

UNITED STATES AIR FORCE ARMSTRONG LABORATORY

1996 TOXIC HAZARDS RESEARCH

ANNUAL REPORT

Darol E. Dodd

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The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care* and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1996, and the Animal Welfare Act of 1966, as amended.

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FOR THE COMMANDER

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PREFACE

The 33rd Annual Report of the Toxic Hazards Research Unit (THRU) presents research and research support efforts conducted jointly by ManTech Environmental Technology, Inc. and Geo-Centers, 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 Nos. F33615-90-C-0532 and F41624-96-C-9010. This document represents the first annual report for the current THRU contract and describes accomplishments from 01 October 1995 through 30 September 1996.

Operation of the THRU under Contract F33615-91-C-0532 was initiated on 16 January 1991 under Work Unit 7757A001, "Toxic Hazards Research," and operation under Contract F41624-96-C-9010 began on 16 January 1996 under Work Unit 7757A002, "Toxicology Research." This research effort was cosponsored by the Army Medical Research Detachment, Walter Reed Army Institute of Research (WRAIR), and by the Naval Medical Research Institute Detachment/Toxicology (NMRI/TD).

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 Officer Representative. The portion of the work effort sponsored by the Army was under the direction of LTC Roland E. Langford, Detachment Commander, and CAPT Clay R. Miller of the Medical Research Detachment. The portion of the work effort sponsored by the Navy was under the direction of the NMRI/TD Officer-in-Charge, CAPT Kenneth R. Still, MSC, USN. Darol E. Dodd, Ph.D., served as the ManTech Geo-Centers Joint Venture Program Manager for the THRU.

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SECTION 1

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ABBREVIATIONS

Abbreviations

°C	Degrees Celsius
μΑ	Microampere
μg	Microgram
μL	Microliter
μm	Micrometer
Ad	Adenoma
ACCS	Accumulation Site
ACM	Advanced Composite Material
ACSL	Advanced Continuous Simulation Language
ADI	Allowable daily intake
ADN	Ammonium Dinitramide
AFMC	Air Force Materiel Command
AL/OET	Armstrong Laboratory/Occupational and Environmental Health Directorate, Toxicology Division
ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
AST	Aspartate aminotransferase
BBPD	Biologically based pharmacodynamic
BE	Bioenvironmental Engineering
BMD	Benchmark dose
BrCCl ₃	Bromotrichloromethane
Ca	Carcinoma
CCD	Charged-coupled device
CCI ₄	Carbon Tetrachloride
CF ₃ I	Trifluoroiodomethane (Iodotrifluoromethane)
СН	Chloral Hydrate
cm	Centimeter
CNS	Central Nervous System
СО	Carbon Monoxide
CO2	Carbon Dioxide
CPVC	Chlorinated Polyvinyl Chloride

CV	Coefficient of variation
CYP	Cytochrome P450
CYP2E1	Cytochrome P450 IIE1
CYP4A1	Cytochrome P450 IVA1
DCA	Dichloroacetic Acid
DM-HMMS	Defense Management Hazardous Materials Management System
DNA	Deoxyribonucleic Acid
DOD	Department of Defense
DOT	Department of Transportation
ECD	Electron Capture Detector
EM	Electron microscope/microscopy
EPA	U. S. Environmental Protection Agency
EPR	Electron paramagnetic resonance
ERAP	Environmental Risk Assessment Program
ESI	Electrospray ionization
eV	Electron volt
Foci	Altered hepatic foci
ft	Feet
ft²	Feet squared
g	Gram .
GC	Gas Chromatograph
GC/MS	Gas chromatography/mass spectroscopy
GLM	General Linear Model
GLP	Good Laboratory Practices
GST	Glutathione S-transferase
H_2O_2	Hydrogen Peroxide
h	Hour
HM	Hazardous Material
HSC	Human Systems Center
HSR ,	Health and Safety Representative
HW	Hazardous Waste
i.p.	Intraperitoneally
IAP	Initial Accumulation Point
IRP	Installation Restoration Program

