. The interest of this paper is to provide at least some of the data needed for Monte Carlo PBPK modeling.

Of particular interest to the military is the effect of strenuous physical fitness standards and the widely varying occupational requirements on the physical endurance of the military population and the effects of these factors on exogenous chemical exposure. Since the military population is monitored for medical information in a systematic way during their service career, we sampled these records in order to ascertain if any information useful for interspecies extrapolation to man could be found. The choices for examining military subpopulations included samples and assumed characteristics of SEALS (SEa, Air, Land), BUDS (trainee SEALS), divers, aviators and fleet sailors. In addition to deriving data on Navy populations believed indicative of the general Navy population, we were interested in determining if there were sub-populations within the Navy whose physical requirements set them apart from the general population, and thus might serve as indicators of upper and lower limits of attainable characteristics. The general choices for considering military subpopulations included known population samples and assumed characteristics of SEALS (Sea, Air, Land), divers, aviators, and fleet sailors. The SEALS

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and divers were believed to be a trimmer, more athletic community. The aviators were known to have stringent flight physicals they were required to pass periodically. The fleet sailors were chosen to provide a benchmark for comparisons and represent majority of the active duty personnel.

The results of this project will be used to describe the statistical distribution of each subpopulation and to perform Monte Carlo simulations on the subpopulations for the evaluation ofdifferences in pharmacokinetics.

METHODS

Data Collection

The populations and sample sizes used in this report were largely dependent upon the availability of data from the various communities within the Navy. Data were obtained from the Navy research/data holding facilities mentioned in the acknowledgments. Sample sizes were chosen to avoid statistical inadequacy. In selecting the number from each community there was no intention of having a "proportionate sample" of the communities in the Navy. For mixed-sex populations, aviators, and fleet, every effort was made to ensure that females were adequately represented. These data were obtained from the Navy Form 88 medical records. Although clinical parameters useful in medical diagnosis were available, the data elements we found immediately useful included age, height, weight, and body fat percentage. The balance of the data were not immediately generally applicable to kinetic modeling.

The data for each population was provided in ASCII format on floppy disks. Each dataset was converted into an RS/1 table. A column was added to each table which indicated the membership in a population. In several cases, the body weight had to be converted from pounds to kilograms, and the body height was converted from inches to centimeters. Since the data came from different locations, the variables from each dataset were not the same; therefore, the common variables were identified and separated into another table. The tables were then merged into one table.

Outliers

Descriptive statistics (n, mean, median, std, min, max) were used to screen for outliers among the groups. Age, height, weight, and body fat were the variables examined using multivariate analyses. Principal component analysis (reference-statistical text) was the key method used. Factors (i.e., axes) representing the structure of the original variables were determined according to Barnett and Lewis (1984). The Mahalanobis distances from each point to the centroid which was the mean of each variable for the original data were computed. The Mahalanobis distances from each case to the factor scores were also computed. The difference in the scores was then determined. These distances have a chi-squared distribution for large samples. If the chi-square result for a point is statistically significant ($p < 0.05$), the point is considered an outlier. Outliers were eliminated if a data point appeared to be an obvious data entry error.

Population Distribution Estimates

The determination of a distribution requires measures of the following components: central tendency (mean and median), variation (standard deviation, minimum and maximum), correlation among the variables (correlation and covariance), and the theoretical distribution (normal, log-normal and others). The distributional properties of body fat (%), body weight

(kilograms) and body height (centimeters) were calculated for each population. The Wilk-Shapiro ($5 \leq n \leq 2000$) test of normality (Dixon et al., 1990) or the skewness-kurtosis chi-squared test of normality ($n > 2000$) were used to test the normality of the variables. If a variable was not normal by the above test, the log transform was applied to the variable, and the test of normality was applied to the variable.

Variable Correlational Structure

Defining the distribution of a population requires the following measures: central tendency (i.e., mean and median), variability (i.e., standard deviation, minimum and maximum), relations between variables (i.e., correlation and covariance), and classification (i.e., normal or log-normal). The values of the statistics of the variables for each population were calculated. The Wilk-Shapiro ($5 \leq n \leq 2000$) test of normality (Dixon et al., 1990) or the skewness-kurtosis chi-squared test of normality ($n > 2000$) from Dixon et al., (1990) were used to examine the normality of the variables. If a variable was not normal according to these tests, the log transformation was applied and the test of normality was conducted.

The Pearson Product Moment Correlation Coefficients were found for the following variables: age (years), body fat (BF), body weight (BWKG in kilograms), and body height (BHCM in centimeters).

Population Equality

The equality of population means was tested using a one factorial (populations) multivariate analysis of variance on body fat, body weight, and body height. The assumption of equality of population variances was tested using a Levene's test for variances (Levene, 1960). If the Levene's test was significant ($p < 0.05$) for a particular variable , a Box-Cox transformation (Box, 1964) was applied to the variable. Then a one factorial multivariate analysis of variance was applied to the transformed and non-transformed variables. Bonferroni multiple comparisons were used for pairwise comparisons (Rosner, 1990).

RESULTS

Outliers

Since the data were generated by other laboratories within the Navy, it was necessary to unify fields, names, and specifications. Some outliers were identified when the multivariate test for outliers (principal component analysis) was applied to each community. Table 8.1-1 presents the ultimate sizes of the samples along with the original sample size in parentheses. The percent column indicates the percentage of sample size which were outliers (i.e., beyond three standard deviations from the mean). No outliers were removed. The only values removed were extreme values that were held to be obvious typographical errors. Other outliers were not removed since there was no justification for removal.

TABLE 8.1-1. OUTLIERS RESULTS FOR EACH POPULATION (P < 0.01)

	SAMPLE SIZE	PERCENT
BUDS	39	3
SEALS	48	4
DIVERS	144	8
MALE AVIATORS	150(192)[a]	7
FEMALE AVIATORS	38(58)[a]	11
MALE FLEET	2411	8
FEMALE FLEET	317(318)[b]	8

a = original sample size. Sample size was reduced because there were missing data.
b = original sample size. Sample size was reduced because there was an outlier which was an apparent entry error.
c = the percent is the number which are larger in absolute value than three standard deviations from the sample size.

The females from the fleet were reduced by one since one value (i.e., 1.6) was removed. This was believed to be an entry error since all other values received were presented in whole values. The aviators, both males and females, were reduced somewhat due to missing objective data points (i.e., body fat).

Determine the Distributions of the Population

Body fat was normally distributed (i.e., for BUDS, male aviators, females aviators, fleet males, and divers) or log-normally distributed (SEALS and fleet females) and is presented in Table 8.1-2. The SEALS showed a different distribution for body fat than did the BUDS who are trainees prior to becoming SEALS.

TABLE 8.1-2. DISTRUBUTION TEST FOR AGE (YEARS)

	DISTRIBUTION[a]	W STATISTICS[b]	p-value
BUDS	NOT NORMAL	0.8943	0.0012
SEALS	NOT NORMAL	0.9400	0.0240
DIVERS	NOT NORMAL	0.9718	0.0041
MALE AVIATORS	NOT NORMAL	0.8850	0.0000
FEMALE AVIATORS	NOT NORMAL	0.8384	0.0000
MALE FLEET[c]			
FEMALE FLEET	NOT NORMAL	0.9526	0.0000

a = the distribution is truncated normal since there is no data less than eighteen years of age.
b = the Shipiro-Wilk W statistics except the male fleet which has a large sample size (N > 2000).
c = data unavailable.

Describing the populations

The ages of the communities reflect the training required for entry into each of the populations. Those communities with several month-long entry courses following college (i.e., aviators) will often have a slightly older population. The SEALS with a series of courses and accomplishments may have a slightly older population, whereas the BUDS, because these individuals are in-training to become SEALS will be somewhat younger.

Height was normally distributed for all populations. These results are presented in Table 8.1-3.

TABLE 8.1-3 DISTRIBUTION TEST FOR BODY HEIGHT (CENTIMETERS)

	DISTRIBUTION	W STATISTIC[a]	p-value
BUDS	NORMAL	0.98	0.86
SEALS	NORMAL	0.98	0.79
DIVERS	NORMAL	0.98	0.66
MALE AVIATORS	NORMAL	0.97	0.06
FEMALE AVIATORS	NORMAL	0.96	0.18
MALE FLEET	NORMAL	5.90(2)[b]	0.05
FEMALE FLEET	NORMAL	2.73(2)[b]	0.25

a = the Wilk-Shapiro test of normality test statistic.
b = skewness-kurtosis test of normality using a chi-square (df).

Table 8.1-4 presents body weight which was either normally distributed (i.e., BUDS, SEALS, male and female aviators, and divers) or log-normally distributed (i.e., male and female fleet sailors).

TABLE 8.1-4. DISTRIBUTION TEST FOR BODY WEIGHT (KILOGRAMS)

	DISTRIBUTION	W STATISTIC[a]	p-value
BUDS	NORMAL	0.99	1.00
SEALS	NORMAL	0.96	0.13
DIVERS	NORMAL	0.98	0.22
MALE AVIATORS	NORMAL	0.98	0.61
FEMALE AVIATORS	NORMAL	0.96	0.12
MALE FLEET	LOG-NORMAL	1.96(2)[b]	0.37
FEMALE FLEET	LOG-NORMAL	0.99	0.78

a = the number which is obtained after a statistical test is done.
b = skewness-kurtosis test of normality using a chi-square (df).

Body fat is summarized and presented in Table 8.1-5. The means of body fat separated into three groups: (a) BUDS, (b) SEALS, male aviators, male fleet sailors, divers [which were not different from each other], and (c) female aviators and female fleet sailors [which were not different from each other]. There was a statistically significantly difference ($p<0.05$) between the the subpopulations. The BUDS clearly had smallest mean body fat. The other male groups were not different from each other, the females clearly had the larger body fat mean than the males. The difference between females and males was attributed to the differing maximum body fat limits for females (33%) compared to males (26%).

TABLE 8.1-5 DISTRIBUTION TEST FOR BODY FAT (%)

	DISTRIBUTION	STATISTIC[a]	p-value
BUDS	NORMAL	0.96	0.24
SEALS	LOG-NORMAL	0.97	0.40
DIVERS	NORMAL	0.97	0.16
MALE AVIATORS	NORMAL	0.98	0.18
FEMALE AVIATORS	NORMAL	0.96	0.20
MALE FLEET	NORMAL	2.68(2)[b]	0.26
FEMALE FLEET	LOG-NORMAL	0.98	0.29

a = the number which is obtained after a statistical test is done.
b = skewness-kurtosis test of normality using chi-square (df).

TABLE 8.1-6. DESCRIPTIVE STATISTICS AGE (YEARS)

	MEAN	MEDIAN	STD. DEV.	MIN	MAX
BUDS	22	21	2	19	28
DIVERS	25	24	4	18	37
SEALS	26	25	4	19	36
MALE AVIATORS	32	30	8	21	51
FEMALE AVIATORS	28	26	6	21	45
MALE FLEET	30	28	7	18	56
FEMALE FLEET	29	28	6	18	50

TABLE 8.1-7 DESCRIPTIVE STATISTICS BODY HEIGHT (CENTIMETERS)

	MEAN	MEDIAN	STD. DEV.	MIN	MAX
BUDS	178[b]	178	6	165	192
SEALS	178[b]	176	5	162	188
DIVERS	176[b]	175	6	161	196
MALE AVIATORS	180[b]	180	6	163	193
FEMALE AVIATORS	166[a]	165	7	155	180
MALE FLEET	178[b]	178	7	152	203
FEMALE FLEET	165[a]	165	7	150	184

a = significantly different than male at $p < 0.01$.
b = significantly different than female at $p < 0.01$.