JP	Jet petroleum (fuel)			
kV	Kilovolt			
kW/m ²	Kilowatt/meter squared			
L	Liter			
L/min	Liters per minute			
LAN	Local Area Network			
LCPH	Long chain petroleum hydrocarbons			
LDH	Lactate Dehydrogenase			
LOAEL	Lowest Observable Adverse Effect Level			
M to D	Moderately to densely			
MCA	Monochloroacetic Acid			
m	Mass loss rate			
m ²	Meter squared			
mg	Milligram			
Mi	Minimally to mildly			
min	Minute			
mL	Milliliter			
mm	Millimeter			
MS	Mass spectrometry			
m/z	Mass to charge ratio			
ng	Nanogram			
nm	Nanometer			
NIOSH	National Institute of Occupational Safety and Health			
NOAEL	No Observable Adverse Effect Level			
O^2	Oxygen			
OSHA	Occupational Safety and Health Act			
PAHs	Polycyclic Aromatic Hydrocarbons			
PBPK	Physiologically based pharmacokinetic			
PBr ₃	Phosphorous Tribromide			
PID	Proportional Integral Derivative			
PKPD	Pharmacodynamic			
PPE	Personal protective equipment			
ppm	Parts per million			
psi	Pounds per square inch			
Q	Irradiance or heat flux (kW/m ²)			
Q1	Quadrupole 1			

xv

Q2	Quadrupole 2		
Q3	Quadrupole 3		
QAA	Quality Assurance Associate		
QAC	Quality Assurance Coordinator		
QTIP	Quick Turn-in Process		
RCRA	Resource Conservation and Recovery Act		
REWG	Rocket Emission Group		
RfD	Reference Dose		
RNA	Ribonucleic Acid		
S	Second		
SEM	Scanning electron microscope/microscopy		
S/N	Signal to noise ratio		
SIDS	Screening Information Data Set		
SOP	Standard Operating Procedures		
SRM	Selective reaction monitoring		
TBA	Thiobarbituric Acid		
TBARS	Thiobarbituric Acid reactive substances		
TCA	Trichloroacetic Acid		
TCE	Trichloroethylene		
TEM	Transmission electron microscope/microscopy		
T _{fl}	Duration of flaming		
THRU	Toxic Hazards Research Unit		
T _{ign}	Time of Ignition		
TMPP	Trimethylolpropane Phosphate		
TPA	Total petroleum hydrocarbons		
TSQ	Triple stage quadrupole		
UD	University of Dayton		
UXO	Unexploded ordnance		
v	Airflow (L/min)		
\overline{V}	Average Airflow Velocity		
WPAFB	Wright-Patterson Air Force Base		
WSU	Wright State University		

SECTION 2

INTRODUCTION

INTRODUCTION

D.E. Dodd

This report presents a review of the activities of the ManTech Geo-Centers Joint Venture Toxic Hazards Research Unit (THRU), for the period 01 October 1995 through 30 September 1996. The THRU is an on-site, contractoroperated, 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 (project requests) provided by the Contract Officer Representative, the THRU also coordinates toxicology conferences, expert workshops, and program reviews. Research support benefiting 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 proliferative hepatocellular lesions following administration of dichloroacetic acid (DCA) and trichloroacetic acid (TCA); development of biologically based dose-response models for lipid peroxidation induced by TCE or bromotrichloromethane; application of physiologically based pharmacokinetic (PBPK) models in predicting the kinetics of halon replacement candidates following short duration exposures; acute toxicity testing of the halon replacement candidate phosphorus tribromide (PBr₃); subacute toxicity testing of surrogate candidates for long chain petroleum hydrocarbons; subchronic toxicity testing of n-Nonane; reproductive toxicity testing of advanced composite materials (ACM); morphometric analysis of smoke particulates following combustion of ACM; and applications of confocal microscopic techniques in toxicology research.

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 developmental toxicity potential of several propellant and explosive candidates using an animal alternative screen employing *hydra attenuata*, and evaluated in rats the potential effects of a simulated Persian Gulf War exposure.

Toxic Hazards Research Unit technical directives that supported the Navy included mechanistic investigation of trimethylolpropane phosphate-induced central nervous system toxicity, and the acute toxicity of jet fuel JP-8 combined with various additive packages. 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 toxicology conferences and workshops, including the series of annual toxicology conferences that have been coordinated by the THRU since 1965. The proceedings of the 1995 toxicology conference, "Risk Assessment Issues for Sensitive Human Populations" were compiled by the THRU and distributed as a publication by the journal *Toxicology* (Vol. 111, Nos. 1-3, 1996). The 1996 toxicology conference "Conference on Advances in Toxicology and Applications to Risk Assessment" was conducted 23-25 April at the Hope Hotel and Conference Center at Wright-Patterson Air Force Base (WPAFB). The proceedings of this conference are being compiled by the THRU for publication as a technical report. In August 1996, the THRU initiated its planning efforts for the 1997 toxicology conference, "Conference on Issues and Applications in Toxicology and Risk Assessment."