TABLE 8.1-8 DESCRIPTIVE STATISTICS BODY WEIGHT (KILOGRAMS)

	MEAN	MEDIAN	STD. DEV.	MIN	MAX
BUDS	76[a]	76	6	63	91
SEALS	79[a]	79	8	67	103
DIVERS	77[a]	76	8	59	98
MALE AVIATORS	81[a]	81	9	54	86
FEMALE AVIATORS	62[b]	62	8	48	83
MALE FLEET	81[a]	79	12	46	143
FEMALE FLEET	62[b]	61	9	41	96

a = significantly different than female at $p < 0.01$.
b = significantly different than male at $p < 0.01$.

TABLE 8.1-9. DESCRIPTIVE STATISTICS BODY FAT (%)

	MEAN	MEDIAN	STD. DEV.	MIN	MAX
BUDS	10^{abcdeg}	11	2	5	15
SEALS	14^{cef}	14	3	9	26
DIVERS	13^{cef}	13	4	4	23
MALE AVIATORS	15^{cef}	14	4	2	30
FEMALE AVIATORS	22^{abdfg}	22	6	10	36
MALE FLEET	16^{cef}	16	5	1	33
FEMALE FLEET	24^{abdfg}	24	6	10	44

a = significantly different than SEALS at $p < 0.05$.
b = significantly different than male aviators at $p < 0.01$.
c = significantly different than female aviators at $p < 0.01$.
d = significantly different than male fleet at $p < 0.01$.
e = significantly different than female fleet at $p < 0.01$.
f = significantly different than BUDS at $p < 0.01$.
g = significantly different than divers at $p < 0.01$.

Structure of Correlations Between Variables

Tables 8.1-10 through 8.1-16 present results of analyses of correlations between the four variables for each of the seven populations. All four variables are presented although only three show on an axis at a time. As with most statistical presentations of matrices, only the top half is shown since the lower portion is symmetric.

TABLE 8.1-10 PEARSON CORRELATION COEFFICIENTS FOR BUDS

	BODY FAT	BODY WEIGHT	BODY HEIGHT
AGE	0.3120	0.1679	0.0822
BODY FAT		0.3194^{a}	0.0196
BODY WEIGHT			0.6718^{b}

a = significant at $p < 0.05$.
b = significant at $p < 0.01$.

The BUDS show body weight correlated with body fat and highly correlated with body height.

TABLE 8.1-11. PEARSON CORRELATION COEFFICIENTS FOR SEALS

	BODY FAT	BODY WEIGHT	BODY HEIGHT
AGE	0.2919[a]	0.0926	0.0602
BODY FAT		0.6301[b]	0.1034
BODY WEIGHT			0.5560[b]

a = significant at $p < 0.05$.
b = significant at $p < 0.01$.

The SEALS present age correlated with body fat, and body weight was highly correlated with body fat and body height.

TABLE 8.1-12. PEARSON CORRELATION COEFFICIENTS FOR DIVERS

	BODY FAT	BODY WEIGHT	BODY HEIGHT
AGE	0.2006[a]	0.1503	0.1123
BODY FAT		0.5867[b]	-0.0770
BODY WEIGHT			0.5175[b]

a = significant at $p < 0.05$.
b = significant at $p < 0.01$.

The divers show body fat highly correlated with body weight and correlated with age. Body weight was highly correlated with body height.

TABLE 8.1-13. PEARSON CORRELATION COEFFICIENTS FOR MALE AVIATORS

	BODY FAT	BODY WEIGHT	BODY HEIGHT
AGE	0.3102[a]	0.0589	0.0159
BODY FAT		0.5536[a]	0.0990
BODY WEIGHT			0.5132[a]

The male aviators show age highly correlated with body fat. Body weight was highly correlated with body fat and body height.

TABLE 8.1-14. PEARSON CORRELATION COEFFICIENTS FOR FEMALE AVIATORS

	BODY FAT	BODY WEIGHT	BODY HEIGHT
AGE	-0.1520	-0.1108	0.0913
BODY FAT		0.6992[a]	-0.1955
BODY WEIGHT			0.2469

a = significant at p < 0.01.

The female aviators present body fat was highly correlated with body weight.

TABLE 8.1-15. PEARSON CORRELATION COEFFICIENTS FOR MALE FLEET SAILORS

	BODY FAT	BODY WEIGHT	BODY HEIGHT
AGE	0.2934[a]	0.1417[a]	0.0224
BODY FAT		0.6642[a]	0.0452[b]
BODY WEIGHT			0.5459[a]

a = significant at p < 0.01.
b = significant at p < 0.05.

The male fleet sailors present body weight highly correlated with age, body fat, and body height. Body fat was highly correlated with age and also was correlated with body height.

TABLE 8.1-16. PEARSON CORRELATION COEFFICIENTS FOR FEMALE FLEET SAILORS

	BODY FAT	BODY WEIGHT	BODY HEIGHT
AGE	0.0519	0.0506	-0.0151
BODY FAT		0.6420[a]	-0.0235
BODY WEIGHT			0.5451[a]

a = significant at p < 0.01.

The female fleet sailors present show body weight highly correlated with body fat and body height.

DISCUSSION

In undertaking this work, we were interested in determining if some of the military occupational specialties might produce subpopulations of individuals that would be useful for estimating extremes of human population differences. We hypothesized that such populations might be found among the aviators, BUDS, SEALS, or divers. This seems to be the case for BUDS, who are statistically significantly lower in body fat percentage than are the other Navy populations. Analysis of the data obtained in this study indicates that Navy aviators and Navy divers are not statistically significantly different than fleet sailors in their body fat age and weight relationships. Navy females have higher body fat percentages than do their male counterparts at any given age. This latter finding is not surprising and is consistent with the general observations of the human .

The data we have obtained also suggest that while the "standard 70-kg man" is a useful surrogate for the human population in health hazard risk assessments targeted at determining a median effect for the human population, this approximation significantly underestimates the height, weight, and body fat of the average Navy male sailor. The reference more closely estimates Navy female sailors' height and weight, but underestimates their percentage body fat. It is anticipated that such mis-estimates would impact on predicted pharmacokinetics of exogenous xenobiotic chemicals. For chemicals that are highly fat soluble, internal uptake and storage will be higher than predicted and storage half-times should be significantly longer. Similarly, for highly water soluble (lipophilic) chemicals, uptake will be less than predicted and clearance will be shorter. The underestimate of size for mature adult males will result in smaller physiological compartments in physiologically based models, and thus overprediction of peak compartment concentrations of exogenous chemicals.

Age and body height are not commonly used in a PBPK modeling, but these data suggest that these parameters could be used along with appropriate mathematical formulae as a basis for including population effects in such models, albeit with some loss of accuracy. Age, height, and weight have the advantage that they are more readily assessable than body fat. No attempt should be made to incorporate the age and body height in the PBPK model itself. The Monte Carlo simulations of body fat and body weight which are used as PBPK model parameters should be found using the multivariate normal distribution.

Since body fat and body weight were correlated with age, the Navy should consider age- and body-height dependent body fat standards. When age increased, the body fat had a tendency to increase. Since most of the participants in the study were still in the Navy, it implies that the increase in body fat did affect the performance of an individual.

The study indicated that the lowest to highest body fat was the trainee, male Navy personnel, and female Navy personnel. The female Navy personnel had a body weight of about 62 kilograms; whereas, the male Navy personnel had a body weight in the high 70 kilograms or low 80 kilograms range. The female personnel body height was about 10 centimeters lower than male personnel.

ACKNOWLEDGMENTS

The authors gratefully acknowledge and thank the various commands and individuals that shared their data. Without these contributions of time, effort, and patience this work would not exist. This work was accomplished without involving a single new volunteer; all of the data previously existed and was gathered for other reasons. Many have tolerated multiple

requests for data in various forms; this has been due to our compulsive efforts to achieve numbers of individuals to take each variable into the shoulders of the Student's t distribution curve for each population. Particular thanks go to Jim Hodgdon and Marcie Beckett of Naval Health Research Center, San Diego, CA for multiple consultative sessions. Thanks, also, to Jim Kiesling of Naval Aviation Medical Institute, Pensacola, FL for various passes at his database.

Thanks, too, to Frank Goforth for his set of BUDS and SEALS data, which was the cornerstone from which this project was launched. We wish to thank Captain Frank Kelley at the Naval Hospital, San Diego, CA for his help gathering the data for the fleet sailors from Naval Station on 32nd Street, San Diego, CA. Thanks, also, to CDR Marcinik of Naval Medical Institute, Bethesda, MD for the divers' data.

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8.2 DATA COLLECTION INTERFACE FOR ISOLATED PERFUSED RAT LIVER: RECORDING OXYGEN CONSUMPTION, PERFUSION PRESSURE AND pH

J. Wyman, J. S. Stokes, M. Goehring[1], M. Buring,[1] and T. Moore[1]

ABSTRACT

A computer-aided data collection system was developed for use with an existing isolated rat liver perfusion apparatus. The system monitors and records perfusion time, oxygen consumption, perfusion pressure, and pH. Maintenance of perfusate pH is also accomplished using an electronically controlled solenoid valve for delivery of 95% O_2/5% CO_2 gas to the perfusion gas exchange system. Descriptions of software and hardware required to develop this system are provided.

INTRODUCTION

Isolated organ perfusion is a well established technique used routinely in many laboratories. Organs which are commonly perfused are lung (Ryrfeldt and Moldeus, 1993; Hulsmann and de-Johgste, 1993), liver (Gores, et al., 1986), kidney (Lieberthal, 1991; Nagami, 1990), heart (Anderson, et al., 1990; Ferrari, et al., 1993-), and stomach (Waisbren, et al., 1994). Advantages of isolated organ perfusion include elimination of non-tissue effects (humoral/endocrine control and neuronal regulation), maintenance of tissue architecture and physiologic cell-to-cell interaction, and the ability to administer xenobiotics and/or manipulate environmental conditions (such as pH or temperature) at levels which may be lethal *in vivo*.

Although the use of isolated perfused organs provides numerous advantages, a disadvantage common to all isolated organ preparations is that the tissue, with time, will become non-viable. A slow decline toward cell death begins at the time the tissue is isolated from the host organism. Death of an isolated tissue may result from a variety of insults including physical damage because of elevated perfusion pressure, inadequate perfusion causing insufficient oxygenation and loss of homeostasis through electrolyte and pH imbalances. The functional longevity of a particular organ preparation will depend on the tissue being perfused and its demand for oxygen, the physiologic function being monitored to assess viability, and the isolation and perfusion techniques being employed.

A variety of physiological and biochemical parameters have been used to assess viability of the isolated preparation (Sweeny and Diasio, 1991). Typically, the viability of isolated organs during perfusion has been assessed by monitoring perfusion pressure (i.e., resistance to flow), release of cellular enzymes, changes in perfusion ion composition and secretion of tissue specific substances (e.g., bile flow and composition by the isolated perfused liver), and/or measurement of oxygen consumption over time.

The collection and analysis of data to assess viability of isolated organs are often done manually and can be time consuming and tedious. The current paper describes data collection and analysis controlled by a computer program linked to a system designed to allow perfusion of isolated rat livers. The specific parameters monitored during a 2.5-h perfusion included pH, perfusion pressures (pre- and post-liver), and oxygen consumption. In addition, the computer program controls the pH of the

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perfusate by adjusting the delivery of O_2 and CO_2 into the perfusion system "lung." Although the perfusion system described is for that of the isolated rat liver, the data collection system can be applied to a variety of perfusion systems used for many different types of tissue.

EXPERIMENTAL PROCEDURE

System Design

The focus of this paper is to provide a description of the computer interface which enables the collection of data on viability parameters for the isolated perfused liver. To better understand the application and design of the automated portion of the system, a brief description of the general perfusion system is provided. The isolated perfused rat liver system used in our laboratory (Figure 8.2-1) was originally developed by Gollan et al., 1981. The perfusion circuit is maintained at a constant

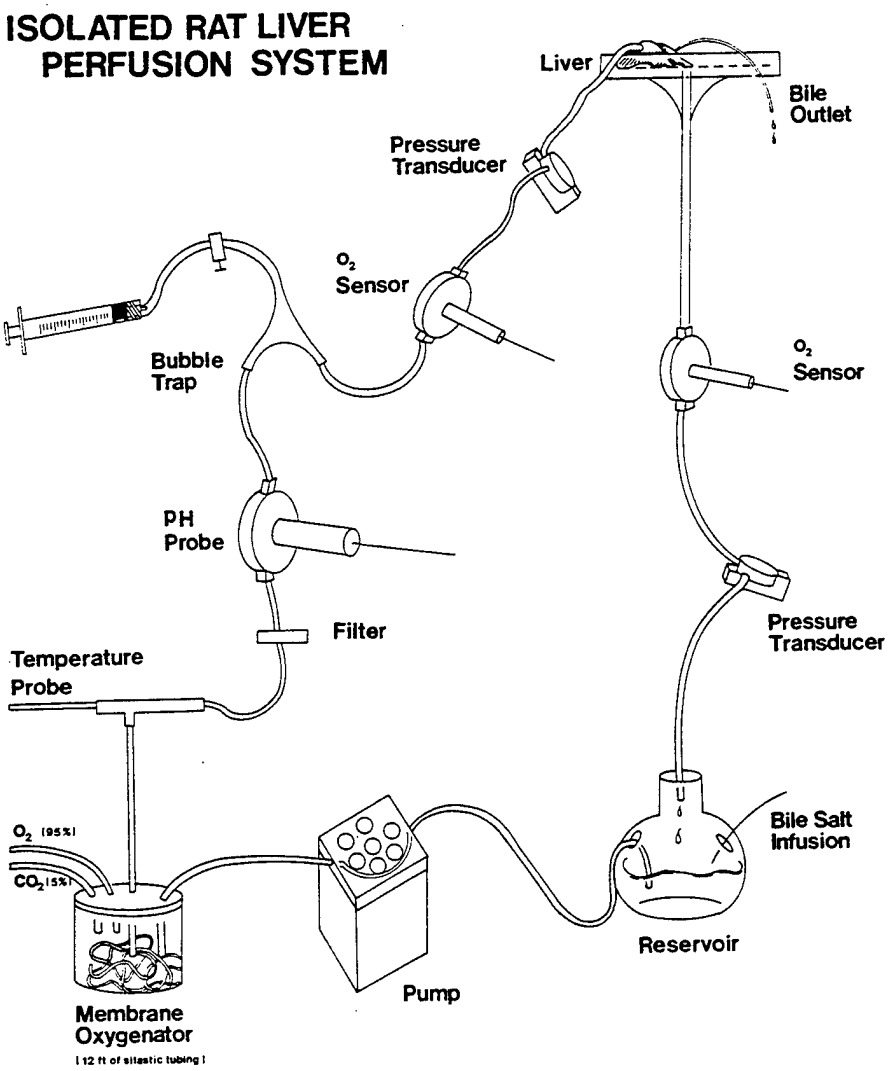

Figure 8.2-1. Diagram of the Isolated Perfused Liver System. This system was housed in a two-compartment Lucite® cabinet.

temperature (37 °C) inside a two-compartment, Lucite® cabinet. Recirculation of perfusate is accomplished using a peristaltic pump (LKB Multiperpex 2115, Bromma, Sweden). (Note: The authors understand that this pump is no longer manufactured. An alternative pump that has proven satisfactory, [Bras, personal communication, 1994] is the Watson-Marlow Model 5300 pump [Watson-Marlow Ltd., Falmouth, Cornwall TR11 4RU, England]).

The following is a description of the perfusion circuit sequence of circulation. The perfusate is in a glass reservoir which sits on a stir plate and allows continuous mixing of the perfusate. The reservoir has two side ports through which perfusate samples can be added or removed, and through which bile salts (sodium taurocholate, 18 mg/mL) are infused at a rate of 1.0 mL/h. Leaving the reservoir, the perfusate moves through the pump to a membrane "lung" (Hamilton, et al., 1974) (made of 12 ft coiled Silastic tubing, 0.147 cm i.d., 0.196 cm o.d., Dow Corning Corp., Midland, MI) and on to a temperature-sensitive electrode (#403 temperature probe, Yellow Springs Instruments [YSI], Yellow Springs, OH). The electrode is connected to a thermostat and the perfusate is maintained at 37 °C through temperature controllers (Model 63 RC, Yellow Springs Instruments) and fans (Pamotor Miniature Fan, Model 3000, Burlingame, CA) placed in both compartments of the cabinet. Perfusate moves next through a stainless steel filter screen (Milipore) to an inverted "Y" bubble trap. Before the perfusate reaches the liver it travels through a "pre-liver" oxygen-sensitive probe (connected to an oxygen monitor) and a pressure transducer. The perfusate enters the liver through a 16-gauge catheter inserted in the portal vein. Upon leaving the liver, the perfusate passes through the "post-liver" oxygen probe connected to the oxygen monitor and to the down-line pressure transducer before returning to the reservoir.

The perfusate is oxygenated with a mixture of 95% O_2 and 5% CO_2 delivered (approximately 2 liter/min) to the screw top jar which holds the Silastic tubing. Due to the reduced oxygen carrying capacity of a hemoglobin-free perfusion medium, the delivery of O_2 to the liver is enhanced by an increased flow rate (25 mL/min). Rat hepatic blood flow rates *in situ* have been determined to be from 0.5 to 2.0 mL/min per g liver (Steiner and Mueller, 1961). In addition to the 95% O_2 /5% CO_2, the "lung" is also connected to 100 % O_2 , and the pH of the perfusate is maintained ± 0.1 pH units) by varying the relative delivery of the two gases through a computer modulated (discussed below) solenoid valve (Model B2DA1052, Skinner Valve Div., Honeywell, Inc., New Britain, CT). The YSI O_2 electrodes and pH electrode were modified to allow flow-through measurements by mounting the electrodes in Plexiglas® housing containing a "T" canal.

Hepatectomy

Animals are anesthetized and livers are removed and prepared for perfusion as previously described (Gollan, et al., 1981; Hems, et al., 1966). Diethyl ether is used as an anesthetic in our laboratory because of its minimal effects on hepatic detoxification pathways. Fischer 344 rats, weighing between 200 and 350 g are typically used . To allow transfer of the liver to the perfusion cabinet, the bile duct is cannulated using PE-10 tubing, the portal vein and inferior vena cava (above the diaphragm) are both cannulated with 16 gauge catheters (Angiocath®, Deseret Pharmaceuticals, Sandy, UT). The liver is flushed with 3.0 mL sterile heparin (1000 units/mL) (Elkins-Sinn, Cherry Hill, NJ)/saline (.154 M) (50/50 mix) followed by continuous perfusion with Krebs-Henseleit-Ringer bicarbonate solution (Dawson and McKenzie, 1969) containing 11.5 mM glucose. Following dissection from the rat, the liver is rinsed with warm saline and transferred to the perfusion cabinet where it is placed on a nylon mesh inside an open Petri dish. The placement of the liver takes place in two steps (see Figure 8.2-1). First, the vena cava Angiocath® is connected, using a Luer lock hub, to tubing exiting the reservoir and Petri dish. Once connected, this tubing is pulled through the neck of the Petri dish until the liver will lie flat in the dish. The second

step in the placement requires disconnecting the portal vein Angiocath® from the Krebs-Henseleit-Ringer solution and reconnecting the Angiocath®-Luer lock hub to the perfusion circuit. The time required for surgery is normally 15-20 min.

Although a variety of perfusion media have been used, we routinely perfuse with Krebs-Henseleit-Ringers medium which is well oxygenated and carefully pH controlled. Depending on the particular xenobiotic being studied, 2 to 5% bovine serum albumin (Gores et al., 1986; Gollan et al., 1981) may be included in the perfusion medium to enhance solubility and delivery of the xenobiotic, as well as to improve the overall longevity of the preparation by maintaining oncotic pressure.

Viability Parameters Monitored

In addition to oxygen consumption and pressure measurements during a perfusion, bile flow and release of hepatic enzymes into the perfusate are monitored. Bile flow is determined by collecting bile in conical tubes and weighing the tared tubes at appropriate intervals. Enzyme activity (lactate dehydrogenase of sorbitol dehydrogenase) is determined using a CobasBio® centrifugal analyzer (Roche Analytical Instruments, Inc.) with enzyme assay kits obtained from Sigma Chemical.

Data Collection Design

The goal was to acquire and digitize two analog signals from an instrument connected to two oxygen sensing probes, to display the values on a personal computer monitor along with the time of day, and to record these values in a data file. This was later expanded to include a pH input, two pressure inputs, and an output to control the pH level. The CRT display for the data presentation is shown as Figure 8.2-2.

Oxygen Consumption Calculations and Hardware

The solubility of O_2 in the perfusate (mL/mL perfusate) was calculated from values provided by YSI, Yellow Springs, OH, obtained from (Umbreit et al., 1964). The solubility of O_2 in the perfusate was linear over a concentration from 0 to 100% in the gas phase. Since the YSI O_2 monitor reports O_2 concentration as a percent, the volume of O_2 dissolved in the perfusate was determined from linear regression analysis using the following equation:

$$\text{mL } O_2/\text{mL perfusate} = 0.240\ (\%\ O_2 \text{ monitor reading}) + 4.74 \times 10^{-3}$$

Prior to each perfusion, the cellophane membrane for each O_2 electrode was replaced as described by the manufacturer. Calibration of the electrodes was performed by placing electrodes in Krebs medium which was saturated with air, followed by saturation with 100% O_2. Consumption of O_2 (mL/min) was determined by subtracting the calculated values for the pre- and post-liver concentration of O_2 (mL/mL) multiplied by a perfusate flow of 25 mL/min.

PERFUSED LIVER
Project No.

00-00-94 00:00:00
Date Time

Comments:

pH: 7.39 CO2: OFF (or ON)[A]

	Oxygen			Flow Rate: ml/min	Pressure
In:	[B]95.0%	[C]22.8 ul/ml	570.0 ul/min	25.0	22.5 mm Hg
Out:	8.3%	2.0 ul/ml	49.8 ul/min	25.0	18.6 mm Hg
			[D]520.2 ul/min consumed		

2FLOW- 3FLOW+ 4WATCH 5RECORD 6 7 PO OFF 8CALIB 9 10EXIT

Figure 8.2-2. Computer Display During a Typical Control Perfusion.

[a] The solenoid valve which controlled the flow of 95% O_2 /5 % CO_2 was activated electronically by pH output values. The set points for switching the valve open and closed were pH = 7.45 and pH = 7.35, respectively.

[b] Percent saturation of oxygen in the perfusate.

[c] Concentration of oxygen calculated from (a) as described by (Umbriet, et al., 1964).

[d] Calculated volume of oxygen consumed based on a perfusate flow rate of 25 mL/min.

Opto22 analog to digital (A to D) hardware was selected to acquire and digitize the analog signals because it is easy to implement and modify (one plug-in input module for each input/output signal), and because of its reasonable cost. An eight position mother board (model PB8AH with model B2 "Brain Board") had sufficient capacity to accommodate all signals needed with additional room for expansion. A schematic of hardware component connections is shown as Figure 8.2-3.

The oxygen analyzer (Model 5300, Yellow Springs Instruments, Yellow Springs, OH) provided a 0 to 1 volt analog signal with common (chassis ground) connected to the "+" or red output terminal. Opto 22 model AD-13T 0 to 100 mV electrically isolated input modules were used for the two oxygen analog inputs. To scale the 0 to 1 volt signal down to a 0 to 100 mV range (10:1), two adjustable resistive voltage dividers were used. The resistive divider, which appears to the AD-13T module as an approximately 9.09 kilo-ohm impedance, and to the YSI oxygen analyzer as a 100 kilo-ohm load, needed to be of sufficiently high impedance so as not to load the oxygen analyzer (> 20 kilo-ohm), and of sufficiently low impedance as to not be affected by the AD-13T input module input impedance. The AD-13T (maximum recommended 10K source resistance) has a 100 megaohm input impedance. With 1.000 volt provided by a Data Precision 8100 DC Secondary Voltage Standard applied to the resistive divider input, each voltage divider was adjusted to provide a 100 mV output as measured with a digital voltmeter (Fluke 8024A) .Hardware for pH and Pressure Measurements.