The execution of the THRU contract Statement of Work involves the integrated effort of a multidisciplinary staff of scientists, research technicians, and administrative and research support personnel. Sections 3 through 9 of this

report emphasize the technical activities of the THRU. Sections 10 and 11 present highlights of the conferences and research support activities, including research engineering/fabrication, mathemátics and statistics, pathology support, quality assurance, and health and safety. Section 12 of this report is a set of appendices that describe the THRU organization, its personnel, and its awards, publications and presentations. Personnel from Geo-Centers, Inc. joined the THRU's Joint Venture Contract in September 1996.

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 (project 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 project requests and are published as technical reports or peer-review publications (refer to "Products List for 1995-1996" in Section 12). 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.

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SECTION 3

TRICHLOROETHYLENE CARCINOGENICITY PROJECT

3.1 ELECTROSPRAY ANALYSIS OF BIOLOGICAL SAMPLES FOR TRACE AMOUNTS OF TRICHLOROACETIC ACID, DICHLOROACETIC ACID, AND MONOCHLOROACETIC ACID

W.T. Brashear and C.T. Bishop¹

ABSTRACT

Trichloroethylene (TCE) has been identified as a widespread groundwater contaminant. Trichloroacetic acid (TCA) and dichloroacetic acid (DCA) are toxicologically significant metabolites of TCE which produce tumors in $B_6C_3F_1$ mice. A sensitive method for measuring these metabolites in plasma has been developed to obtain pharmacokinetic data from TCE exposure. This is particularly important since DCA is more potent at producing hepatoproliferative lesions than TCA. At present, it is unclear whether DCA is produced by humans. Existing gas chromatographic methods cannot detect DCA at low ng/mL levels. A Finnigan TSQ 700 mass spectrometer with electrospray ionization was used to measure TCA, DCA, and monochloroacetic acid (MCA) in plasma. The mass spectrometer was operated in negative ion tandem mass spectrometric mode. The limit of detection for TCA and DCA was 4 ng/mL, and the limit of detection for MCA was 25 ng/mL. Plasma samples from human subjects exposed to 100 ppm TCE for 4 h contained TCA at concentrations as high as 10 µg/mL, DCA concentrations were less than 5 ng/mL, and MCA was not detected (less than 25 ng/mL).

INTRODUCTION

Trichloroethylene (TCE), a widely used degreasing and cleaning solvent, is an environmental contaminant 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 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) and are toxicologically significant metabolites of TCE. DCA is of particular concern since it appears to be a more potent hepatoproliferative agent than TCA in $B_6C_3F_1$ mice (Bull et al., 1990). However, DCA has not been found in humans, and it is not clear whether DCA is formed by humans (Hathway, 1980). DCA has been reported in blood from $B_6C_3F_1$ mice exposed to TCE after oral gavage (Templin et al., 1993), but has not been found in the blood of Sprague-Dawley rats exposed to TCE via oral gavage (Larson and Bull, 1992). With equivalent doses of TCE, mice higher peak blood levels of both TCA and DCA than rats. This species difference has been attributed to a higher rate of TCE metabolism, which is supported by higher peak blood concentrations of TCA and DCA in mice (Larson and Bull, 1992). To evaluate the significance of DCA as a metabolite of TCE, the question

¹Armstrong Laboratory, Toxicology Division, Wright-Patterson AFB, OH.

of whether DCA is a metabolite of TCE in species other than mice needs to be investigated. The concentrations of DCA reported in biological samples are much lower than TCA (Larson and Bull, 1992; Dekant et al., 1984). This suggests that improving the limit of detection may help clarify the role of DCA as a metabolite of TCE. Most gas chromatographic methods with electron capture detection (GC/ECD) have a limit of detection of approximately 1 μ g/mL (Larson and Bull, 1992; Ketcha et al., 1996). In addition to this, it has been shown that TCA can be converted to DCA in fresh blood by strong acid (Ketcha et al., 1996). Biological samples need to be acidified to derivatize and extract TCA and DCA. However, this conversion which is catalyzed by reduced hemoglobin may lead to erroneous pharmacokinetic data. Here, a more sensitive analytical method has been developed using electrospray ionization mass tandem mass spectrometry (ESI/MS/MS) for the analysis of TCA, DCA, and MCA in biological samples. This method has a 4 ng/mL limit of detection for DCA and uses plasma samples in order to prevent any artifactual conversion of TCA to DCA.

MATERIAL & METHODS

Chemicals & Equipment

TCA (99+%), DCA (99+%), and MCA (99+%) were obtained from Aldrich Chemical Company (Milwaukee, WI). Sulfuric acid and acetic acid were obtained from Fisher Scientific (Fair Lawn, NJ). Diethyl ether was obtained from Baxter Healthcare Corporation, Burdick & Jackson Division (Muskegon, MI). A Thermolyne model 16700 vortex mixer was used to vortex and extract samples (Dubuque, IA). An N-Evap analytical evaporator from Organomation Inc. (Berlin, MA) was used to evaporate ether extracts. Samples were analyzed on a Finnigan TSQ 700 mass spectrometer equipped with a Finnigan Electrospray interface (Finnigan MAT, San Jose, CA) and an LDC 4100 MS solvent delivery system (Thermo Instrument Systems Inc., Riviera Beach, FL).

ASSAY PROCEDURE

TCE Exposure

Human volunteers were exposed to 100 ppm TCE via inhalation for 4 h. A baseline blood sample was collected prior to exposure. Plasma samples were collected during the exposure and at timed intervals up to 94 h from the start of the exposure. Samples were collected during the exposure at t = 0.5, 1, 2, 3, and 4 h. Following the exposure, samples were collected at t = 4.25, 4.5, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, 46, 70, and 94 h. The blood samples were collected from an indwelling venous catheter using a heparinized syringe. Plasma was immediately separated by centrifugation and frozen at -80 °C until the time of analysis. Sample sets from 16 volunteers were analyzed for TCA, DCA, and MCA by tandem ESI/MS/MS.

Preparation of Samples

Plasma samples were prepared for electrospray analysis. A 0.5 mL aliquot of plasma was combined with 0.1 mL of water. This was acidified with 0.5 mL of 10% sulfuric acid, allowed to stand at room temperature for 1 h, and extracted into 2.5 mL of diethyl ether by vortex mixing vigorously for 1 min at room temperature. The sample was frozen at -20 °C for 1 h, thawed, and then centrifuged at 4000 xg for 45 min. The ether layer was removed and evaporated to dryness under nitrogen. The evaporated extract was reconstituted in 0.5 mL of 75% methanol, 24% water, 1% acetic acid. The reconstituted extract was centrifuged at 2000 xg for 20 min to remove any undissolved material and the clarified liquid was analyzed by electrospray mass spectrometry. Samples above 100 ng/mL were diluted to bring them within the range of the standard curve.

Standard curve samples were prepared from control plasma. A 0.1 mL aliquot of a standard solution of TCA/DCA/MCA was added to a 0.5 mL aliquot of human plasma. The sample was then acidified and extracted as described in the above paragraph. Standards and quality control samples were prepared at levels of 0, 5, 10, 50, and 100 ng/mL for DCA and TCA, and 0, 25, 50, 250, and 500 ng/mL for MCA.

The extraction efficiency was determined by comparing the slopes of two standard curves. The slope of extracted plasma samples spiked with TCA, DCA, and MCA was compared with unextracted standards in matrix. The unextracted standards in matrix were prepared from blank plasma samples extracted with ether, and reconstituted with standard solutions of TCA, DCA, and MCA in mobile phase (75% methanol, 34% water, 1% acetic acid). The matrix effect was evaluated by comparing the slope of unextracted standards in mobile phase with the slope of unextracted standards in matrix.