The analog output from the Orion model 611 pH meter was connected to a Opto22 AD13T 0-100 millivolt isolated input module. Prior to each day of perfusion, pH electrodes were calibrated at pH = 7.00 using a standard calibration solution.

The Hewlett-Packard model 1290C Universal Quartz Transducer pressure transducers used to measure blood pressure can accept either an AC or DC excitation voltage. When used with a DC excitation (range 5 to 10 volts DC) they produce an output of 5 microvolts per volt of excitation per mm Hg applied pressure. A DC excitation voltage of 8 volts was chosen, as this will provide 40 microvolts of output per mmHg of applied pressure. Each transducer signal is applied to an Opto22 AD-9T 0-50 millivolt input module. The AD-9T has a resolution of 4096 counts for its 0-50 millivolt input giving us 12.21 microvolt/digit resolution.

A precision 8 volt DC source was designed using a National Semiconductor LM399H 6.95 volt precision reference and operational amplifier voltage follower/amplifier to provide the increase to 8 volts and supply the current needed by the 1290C transducers. The 1290C transducers appear as a 1500 ohm load and a 800 microamp nominal constant current sink, giving a total nominal current drain on the 8 volt source of 6.13 milliamps for each transducer.

Calibration of the pressure transducers was accomplished at the beginning of each day of perfusions by connecting the flow-through cell of the transducer to a blood pressure bulb and gauge. Typically, the set point for the pressure within the cell would be 50 torr.

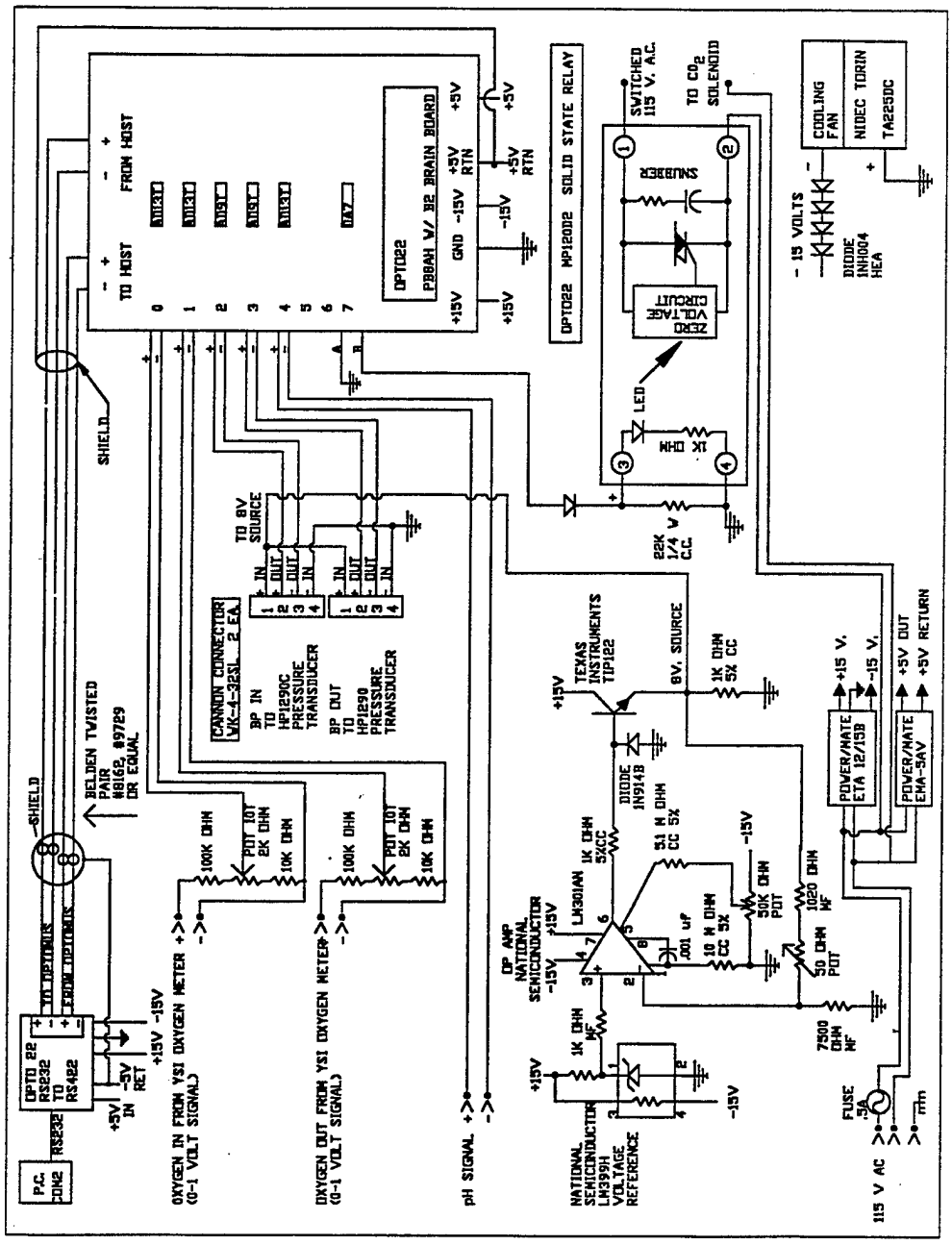

Figure 8.2-3. Schematic of Hardware Connections for Data Collection Interface

Hardware for Control of CO_2

An Opto22 model DA7 -10 to +10 volts DC output module was used to control an Opto22 MP120D4 solid state relay switching 115 volts AC to control a solenoid in the CO_2 line. The target pH was 7.4, so the solenoid was turned on when the analog signal from the pH meter indicated the pH was > 7.5 and turned off when the pH was < 7.3. The + and - 15 volt power and + 5 volt power are provided by a PowerMate® ETA 12/15B (+/- 15 volts @ .5 amp) and a PowerMate EMA 5AV (+5 volts @ 1.2 amps).

Cooling Fan

A Nidek Torin model TA225DC (12V, .08A) fan and a vent hole were added to cool the previously closed chassis. The 15 volt power was reduced to the required 12 volts with four 1N4004 forward biased power diodes in series. The -15 volt power supply was used because it had the least load.

Personal Computer

The personal computer is connected to the data acquisition system by way of its serial port (COM2:). A standard 25 pin connector (male on one end, female on the other) modem cable is used to connect the personal computer's serial port to the Opto22 AC7 converter which is connected to the Opto22 B2 Brain Board. A call in the BASIC language program (CALL OPTOWARE...) is used to interrogate the Opto22 motherboard and its installed modules. Each module returns a number representing the input signal it sees. For instance, the AD13T (0 to 100 millivolt input) module would return the output shown in Table 8.2-1.

TABLE 8.2-1. AD13T MODULE: APPLIED VOLTAGES AND CORRESPONDING OUTPUT NUMBERS.

Applied voltage:	Number returned:
0 millivolt	10
25 millivolt	1,024
50 millivolt	2,048
75 millivolt	3,072
100 millivolt	4,096

Values for Perfusion Viability Measures

For non-treated livers, oxygen consumption ranged from 500 to 700 mL/min/g liver, or under the conditions of temperature and pressure (37 °C and 740 torr, respectively), approximately 1.9 to 2.6 mmol O_2/min g liver. These values compare favorably with the range of 2.0 to 2.64 mmol min/g liver previously reported (Sugano et al., 1978; van Dyle et. al., 1983).

Figure 8.2-4 depicts the level of LDH activity over time for naive livers from Fischer 344 rats. The release of LDH, and other hepatic enzymes (not shown) begins to increase rapidly after approximately 2 h perfusion. Release of hepatic enzymes

indicates a loss of integrity of the cell membrane, but is not a definitive measure of cell death. However, because of elevated perfusate enzyme levels, liver perfusions are typically not performed for periods longer than 2.5 h in our laboratory. We have found that if longer perfusion times are required, a perfusate with greater oxygen carrying capacity can be substituted for the Krebs-Henseleit-Ringer solution, such as a hemoglobin, heparinized red blood cell preparations, or a perfluorocarbon emulsion. Use of a fluorocarbon emulsion has reportedly enabled perfusion periods up to 20 h, with release of liver enzymes occurring after 10 h perfusion. (Krone et al., 1974).

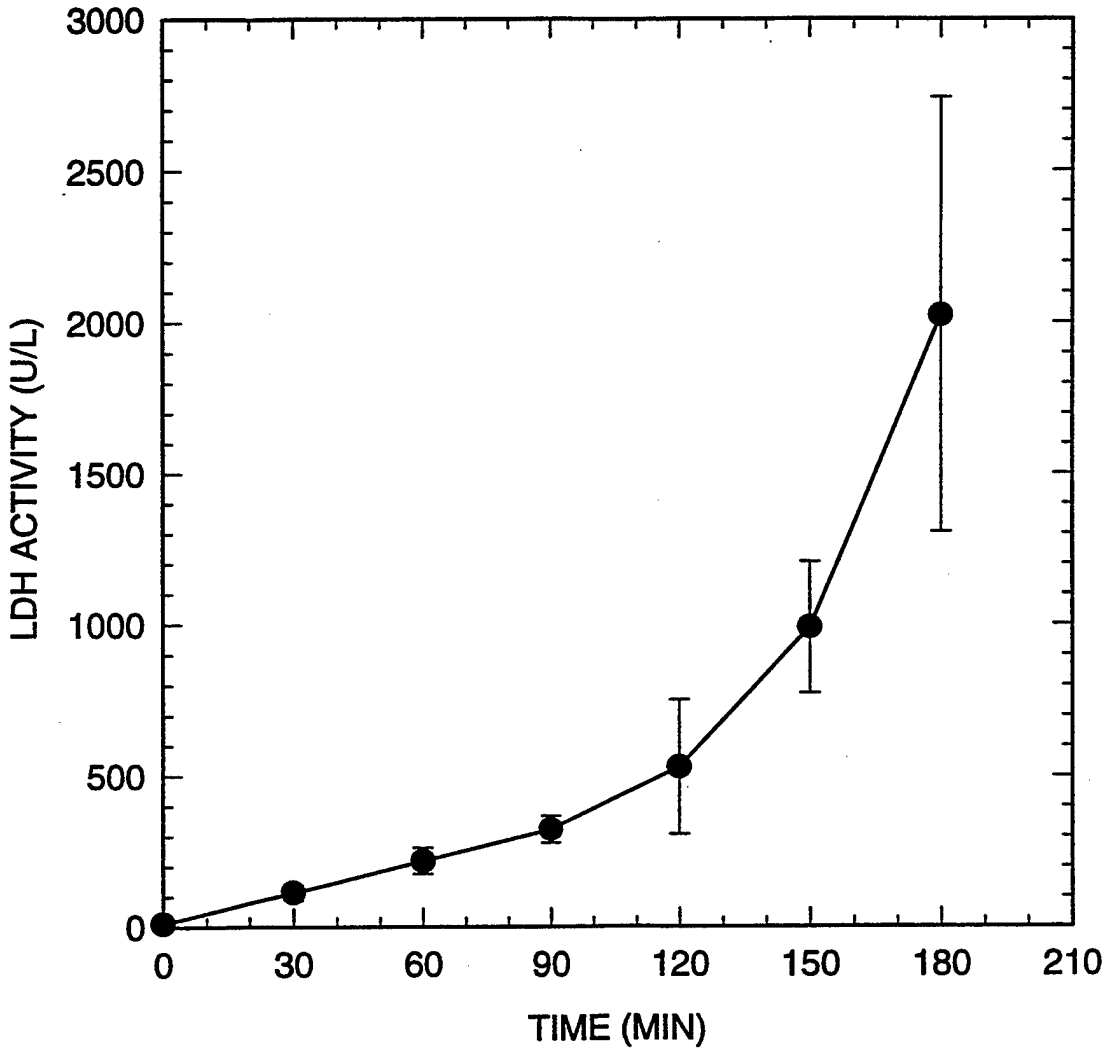

Figure 8.2-4. Temporal Release of Lactic Acid Dehydrogenase by Perfused Rat Livers. Values represent the mean ±S.D. for five determinations. See Materials and Methods for assay conditions. Livers were perfused in a recirculating mode.