Electrospray Ionization Mass Spectrometry

The ESI/MS/MS analysis was performed on a Finnigan TSQ 700 mass spectrometer operating in the negative ion mode. The spectrometer was equipped with a Finnigan electrospray interface and an LDC 4100 MS solvent delivery system. The ESI inlet was equipped with a 10 μ L injection loop, the flow rate was 100 μ L/min, and the inlet capillary was held at 150 °C. The nitrogen sheath gas for the ESI interface was set at 40 psi and no auxiliary gas flow was used. The electrospray voltage was set at 6 kV and current was 2 μ A. Argon was used as the collision gas and the collision cell was operated at 1.5 millitorr, with an offset of 150 eV.

DCA was detected via selected reaction monitoring (SRM) MS/MS in the neutral loss mode. The first quad, Q1, was set at 127 m/z and the third quad, Q3, was set at 83 m/z; the scan time for the SRM was 0.14 s. This corresponded to the neutral loss of CO_2 from DCA (Figure 3.1-1). TCA underwent the analogous reaction with Q1 set at 161 m/z and Q3 held at 117 m/z. However, MCA was analyzed with Q1 set at 153 m/z and Q3 set at 93 m/z. This SRM corresponded to the loss of acetic acid from the negatively charged (MCA•AcOH) adduct.

The peak areas were manually integrated using the Finnigan CHRO program supplied with their proprietary ICIS software. First and second order polynomials, without weighting, were used for standard curves of TCA, DCA, and MCA.



Figure 3.1-1. Configuration of mass spectrometer to analyze biological samples for DCA. DCA was detected via selected reaction monitoring (SRM) MS/MS in the neutral loss mode. The first quad, Q1, was set at 127 m/z, and the third quad, Q3, was set at 83 m/z. The collision cell, Q2, contained argon. This corresponded to the neutral loss of CO₂ from DCA.

RESULTS

Standard curves obtained from plasma were linear up to 100 ng/mL for TCA and DCA. The detection limit was 4 ng/mL for TCA and DCA (S/N > 3, 99.9 %). The detection limit for MCA was 25 ng/mL. The electrospray signal from a series of extracted plasma samples spiked with DCA is shown in Figure 3.1-2. A typical standard curve for DCA and TCA ranging from 0 to 100 ng/mL is shown in Figure 3.1-3. The peak areas have been plotted against concentrations of DCA and TCA in ng/mL. Typical curves for DCA and TCA were: y=871.3x-1015, $r^2>0.994$; and y=1383x-153.7, $r^2>0.999$, respectively. As shown in Figure 3.1-1, TCA gave a stronger electrospray response than DCA. The response from MCA was considerably weaker than either DCA or TCA, and MCA standards were five times higher than TCA and DCA. A typical standard curve for MCA is shown in Figure 3.1-4. Due to the higher concentrations, a second order polynomial fit the MCA data and a typical curve is; $y=0.2713x^2+802.7x-4972$, $r^2=0.999$.

The precision and accuracy of the method were determined. The intra-day and inter-day variability are shown in Table 3.1-1. TCA, DCA, and MCA were extracted and analyzed in plasma samples at low, medium, and high concentrations. Because of a relatively weak response, MCA was analyzed at concentrations five times higher than TCA and DCA. The precision samples for intra-day variability gave results which were within 95% of their expected values. However, the medium and high level inter-day precision samples gave results which were lower than their expected values by 5-20%.

ТСА		Actual Concentration ng/mL	Number of Replicates	Accuracy (mean % found/added)	Precision (% CV)
	Intra-day	10	14	86	58
	-	50	15	96	25
		100	14	105	31
	Inter-day	10	15	91	63
		50	16	94	41
		100	16	89	28
DCA					
	Intra-day	10	15	101	34
		50	16	96	25
		100	15	101	33
	Inter-day	10	13	103	37
		50	15	85	42
		100	15	81	31
MCA					
	Intra-day	50	8	86	27
		250	18	91	31
		500	17	95	26
	Inter-day	50	8	107	29
		250	18	79	40
		500	18	80	37

TABLE 3.1-1.INTRA-DAY AND INTER-DAY VARIABILITY FOR MCA, DCA, AND TCA AS
DETERMINED BY ESI/MS/MS

The extraction efficiency of TCA, DCA, and MCA from human plasma with 10% sulfuric acid was determined to be 75%. The plasma matrix was found to suppress the electrospray signal for TCA, DCA, and MCA by 50%.