The amount of bile collected from naive livers begins to decline after 2.5 h (Figure 8.2-5). After the first 30 min of acclimation, bile flow stabilized around the 400 µL/h level for a period of 2 h. Expressed as µL/min/g liver, bile flow was in the range of 0.6 to 0.8 µL/min/g liver, which is lower than values previously reported in the literature (Boyer and Klatskin, 1970).

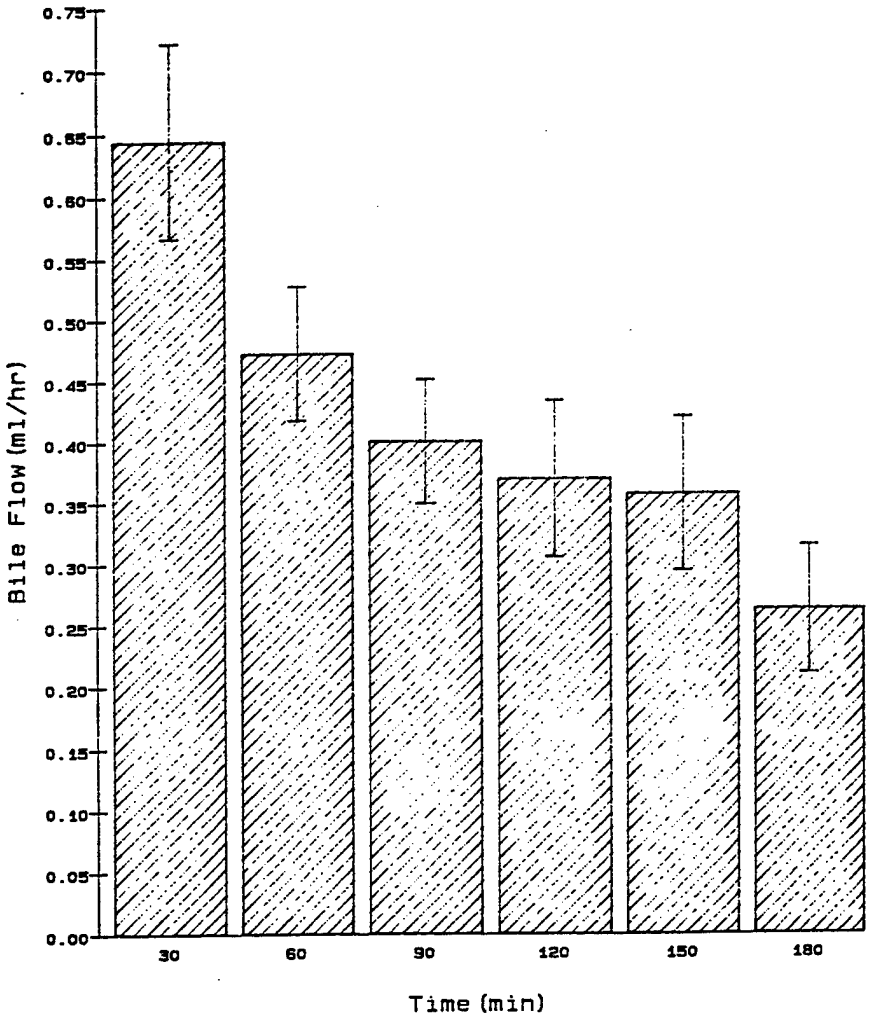

Figure 8.2-5. Cumulative Bile Flow from Isolated Perfused Livers of Fischer 344 Rats. Flow determinations are based on weights of bile collected during 30 min intervals. The volume of bile was converted from the weight by assuming a density = 1. Data are reported as the mean ±S.D. (n = 5).

The values reported (livers from Sprague Dawley rats) were on the order of 0.8 to 3.12 μL/min/g liver. An explanation for lower bile flow values may lie in the particular strain of rat being used. Under the same experimental conditions, we have observed that choleresis is variable from one strain of rat to another, with Sprague Dawley rats producing more bile than Fischer 344 rats (unpublished results). Previous studies have shown differences in the biliary excretion rates of sulfobromophthalein and bilirubin among different strains of rat (Klassen et al., 1969).

The perfusion system described here is a constant flow, variable pressure design which uses a peristaltic pump to control the flow. The perfusion pressures which were typically observed in a non-treated liver were in the range of 3 to 10 mm/Hg, which is consistent with hepatic perfusion pressures of 8 to 10 mm/Hg which are observed *in vivo* (Gores et al., 1986).

SUMMARY

Isolated organ perfusion with computer-aided data collection allows close control of viability parameters such as O_2 consumption, pH, and perfusion pressures. The monitoring of oxygen consumption using Clark electrodes (Sweeny and Diasio, 1991) is easier than previous methods of drawing pre- and post-liver samples and performing blood gas analysis (Gores et al., 1986). The addition of computer-aided data collection to flow-through Clark electrodes, as described in this paper, is an easier method and provides a more complete record. Similarly, the use of in-line pressure transducers with computer data collection provides an easy method of monitoring and retaining pressure measurements. The use of a computer-controlled solenoid valve to regulate the flow of 95% O_2/5% CO_2 allows close control of perfusate pH. Without the valve, the pH must be closely monitored and the flow of gases continually adjusted to maintain control. Because data collection is easier and viability parameters can be more tightly controlled, as compared to manual adjustment methods, the overall reproducibility of results from one preparation to the next is improved.

The computer program controlling these processes is available upon request. The program was written for use with the isolated perfused rat liver using the hardware described above. Since the reader's specific application and choice of hardware may differ from that chosen by the authors, certain portions of the program may require specific re-writes to adapt it to the needs of the reader. A list of software and hardware used and possible vendors, the program code, and the specific sequence of steps for operating the program are provided as an appendix to this paper.

ACKNOWLEDGMENTS

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APPENDIX 8.2-A

Hardware Components:

Opto22 model PB8AH, 8 position motherboard with B2 Brain Board

Opto22 model AC7, RS-232 to RS-422/485 converter

PowerMate® models ETA 12/15B and EMA 5AV power supplies

Nidek Torin, model TA225DC fan

Opto22 I/O (Input/Output) modules

AD9T, 2 ea.

AD13T, 3 ea.

DA7, 1 ea.

Chassis, misc. connectors/hardware

Software:

Microsoft Quick Basic Compiler Ver. 4.5; Opto22 Optoware Analog/Digital Driver Ver. 4.04

Vendors addresses:

Opto22 (800)321-6786
43044 Business Park Drive
Temecula, CA 92590-3665

Nidek Torin (203)482-4422
100 Franklin Drive
Torrington, CT 06790

Power/Mate (800)843-8544
2727 Kurtz Stret
San Diego, CA 92110

Microsoft (800)426-9400
16011 NE 36th Way
Box 97017
Redmond, WA 98073-9717

Program Operation:

The data collection program was initialized using the following sequence of steps: At the C:\ prompt, type "g" (without quotation marks), then return; enter flow rate value, then return (the authors use 25 mL/min); to begin calibration of the pressure transducers, key F7 followed by F8 (both in order); then 50 enter (set point chosen arbitrarily). Pressure transducers are independently calibrated using the bulb and gauge from a blood pressure cuff (sphygmomanometer). Starting with "pressure in" (top compartment of the cabinet) the bulb and transducer are pressurized to 50 mm/Hg; then, press return. This bulb and gauge procedure is repeated to calibrate the "pressure out" (lower compartment transducer) and then F7 and F8 are pressed again to turn off calibration mode and return to the main program. Once transducers, pH and O_2 probes (discussed above) have been calibrated, the data collection program is started by pressing F5. The program display layout is shown in Figure 8.2-2. At the end of the perfusion, data collection is stopped by pressing F10.

SECTION 9

CONFERENCE SUPPORT

9.1 1995 TRI-SERVICE TOXICOLOGY CONFERENCE ON RISK ASSESSMENT ISSUES FOR SENSITIVE HUMAN POPULATIONS

L. A. Doncaster

The Conference on Risk Assessment Issues for Sensitive Human Populations was held at the Hope Hotel and Conference Center, Wright-Patterson Air Force Base, OH, from 25 through 27 April 1995. The conference was sponsored by Tri-Service Toxicology, Wright-Patterson Air Force Base; the Office of Naval Research, United States Environmental Protection Agency; and the Division of Toxicology, Agency for Toxic Substances and Disease Registry; and was coordinated by ManTech Environmental Technology, Inc., Toxic Hazards Research Unit.

The goals of the conference were (1) identification of sensitive populations in the risk assessment process; (2) understanding biological differences and species variability; and (3) examination of the mechanisms for dealing with sensitivities in hazard identification, dose response, and exposure characterization. The conference was divided into five half-day sessions, excluding evening poster and database sessions.

Session I of the conference dealt with the biological variability in risk assessment for human populations and included presentations on human interindividual variability in susceptibility to toxic effects (D. Hattis); developmental variability in toxicology (M.E. Mortensen); the influence of sociodemographic factors on susceptibility to toxic chemicals (K. Sexton); a quantitative cancer assessment for vinyl chloride - indications of early-life sensitivity (V.J. Cogliano); efficient tissue repair that underlies the resiliency of postnatally developing rats to chlordecone plus CCl_4 hepatotoxicity (H. M. Mehendale); and phenotypic variation in xenobiotic metabolism and adverse environmental responses (S.A. McFadden).

The subject of Session II was multiple chemical sensitivity (MCS) - clinical, experimental, and theoretical considerations. Included were discussions on chemical sensitivity - symptom, syndrome, or mechanism for disease (C.S. Miller); neurobehavioral models of multiple chemical sensitivity - kindling, kindling-like phenomena, and sensitization (J. Rossi); clinically relevant EEG studies and psychophysiological findings - possible neural mechanisms for multiple chemical sensitivity (I.R. Bell); a potential animal model of MCS with cholinergic supersensitivity (D. Overstreet); and a proposed animal neurosensitization model for MCS in studies with formaldehyde (B. Sorg).

Session III was concerned with the responses of special human subpopulations to toxicants and included presentations on the use of the non-smoking Utah Mormon population in the study of health effects of pollutants (C. A Pope); the Navajos and their experience of uranium mining (L. Abel); manganese biokinetics and toxicity - the influence of iron status (C.L. Kean); environmental justice issues - methylmercury exposure among subsistence fishing and hunting subpopulations (K. R. Mahaffey); the sensitivity of children to heavy metal toxicity (H. Choudhury); methylmercury poisoning in Iraq - an exercise in defining sensitive human subpopulations (J. L. Cicmanec); the biochemical genetics of multiple chemical sensitivity (P.P. Wilcox); and the role of nutrition in the survival after hepatotoxic injury (S. Chandra).

Presentations in Session IV dealt with occupational and environmental exposure case studies and included discussions on an overview of diisocyanate-induced occupational asthma (J. A. Bernstein); histamine releasing factors in diisocyanate-induced occupational asthma (Z.L. Lummus); the development of non-invasive biomarkers for carcinogen-DNA adduct analysis in

occupationally exposed humans (G. Talaska); possible health risks from low level exposure to beryllium (A.W. Stange); the sampling and analysis of airborne resin acids derived from heated colophony (rosin) flux - quantification of exposure to sensitizing compounds liberated during electronics soldering (P.A. Smith); exposure to anthropogenic mercury emissions in the United States (G.E. Rice); and the exposure-response functions in Air Force toxic risk modeling (L.L Philipson and J.M. Hudson).