The analysis of plasma samples from 16 volunteers exposed to 100 ppm TCE by inhalation for 4 h contained TCA at levels of about 10 μ g/mL. The average peak DCA level was less than 5 ng/mL, and MCA was not detected. This data is shown in Figure 3.1-5. Samples from four individual volunteers had peak DCA levels above 10 ng/mL (12-14 ng/mL). These peak DCA levels occurred during the 4-h TCE exposure or less than 1 h after the exposure phase of the study. From the averaged data of the 16 volunteers, the peak DCA level of 3 ng/mL occurred at 2 h

(during the TCE exposure). The average DCA level remained elevated at 2 ng/mL up until 5 h (1-h postexposure). As previously stated, the detection limit for DCA is 4 ng/mL. Values less than 4 ng/mL were not used in computing the average DCA concentrations. The peak level of TCA occurred at 8 h (4-h postexposure) and remained elevated at approximately 10 μ g/mL throughout the 94-h sample collection time.



Figure 3.1-2. Electrospray SRM signal (m/z 127→83) for extracted plasma standards spiked with 0, 5, 10, 50, and 100 ng/mL DCA. The relative signal intensity is plotted against the scan numbers.



Figure 3.1-3. Standard curve of DCA and TCA in plasma. The area of the electrospray signal of the plasma extracts are plotted against concentration.



Figure 3.1-4. Standard curve of MCA in plasma. The area of the electrospray signal of the plasma extracts is plotted against concentration.



Figure 3.1-5. Average plasma concentrations of DCA and TCA vs. time (n=16). Volunteers were exposed to 100 ppm TCE for four hours starting at t=0. Plasma samples were collected up to t=94 h.

DISCUSSION

The assay developed for the analysis of TCA, DCA, and MCA has reduced the limits of detection for these chlorinated carboxylic acids in plasma. The limit of detection for TCA and DCA is 4 ng/mL, and the limit of detection for MCA is 25 ng/mL. The extraction efficiency of these acids from acidified plasma into diethyl ether is 75%. However, the electrospray signal of the acids is suppressed approximately 50% by the plasma matrix. The suppression of the electrospray signal by the plasma matrix decreases the signal to noise level and limits the sensitivity and precision of the assay. The intra-day and inter-day variability had coefficients of variation that averaged 32% and 39%, respectively. This small difference suggests that most of the variability was due to noise in the electrospray signal of the analytes in plasma. The values used for obtaining the low concentration precision data for TCA, DCA, and MCA were close to the limits of detection. This is seen in the highest coefficients of variation at the lowest concentrations. The medium and high concentrations of TCA, DCA, and MCA were above the limit of quantitation (3.3 times the limit of detection) and had lower coefficients of variation (Karnes et al., 1991).

The method had good accuracy for the intra-day precision samples. These samples came within 90% of the expected values. The method gave lower than expected values for the inter-day precision samples at medium and high concentrations. This may have been due to a number of reasons, such as protein binding of the analyte during storage (Templin et al., 1993), absorption of the analyte onto the plastic (polyethylene) tubes used to store these samples, or decreased signal from the analyte after repeated injections.

Small amounts of DCA were found in human plasma samples collected from volunteers exposed to 100 ppm TCE via inhalation for 4 h. No evidence of MCA was found, and TCA concentrations as high as 10-12 μ g/mL were detected. The maximum average concentration of DCA from the 16 volunteers was 3 ng/mL. This occurred after 2 h of TCE exposure and decreased immediately after exposure. The observation that DCA decreased soon after the TCE exposure ended suggests that DCA may be rapidly metabolized in humans. This is consistent with *in vivo* and *in vitro* animal studies which indicate that DCA is rapidly metabolized (Larson and Bull 1992; Lipscomb et al., 1995); however, it is also possible that DCA may not be produced in sufficient quantities to be detected.

All of the individual samples in which DCA was detected were found to contain low levels. The samples which had detectable levels of DCA were frequently close to the lower limit of detection (4 ng/mL), and only a small number of samples had DCA concentrations greater than 10 ng/mL. The appearance and disappearance of DCA from the plasma samples seemed to follow the time course of a TCE metabolite.

However, further investigation of DCA production in man would require a more sensitive analysis for DCA in plasma. The levels of DCA found in this study indicate that DCA is not a predominant metabolite. It is possible

that DCA is produced and rapidly degraded to glyoxalic acid and glycolic acid (Lin et al., 1993); however, this has not been proven, and the detection of small amounts of DCA may be artifactual.