Session V discussed incorporating susceptibility into risk assessment and included presentations on the incorporation of information about susceptible populations into risk assessments (J. Grassman); noncancer toxicity of trichloroethylene - evaluating adverse effects and sensitive populations in the development of oral dose-response values (H.A Barton); the impact of human variability and parameter uncertainty on risks predicted by a physiologically based pharmacokinetic (PBPK) model for chloroform (B.C. Allen); the risk assessment policy for evaluating reproductive system toxicants and the impact of responses of susceptible populations (G.B. Briggs); the use of PBPK modeling to investigate individual versus population risk (H.J. Clewell); and the interindividual variations in susceptibility - risk assessment and risk management (R.J. Preston).

Evening poster and database sessions provided additional scientific information exchange in an informal manner. There were 32 oral presentations, 47 poster presentations, 5 database presentations, and 254 participants in the 3-day conference. The conference was rated highly by the participants. Proceedings of the conference will be published in *Toxicology* and assigned a Government Technical Report number.

9.2 1996 TRI-SERVICE TOXICOLOGY CONFERENCE ON ADVANCES IN TOXICOLOGY AND APPLICATIONS TO RISK ASSESSMENT

L. A. Doncaster

Planning was initiated in October 1995 for the 1996 toxicology conference, "Conference on Advances in Toxicology and Applications to Risk Assessment." It will be held 23 through 25 April 1996 at the Hope Hotel and Conference Center at Wright-Patterson Air Force Base, OH. The conference will be sponsored by Tri-Service Toxicology, Wright-Patterson Air Force Base; the National Center for Environmental Assessment, United States Environmental Protection Agency; and the Division of Toxicology, Agency for Toxic Substances and Disease Registry. The planning committee for the conference includes representatives from each sponsoring agency and the Toxic Hazards Research Unit (THRU).

The goals of the conference are (1) exploration of new methodologies for human and ecological risk assessments; (2) application of guidelines and models in the risk assessment process; and (3) examination of the issues and approaches for communicating risk.

The THRU's Work Plan, which designated a conference coordinator and described invitation and registration procedures, publication procedures, technical support, and the administration of continuing education credits, was submitted to and approved by the Contract Technical Monitor.

Initial conference announcements have been placed in four major scientific journals and the conference announcement has been mailed to over 2800 individuals.

SECTION 10

RESEARCH SUPPORT

10.1 RESEARCH ENGINEERING/FABRICATION SPECIAL PROJECTS

D.L. Courson, H.F. Leahy, W.J. Malcomb, and W.B. Sonntag

The Toxic Hazards Research Unit (THRU) Research Engineering/Fabrication staff has provided technical assistance on a number of research and special projects. This report describes the devices, instruments, and systems that were designed, fabricated, and applied to support several THRU projects. In addition to performing routine maintenance of specialized research systems at the THRU, major renovations of existing systems were undertaken to address efficiency and safety of operation. Other systems that were developed by the Research Engineering/Fabrication Group are described in separate sections of this annual report.

Construction of the Hydra Farm and Hydra Farm Enclosure

The THRU Research Engineering/Fabrication group (RE/F) was tasked to construct a hydra farm and filtration system. The task consisted of the construction of shrimp sieves, farm screens, test dish holders, a filtration system, and a hydra aquarium (farm). This system is to support the establishment of a hydra assay in support of the Defense Women's Health Research Program.

Research Engineering/Fabricaiton staff also was tasked to assist in cleaning Building 429 during development of the hydra laboratory. The RE/F group also fabricated a containment unit/enclosure for the hydra farm. To maintain an operating temperature of 18 to 22 °C, a Koldwave water-cooled air conditioning/heat pump unit was purchased and installed for temperature control of the hydra farm enclosure.

The hydra farm consists of a plexiglas aquarium (2 in. × 13 in. × 48 in.) standing on 6 in. legs with a dust cover lid, and was constructed to house hydra in a clean, natural environment. On one end, two ports allow water to enter the farm where a deflector screen slows the flow of the water into the farm. A dam placed in back of the deflector screen allows the stream of water to run smoothly into the aquarium and not disturb the hydra. Constant fresh air is pumped through concentrically placed holes in the floor of the farm allowing hydra to propagate freely throughout the aquarium. A gate was placed at the opposite end of the farm to allow the farm to be connected to a filtration system.

The filtration system was designed to separate hydra from the farm, to clean the habitat, and to recirculate the hydra medium. This filtering chamber, or reservoir (6¾ in.× 13¾ in. × 16 in.), is divided into three separate chambers. The first chamber was designed to let the media flow through a farm filter (140 × 140 mesh) to screen out any loose hydra, shrimp, or debris. The flow continues into a second chamber where charcoal or filter floss can be contained. As the media flows across this chamber it enters the third chamber where a pump circulates the filtered media back into the farm. Hydra require a cool environment to thrive. To insure this a constant-flow water chamber was fabricated underneath the aquarium to use as an emergency cooling system. Two complete hydra farm systems were constructed.

Design and Construction of a Stainless Steel Nose-Only Inhalation Chamber

The Tri-Service Toxicology's Pharmacokinetics Group requested the THRU to design and fabricate a nose-only inhalation chamber out of stainless steel. This chamber design is similar to a Cannon chamber(Cannon, 1983) and will be connected in-line with the Leach chamber system, (Leach, 1963).

The main chamber body (3.5 in. x 3.0 in.) was constructed with 12 ports in 3 sets that were 90° apar, and offset 45°. The airtight inhalation system has an intake flow going into a 1 in. stainless steel inner tube that follows an in-line port matching the outside chamber and connected by ¼ in. tubes.

Plethysmograph tubes are linked together and exhausted through the bottom of the chamber. The chamber is attached to a 6 in. high turntable stand which alllows the chamber to rotate 360°. Two Legris fittings are used to join this system to the Leach system.

Fifteen nose-only Plexiglas rodent restraining tubes were constructed. The tubes were built with a taper nose port to fit the chamber's O-rings. The restraining tube dimensions are 3 in..x 2½in. x 10 in. length with a septum port centered in the mechanism.

The back plug was designed to slide on two O-rings to secure the rodent in the proper location. The plug has two ports. The first port is a stainless steel tube for holding the tail and to control the temperature. The second port is to hold the animal's tail outside of the restraining tube for tail vein blood sampling. This system was downsized to allow for use within an exhaust hood. Two chamber systems were fabricated.

Design and Construction of a Transportable Projector and Accessories Case

The AL/OET Division Director requested the design and fabrication of a projector case for shipping and storing a projector with accessories. The original manufacturer's case was bulky, heavy, and inconvenient to use. A special vehicle and two couriers had to be used to ship the original case.

The new prototype has been made with Garolite (approximately one-half the weight of aluminum) with 1/8 in. x 3/4 in. aluminum angles for framing. The inside of the case was lined with 1 in. foam to secure the projector and accessories. The case was made with side leather handles for handling and carrying. The total size (33 in. x 23 in. x 11 3/8 in.) will fit into any vehicle for transporting.

The projector fits into the bottom section of the case that is hinged for easy accessibility. An adjustable spring locking hasp is mounted on the sides to lock the door in place and secure the projector during shipping. The top section is hinged on the opposite side of the bottom door to easily reach the accessories. The case is connected with an adjustable clamp and hasp including a combination lock for security. The case is mounted on four sets of freely rotating wheels with a suitcase handle on the top for ease in handling during long walks.

Design and Construction of a Combustion Atmosphere Inhalation Exposure System

The THRU RE/F was tasked to design two combustion atmosphere exposure systems and to dismantle Phase 1 of the B2 airplane system used previously. These exposure systems will be used to evaluate off-gassing and toxicity of specific materials of combustion products. Evaluations to be monitored in the F22 airplane system are animal respiration rates, gas concentrations, airflow rates, and system temperatures. Fabrication of the B2 system will commence following the F22 project and will be expanded to include measurements of smoke opacity, particle distribution and size. Both systems will operate at 38.5, 57.2, and 84.2 kWm/2 (heat flux), and 0.25, 0.50, 0.75, and 1.50 in. of water. Heat flux numbers correlate to 625, 770, and 880 °C. Pressure values correspond to 12, 13, 14, and 20 cubic feet per minute (cfm). The parameters selected are equivalent to expected settling velocities and rate of dispersal of materials should an airplane impact within a populated area.

During fabrication of these systems, a strategy was initiated to upgrade the Combustion Toxicology Program with more advanced technologies. A Data Acquisition System (DAS) and a personal computer capable of manipulating and collecting data were purchased. Additionally, mass flow controllers were procured to perform in conjunction with the DAS and control all main and sampled airflows.

Selected personnel were trained with the new DAS software and Man Machine Interface equipment. Due to budget constraints, implementation of these devices combined with the mechanical system has been gradual. The first of the two systems will not be operational until early 1996.

Several data collection methods are being developed to respond to the customer's initial request; i.e., to be able to measure animal respiration rates and respiration waveforms. Development of these sub-projects will be achieved through the use of low frequency transmitters and pressure or millivolt transducers. The technology is available to accomplish these goals using additional software and hardware.

Support of Quadricyclane Inhalation Toxicity and Persian Gulf Studies

Five of the six 690-L Wahmann chambers were refurbished in anticipation of the Quadricyclane 90-day and Persian Gulf studies. New seals were placed in the top and bottom fittings of each chamber. This included replacement seals in the doors. The chambers were inspected and tested for leaks. Primary and secondary intake and exhaust plumbing received new shrink- wrap sleeves. The manifold of the chamber's washdown system was replaced with a stainless steel version. The old PVC manifold had developed structural cracks. The fifth chamber was set aside to test a JP-4 jet fuel vapor delivery system for the Persian Gulf Study. A stainless steel pan with a thermostatically/PID-controlled heater was constructed. It is anticipated that sand will be placed in the pan and supersaturated with JP-4 jet fuel. The vapors will then be delivered to the chamber atmosphere.

Miscellaneous Projects

Other projects that involved design and fabrication this past year included shock cages and a heating pan for the Persian Gulf study and the re-design and construction of ten plethysmographs to fit a Cannon chamber. These restrainers are more adaptable, require less animal training, and result in less stress in the laboratory animals. Two Wahmann chamber doors

were manufactured. A media scraper, shelving, electrical panels, and brass connectors were also constructed. Two different types of cages (pigeon behavior cages and Faraday cages) were produced for the U.S. Navy laboratories.

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10.2 MATHEMATICAL AND STATISTICAL SUPPORT

C.D.Flemming

The THRU's Biometry staff was actively involved in the support of several research projects including the development of software to interface Image Analysis data with statistical packages, the development of a method to statistically process behavorial toxicology data, the development of a method to analyze percent of control data, and the development of methods to analyze combustion toxicology data.

There were several papers that the Biometry staff reviewed for publication. The Biometry staff also coauthored seven posters for the annual meeting of the Society of Toxicology.

The THRU's statistician assisted various Tri-Service Toxicology personnel in the use of RS/1, SAS, BMDP, and other software packages and aided several investigators with the development of protocols. Power analysis was used on several protocols. The statistician was involved with the incorporation of mathematics and statistics in research plans that will also be applied to new research projects.

Programming in FORTRAN, BMDP, and RPL was performed when required. Informal training in mathematics, statistics, programming, and computers was provided to individual Tri-Service Toxicology personnel.

To complete the Naval Subpopulation project, the following items need to be completed: perform a structional equation analysis, use the data in a multivariate Monte Carlo simulation using established PBPK models, compare the results of the simulation, analyze submarine data, and add this information into an existing paper.

10.3 PATHOLOGY SUPPORT (NECROPSY, HISTOLOGY, AND ELECTRON MICROSCOPY)

J.R. Latendresse and J.W. Lane

Necropsy support was provided in accordance with protocol requirements, standard operating procedures, or as determined by the veterinary pathologists. Routine, accepted methods of anesthesia were followed for terminal bleeding or euthanasia of laboratory required tissues for light microscopic examination.

Necropsy procedures included terminal bleeding or euthanasia of laboratory animals, determination of terminal body weights, detailed dissection, weighing of required organs, and collection and fixation of gross lesions and other processing of tissues included trimming, orientation of tissues in embedding cassettes, paraffin embedding, microtome sectioning of tissues to specified thickness, and applications for routine or special staining and coverslipping. Uniformly processed, high-quality slides were prepared for review by veterinary pathologists. A pathology specimen archive is maintained with controlled access as required by Good Laboratory Practices.

The histology/necropsy support technicians have continued to implement their newly acquired skills in tissue processing required for molecular pathology methods now being used extensively in the laboratory. These new tissue processing requirements include unique fixation protocols, sterile sectioning procedures, and microwave processing to enhance antigen , DNA, and RNA retrieval. The tissue staining methods are also unique. The stains are largely performed on a state-of-the art, computer-based automated stainer operated by laboratory personnel.

This year, pathology support expanded by the addition of an electron microscopist to the Toxic Hazards Research Unit (THRU) staff. The electron microscopy laboratories have been restored to a fully functional working unit following cross-training of two part-time electron microscopy (EM) technicians, updated equipment purchases, and additional EM collaboration with Wright State University and the University of Dayton. This has enhanced our ultrastructural-assessment capability and function utilizing transmission electron microscopy (TEM), scanning electron microscopy (SEM) and qualitative X-ray microanalysis.

Electron microscopy has been integrated into many ongoing research projects as a diagnostic and research support tool. EM personnel provided various ultrastructural and X-ray microanalysis support for the following projects:

1) Peroxisome proliferation data in mouse centrilobular hepatocytes for determination of risk assessment of TCE using TEM.

2) SEM and X-ray microanalysis data to check sample consistency and to assess paramagnetic contamination of electron paramagnetic resonance samples.

3) SEM analysis of combustion materials including particle size analysis.

4) SEM pore size analysis and qualitative X-ray microanalysis of bioceramics in a collaboration with Wright State University and the University of Dayton.

5) TEM study of ultrastructural damage to perfused rat livers.

In addition to providing EM research support, EM personnel also developed and implemented a light microscopy (LM) staining technique for lipofuscin; it was shown that large amounts of lipofuscin were not visible in TCE-treated mouse livers while control tissues such as ovary exhibited positive staining for lipofuscin.

Necropsy, histology, and electron microscopy personnel played a significant role in bringing our laboratories into compliance with the AFMC mandated hazardous material (HM) and hazardous waste (HW) control program. These personnel actively participated in managing HM and HW generated as a result of research activities.

Pathology staff members processed the following research animals and provided the following research support during the past reporting period (Tables 10.3-1 through 10.3-4).

TABLE 10.3-1. TOTAL ANIMALS LISTED BY SPECIES AND RESULTING NUMBEROF SLIDES

Species	Animals	Slides
Rats	1,367	7,236
Mice	218	2,421
Pig	1	3
Monkey	2	24
Dog	6	45
G. Pig	61	9
Frog	2	9
Fox	1	11
Ferret	2	9
Gerbil	4	4

TABLE 10.3-2. NUMBER OF ANIMALS PROCESSED BY MONTH AND RESULTING NUMBER OF SLIDES

Month	Number of Animals (in Species)	Number of Slides (in Type)
October 1994	120 Rats	234 Rat
	36 Mice	7 Mouse
November 1994	90 Rats	511 Rat
	6 Mice	
	1 Monkey	1,241 Mouse

December 1994	62 Rats	463 Rat
	4 Mice	71 Mouse
	3 Dog	1 Dog
	8 G. Pig	8 G. Pig
	2 Frog	----
January 1995	67 Rats	461 Rat
	10 Mice	29 Mouse
	8 G. Pig	8 G. Pig
	2 Frog	9 Frog
	1 Fox	11 Fox
February 1995	243 Rats	728 Rat
	17 Mice	51 Mouse
	22 G. Pigs	----
	1 Dog	1 Dog
	1 Fox	1 Fox
March 1995	59 Rats	1,141 Rat
	17 Mice	30 Mouse
	1 Cat	1 Cat
	1 Dog	1 Dog
	1 Monkey	24 Monkey

TABLE 10.3-2. NUMBER OF ANIMALS PROCESSED BY MONTH AND RESULTING NUMBER OF SLIDES (CONT'D)

Month	Number of Animals (in Species)	Number of Slides (in Type)
April 1995	84 Rats	564 Rat
	14 Mice	778 Mouse
	1 Ferret	9 Ferret
	3 Bull	9 Bull
	1 Monkey	----
	1 Feline	----
May 1995	53 Rats	259 Rat
	16 Mice	800 Mouse
	1 Bull	27 Bull
	2 G. Pig	27 G. Pig
	1 Ferret	1 Ferret
June 1995	84 Rats	259 Rat
	53 Mice	607 Mouse
	1 Bull	30 Bull
	1 Pig	3 Pig
	2 Dog	4 Dog
	2 G. Pig	----
July 1995	265 Rats	1215 Rat
	9 Mice	15 Mouse
	3 Bull	257 Bull
	1 Gerbil	3 Gerbil
	1 Ferret	----
	1 Pig	----
August 1995	265 Rats	381 Rat
	23 Mice	33 Mouse
	1 G. Pig	5 G. Pig
	2 Bull	----
September 1995	149 Rats	890 Rat
	27 G. Pig	9 G.Pig

TABLE 10.3-3. SEM SUPPORT LISTED BY PROJECT

Project Title/ THRU Study Request No.	Sample Type	Number of Samples	Number of Micrographs	Number of Spectra
A05	Combustion Particles	88	490	----
F35	Mouse Liver	20	10	10
Ceramics--WSU/UD	Bioceramics	18	36	18
S18	SFE Combustion	9	50	----
SEMEDS	Fungii	9	14	----
SEM Tech. Training	Various	8	64	32
SEM Maint./Calib.	Standards	4	72	24
TOTALS	----	156	736	84

TABLE 10.3-4. TEM SUPPORT LISTED BY PROJECT

Project Title THRU Study Request No.	Specimen Type	Number of Specimens	Number of Slides	Number of Micrographs
F35	Mouse Liver	2,530	256	396
F35	Mouse Liver Mitochondria Prep	10	6	12
F29-C	Rat Uterine Tissue	60	15	----
F41	Rat Liver	160	20	++
	Rat Skin	30	15	67
TEM Tech. Training	Various	6	----	26
TEM Maint./Calib.	Standards	2	----	70
TOTALS	----	2,798	312	571

note: ++ denotes ongoing study

10.4 ANALYTICAL CHEMISTRY SUPPORT FOR THE ARMY

K. Kuhlmann

The United States Army Medical Research Detachment has an additional mission from the other services in the TriService Toxicology Consortium, in that it has concentrated many of its resources on identifying and quantitating troop field exposures. As a result, personnel training and equipment have focused on industrial hygiene and enviromental issues as well as medical ones.

In addition to a full complement of pumps, impingers, impacters, and other collection apparatus, the laboratory includes the following equipment for sample analysis:

Currently Installed and Operational Instrumentation

1	Perkin-Elmer Qmass 910 Quadruple Gas Chromatograph/Mass Spectrometer
1	Hewlett-Packard 5970 Gas Chromatograph/Mass Spectrometer
1	Perkin-Elmer ATD400 Automatic Thermal Desorbtion autosampler for IH tubes and similar methods of sample introduction
1	Perkin-Elmer 2000 Infrared Analyzer
1	Perkin-Elmer 1650 Infrared Analyzer
1	Perkin-Elmer TGA-7 Thermogravimetric Analyzer with IR interface
1	Perkin-Elmer Autosystem GC with IR interface and FID and ECD detectors
1	Hewlett-Packard 1050 HPLC with Diode Array Detector and Autosampler
1	OI Analytical 4560 Purge and Trap Extractor with autosampler
1	Dynatech Precision Sampling Company Dynasampler 511 liquid autosampler
1	Leeman 3000 Inductively Coupled Plasma (ICP)
1	Microwave Digestor

The Army laboratory possesses the standard equipment necessary to support environmental and industrial hygiene needs. This includes sufficient glassware, precision scales, pH and specific ion meters, etc. The laboratory also maintains a supply of necessary spare parts to allow swift repair in case of equipment malfunction.

All laboratory computers, both those for data acquisition and stand-alone, are networked. This enables the laboratory to use Magneto-optic storage devices for temporary data storage, with final archiving of data on recordable CD-ROMs. This also allows the laboratory to do significant off-line data processing, and enables personnel to do data collation at one central work station.

With the ICP, the Army laboratory has added new capabilities for metals analysis. The organic instrumentation and laboratory personnel have made significant additions to analytical capabilities of non-biological matrices.

The latest addition has been a set of programming tools which allow custom data acquisition as needed. If there is a voltage source, the laboratory can measure it in real-time and save the measurements. Other tools allow programming custom data reduction as needed.

With all instruments now fully functional and custom tools being able to be programmed as needed, the Army laboratory has significantly broadened the analytical capabilities of of the TriService Toxicology Consortium.

10.5 COMPUTER AND ELECTRONICS SUPPORT

J.S. Stokes

The Computer and Electronics Section of the Research Support group provided support for the Armstrong Laboratory/Toxicology Division (AL/OET) VAX minicomputer, personal computers (PC), and data acquisition and telecommunications systems hardware and software utilized by the THRU, Toxicology Division, and Comparative Medicine personnel. The following itemization illustrates a few of the services provided during this reporting period by the Computer and Electronics Support staff.

- Provided telephone and computer support services in the relocation of THRU and other AL/OET personnel and computer systems, as follows:

	To:
Building 79:	
M. Schneider	Building 429, Room 3A
L. Silvers	Building 429, Room 3A
H. Barton	Building 79, Room 154F
J. Frazier	Building 79, Room 154E
L. Steel-Goodwin	Building 824, Room 201
R. Collins	Building 79, Room 116
J. Drerup	Building 79, Room 185
J. Drerup	Building 79, Room 147C
J. Prince	Building 824, Room 201
Building 429:	
S. Godfrey	Building 429, Room 3A
Building 824:	
T. Sterner	Building 79, Room 130
Electronic Paramagnetic Resonance	Building 79, Room 180
L. Steel-Goodwin	Building 79, Room 130

- Set up a shared drive for networked PC users in Buildings 79 and 824 to facilitate transfer of files and data.
- Maintained stock of PC supply items for THRU computer users.
- Provided audio/visual, phone, and computer support for the 1995 Annual Toxicology Conference held at the Hope Conference Center. Researched and procured new amplifier and gated mixer with microphones to improve sound quality. Internet connection was installed for poster/computer session.
- Installed networking software on remaining PCs in Building 79. Converted PC users networked under Lantastic to Windows for Workgroups networking.
- Created spreadsheet to track user community work requests.
- Provided system management for Digital Equipment Corp. VAX computer in Building 79. Also provided support for users of Building 79 VAX computer.

- Attended training classes on MS Word, Excel, Powerpoint, Access, Windows for Workgroups, and Novell Netware 4.1.
- Removed computer viruses from many personal computers and floppy disks.
- Made an audio recording of the pre-bid conference.
- Requested that the remote users in Building 824 be given extensions on the AL/OET phone system in Building 79 to save the cost of commercial phone lines and long distance calls and enhance organizational communication efficiency.
- Provided support for HAZMAT system by connecting users to DM-HMMS system and assisting with access as needed.