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3.2 DISSIMILAR CHARACTERISTICS OF *N*-METHYL-*N*-NITROSOUREA-INITIATED FOCI AND TUMORS PROMOTED BY DICHLOROACETIC ACID OR TRICHLOROACETIC ACID IN THE LIVER OF FEMALE B₆C₃F₁ MICE

J.R. Latendresse and M.A. Pereira¹

ABSTRACT

Dichloroacetic acid (DCA) and trichloroacetic acid (TCA) are metabolites of the industrial solvent and environmental contaminant, trichloroethylene (TCE), as well as contaminants of chlorinated drinking water. Human exposure to these chemicals is of concern because of their carcinogenic potential, all causing liver tumors in mice. Differences in dose-response curves, progression to cancer, and postexposure regression of lesions suggest that TCA and DCA work through different mechanisms. The purpose of this study was to further characterize the proliferative hepatocellular lesions promoted by TCA and DCA utilizing biomarkers of cell growth, differentiation, and metabolism in liver sections to better delineate the distinctions in the mechanism of the two chloroacetates. Fifteen-day old female mice were initiated with 25 mg/kg MNU. The initiated mice were administered DCA or TCA (20.0 mmol/L) in drinking water from age 49 days until euthanasia at age 413 days. The pathologic assessment showed that the foci of altered hepatocytes and tumors occurring in the animals treated with DCA were uniformly eosinophilic and positive immunohistochemically for TGF-a, c-jun, c-myc, CYP 2E1, CYP 4A1, and glutathione S-transferase- π (GST- π). The DCA lesions also were essentially negative for c-fos and TGF- β , but nontumor hepatocytes were consistently TGF- β positive. In contrast, tumors promoted by TCA were predominantly basophilic, lacked GST- π , and stained variably; usually greater than 50% of the tumor hepatocytes were essentially negative for the other biomarkers. These dissimilar characteristics provide a better molecular basis for the distinction in the mechanisms of the two chloracetates, and suggest some potential mechanisms that may contribute to their tumorigenesis.

INTRODUCTION

Dichloroacetic acid (DCA) and trichloroacetic acid (TCA) are metabolites of the important industrial and commercial chemical trichloroethylene (TCE) (Elcombe, 1985; Goldsworthy and Popp, 1987; Dekant et al., 1984). Widespread use of TCE has resulted in the contamination of surface water, groundwater, drinking water, and landfill hazardous waste disposal sites (Coleman et al., 1976; Conglio et al., 1980; Westrick et al., 1984). The United States Air Force is currently involved in a major research effort by both governmental and non-governmental agencies to provide a meaningful scientific basis to assess the human health risk of exposure to

¹Medical College of Ohio, Center for Environmental Medicine, 3000 Arlington Ave., Toledo, OH.

TCE. Additionally, after the trihalomethanes, DCA and TCA are the most common organic contaminants formed as reaction by-products during chlorine disinfection of water containing humic acids and other organic substances (Miller and Uden, 1983; Coleman et al., 1984); they are found in finished chlorinated drinking water with concentrations ranging from 34 to 160 μ g/mL (Uden and Miller, 1983; Krasner et al., 1989).

Administering DCA or TCA in the drinking water to B6C3F1 mice has been shown to cause hepatocellular adenomas and carcinomas (Herren-Freund et al., 1987; Bull et al., 1990; DeAngelo et al., 1991; Pereira and Phelps, 1996; Pereira, 1996). Differences in dose-response curves, histopathology, progression to tumor, and postexposure regression of tumors suggests that TCA and DCA work through different mechanisms (Bull et al., 1990; DeAngelo et al., 1991; Pereira and Phelps, 1996; Pereira, 1996). The mechanism(s) for the hepatocarcinogenic activity of the two chloroacetic acids appears not to result from genotoxic activity, as *in vitro* and *in vivo* tests for genotoxicity have not demonstrated significant activity (Rapson et al., 1980; Herbert et al., 1980; Chang et al., 1992; Waskel, 1978). Also, liver tumors induced by DCA and TCA have been shown not to contain a unique mutation spectrum in the H-*ras* oncogene, but rather mutations in the 61st codon of this oncogene, common to spontaneous tumors in $B_6C_3F_1$ mice (Anna et al., 1994; Ferreira-Gonzalez et al., 1995). Hence, DCA and TCA have been proposed to be non-genotoxic carcinogens that possess tumor- promoting activity. The apparent differences in tumorigenesis between TCA and DCA prompted us to further characterize the tumors promoted by them utilizing molecular biomarkers of cell growth, differentiation, and metabolism to provide a better molecular basis for the distinction in their mechanisms.