10.6 QUALITY ASSURANCE

M. Schneider

PERSONNEL ACTIVITIES

The Quality Assurance Coordinator (QAC) participated in the activities of the Mid West Regional Chapter of the Society of Quality Assurance on the By-Law Committee and with attendance at one Regional meeting. Training covering GLP responsibility for samples, data, and results by study participants, management, and sponsors was received. The QAC attended beginning level training for Access 2.0 database software, and a one-day seminar, "Collaboration and Community in the Workplace," presented by CareerTrack in Dayton, Ohio. The QAC conducted orientation training in the quality assurance program for four new THRU staff.

The QA Associate (QAA) attended intermediate level training for Word for Windows 6.0 word-processing software, and a one-day seminar, "Collaboration and Community in the Workplace," presented by CareerTrack in Dayton, Ohio. The QAA participated on the program management team during the Conference on Risk Assessment for Sensitive Human Populations.

THRU staff involved in research using animals received annual training on the proper care and use of animals presented by Col James R. Cooper, AL/OEVM.

SOP ACTIVITIES

Thirty-three new or revised SOPs were reviewed, prepared for approval, distributed, and placed in the SOP Manuals located in laboratory work areas by the QAA.

STUDY REQUEST AUDITS

The QAC conducted protocol reviews, and procedure, data, and final report audits for THRU Study Requests A02 (TNB), A04 (TNB), A07 (Nitrates), A08 (Unicharge), F28 (TPH), F29 (ADN), F29A (ADN), F29C (ADN), F35 (TCE), F37 (CF_3I), F42 (Quadricyclane), and N18 (JP-8+100).

The QAC acted as ManTech study coordinator for A01 (QA program), A04 (QA Program and fetal evaluation subcontract), and as coordinator for a subcontract with Qualtech, Inc. to conduct a GLP audit for Study Request A02 (TNB).

TECHNICAL EDITING

The QAA was principal editor for the 1994 THRU Annual Report and coordinated processing of the annual report by ManTech Environmental Technical Publications. Five manuscripts for peer-reviewed publication were edited for Study Requests F23 (TCE Metabolism), 009 (Nitrophenol Toxicity), F39 (Pharmacodynamic Description of Biological Effects), A07 (Nitrates), and F38 (Halon 1301 Replacements). Nine technical reports were edited by the QAA for Study Requests A02(TNB SIDS Reproductive), F29 (ADN SIDS Reproductive), F35 (TCE Risk Assessment), A07 (Nitrates), A08

(Unicharge Acute Toxicity), F38 (Halon 1301 Replacements), F39 (Pharmacodynamic Description of Biological Effects), and N16 (Human Health Hazard Assessment).

10.7 HEALTH AND SAFETY

M. Schneider

GENERAL

The Health and Safety Representative (HSR) provided occupational health and safety support for AL/OET, Toxicology Division, in addition to support for ManTech Environmental's THRU staff.

TRAINING

THRU staff who work in laboratory areas received basic training in Wright-Patterson Air Force Base (WPAFB) hazardous materials/hazardous waste (HM/HW) management policies and procedures, the OSHA laboratory chemical standard, and the OSHA bloodborne pathogen standard. THRU staff who were designated as hazardous waste initial accumulation point (IAP) managers or accumulation site (ACCS) managers received specialized training provided by WPAFB Environmental Management (EM) for Environmental Protection Agency Resource Conservation Recovery Act(RCRA) hazardous waste and Department of Transportation (DOT) hazardous materials transportation regulations. The HSR provided safety orientation training for four new THRU staff. The HSR received special training in explosive safety, radiation safety, EM hazardous waste regulations (RCRA 8-hour), DOT hazardous material regulations, EM hazardous materials management system(DM-HMMS), and animal care and use requirements for investigators.

CHEMICAL SAFETY

The transition of the AL/OET hazardous material program into the WPAFB HazMat Cell program was brought to full speed as the result of several inspections by EM. A massive effort is currently underway to bring all AL/OET operations into compliance with WPAFB EM regulations. The chemical inventory that was maintained by the HSR was used as the basis for the transition into the WPAFB HM program. The HSR was assigned a team leader role for the HM transition process. A Quality & Safety staff member was assigned to this team also. The HSR was given responsibility for coordinating the turn-in process for excess hazardous material. The HSR revised the Chemical Hygiene Plan to comply with OSHA and Air Force regulations.

EXPLOSIVES SAFETY

The HSR was assigned responsibility for the AL/OET explosives safety program. Each item stored in the facility required licensing by WPAFB. The HSR provided training for the investigators and technicians using explosive materials.

HAZARDOUS WASTE

The HSR was assigned responsibility for operation of the accumulation site located in Building 77. Through the end of this reporting period, 130 containers of hazardous waste were received at this facility. The HSR was also designated

as alternate for each of the 16 IAPs located in Building 79, and was IAP manager for one site, the chemical store room located in Room 117.

PERSONNEL SAFETY

THRU staff were provided with appropriate safety equipment as required by specific work assignments. Purchase agreements were established with local vendors for safety glasses and safety shoes. The medical surveillance program, which included employee physicals (pre-employment, annual, and exit), emergency care for work related injury, follow-up examinations for work release, consultation for pregnant staff or suspected work related illness, and initiation of Workers Compensation paperwork was continued. Hepatitis B immunization was offered to staff who might encounter bloodborne pathogens in their jobs. All ManTech staff were offered annual physicals. Thirty-two annual physicals and one exit physical were completed during this report period. Two THRU staff required treatment related to on the job injuries. Both were released to return to work. Two staff began the hepatitis immunization series.

Eyewash bottles were installed in locations where hazardous materials were present. Chemical spill kits were located outside laboratories and stocked with appropriate supplies. The HSR coordinated the AL/OET respiratory protection program. A new AL/OET OI was developed with WPAFB Bioenvironmental Engineering (BES). DOD personnel were trained and fit tested for respirator wear by Occupational Health, while Contractor staff were trained and fit tested by the HSR. Permanent welding permits were established for THRU shop areas through the WPAFB Fire Department.

INSPECTIONS

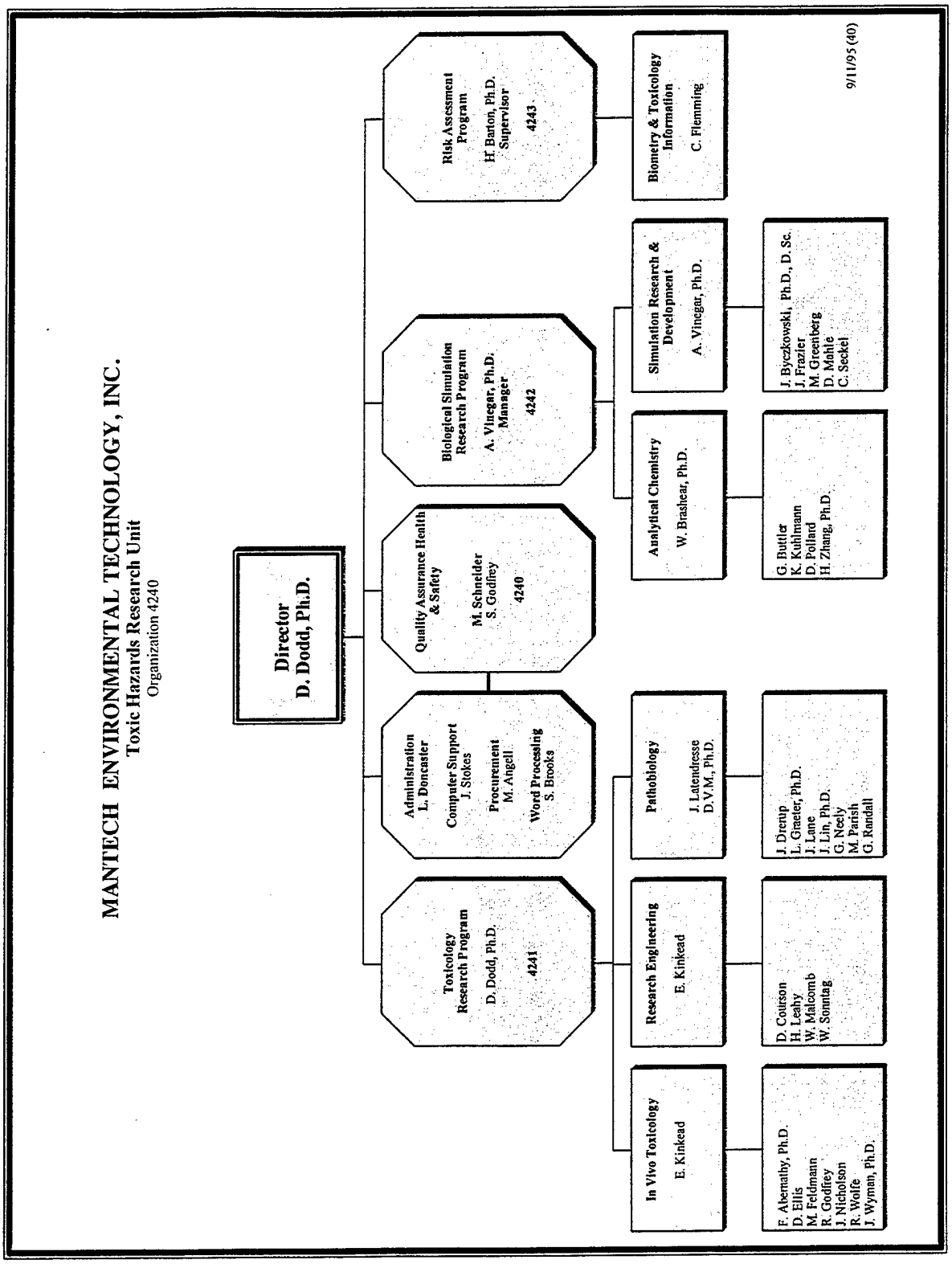
Work areas occupied by THRU staff were subject to annual inspections by Air Force safety personnel from HSC, Brooks AFB, WPAFB (EM, BES, Public Health), and AL North.. Specific programs as Environmental Compliance and Management Program, Radiation Safety, Hazardous Waste (chemical and biological), and Explosives Safety were inspected several times during the year. The HSR accompanied these inspection teams. Response by ManTech to the findings was always timely. The HSR also conducted spot and monthly safety inspections, including testing eye-washes and showers. Issues of immediate concern were addressed with staff and supervisors on-the-spot. Work requests were submitted through the OET Building Superintendent to correct building related safety issues.

SECTION 11

APPENDICES

APPENDIX A

TOXIC HAZARDS RESEARCH UNIT LIST OF PERSONNEL



APPENDIX B

PRODUCTS LIST FOR 1995

October 1, 1994 - September 30, 1995

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Abernathy, Flemming, Sonntag, Measusrement of Cardiovascular Response in Male-Sprague-Dawley Rats Using Radiotelemetric Implants and Tailcuff Sphygmomanometry: A Comparitive Study. 1995. *Toxicol. Meth,* 5:89-98.

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Vinegar, Jepson, Pharmacokinetic Modeling and Its Role in Cardiac Sensitization, 11/17/95, USEPA Conf on Physiological Effect of Alternative Fire Protection Agents, 18-19 Dec, Bethesda, MD.

AWARDS and HONORS

Steel-Goodwin, Pravecek, Hancock, Schmidt, Channel, Bartholemew, Bishop, **Ketcha**, Best Poster (1st Place) presented by the Tri-Service Toxicology Division, 2 Mar 95 **"Trichloroethylene: Free Radical Studies in B6C3F1 Mouse Liver Slices.**

Wyman, Fisher, Prues, **Flemming**, Brendel, Best Poster (2nd Place) presented by the Tri-Service Toxicology Division -2 March 95 "Comparative Toxicity of 2,6-Di-tert-Butyl-4-Nitrophenol and Other Nitrated Phenols in Human and Rat Hepatic Tisue Slices.