MATERIALS AND METHODS

The foci of altered hepatocytes and tumors used in this report were obtained from an initiation-promotion study of DCA and TCA that has been published (Pereira and Phelps, 1996). Detailed experimental design and methods are given there.

Briefly, at necropsy, the liver along with visible lesions was cut into approximately 3-mm pieces, fixed in 10% neutral-phosphate buffered formalin for approximately 18 h, transferred to 70% alcohol, and within 48 h embedded in paraffin. The blocks were sectioned at 5 μ m and stained with hematoxylin and eosin for histopathologic evaluation for foci of altered hepatocytes, hepatocellular adenomas and carcinomas. The 5-micron sections also were processed immunohistochemically for glutathione S-transferase (GST)- π by the avidin-biotin-peroxidase complex method employing rabbit NCL-GST- π (10 μ g/mL) from human chronic lymphoblast leukemic spleen (Novocastra Laboratory, Ltd., Newcastle upon Tyne, UK), goat blocking serum, Vector Kit PK-4001, and counterstained with hematoxylin.

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For immunohistochemical evaluation of the molecular biomarkers other than GST- π , the paraffin-embedded specimens were sectioned at 4 µm and processed with a TechMate 1000 (BioTek Solutions, Santa Barbara, CA) automated immunostaining system using a modification of the method of Iezzoni et al., (1993). This automated procedure allowed all the specimens to be processed and stained at one time in pairs (one each DCA and TCA specimen per slide pair), utilizing batch-prepared reagents and identical stepwise time intervals which minimized procedural variability. Briefly, for antigen retrieval, sections were microwaved in citrate buffer (BioTek Solutions, Santa Barbara, CA), then reacted for 20 min with 3% hydrogen peroxide and blocked with normal serum derived from the same species as the link antibody (BioTek Solutions, Santa Barbara, CA). Slides were then incubated 30 min with the appropriate polyclonal primary antibody (Table 3.2-1).

 TABLE 3.2-1.
 PRIMARY ANTIBODIES USED FOR IMMUNOHISTOCHEMICAL DETECTION OF BIOMARKERS OF CELL GROWTH, DIFFERENTIATION, AND METABOLISM

		Working		
Antibody ^a	Ig class	concentration	Vendor	Vendor's Address
TGF-α	Goat IgG	10 μg /mL	R&D Systems	Minneapolis, MN
TGF-β	Rabbit IgG	20 μg /mL	R&D Systems	Minneapolis, MN
c-jun	Rabbit IgG	3 μg /mL	Oncogene Science	Uniondale, NY
c-fos	Rabbit IgG	3 μg /mL	Oncogene Science	Uniondale, NY
c- <i>myc</i>	Rabbit IgG	20 μg /mL	Upstate Biotechnology	Lake Placid, NY
CYP 2E1	Goat IgG	2 μg /mL	Gentest Corporation	Woburn, MA
CYP 4A1	Goat IgG	2 μg /mL	Gentest Corporation	Woburn, MA
GST-π	Rabbit IgG	10 µg/mL	Novaocastra Laboratory	Newcastle upon Tyne, UK

^a TGF = Transforming growth factor GST = Glutathione S-transferase

Detection of immunoreactivity was performed using biotinylated link antibody (BioTek Solutions, Santa Barbara, CA or BioGenex, San Ramone, CA) and avidin-biotin complex (ABC kit, BioTek Solutions, Santa Barbara, CA or strepavidin-biotin, BioGenex, San Romone, CA) (20 min incubation for each, at room temperature), followed by incubation in hydrogen peroxide/diaminobenzidine (BioTek Solutions, Santa Barbara, CA) for 21 min. Tissues were counterstained with hematoxylin. The controls for non-specific staining included specimens stained exactly like test specimens except the appropriate non-immune serum or non-biotinylated antibody was substituted for the primary antibody or secondary antibody, respectively. None of these negative controls demonstrated significant immunostaining.

RESULTS

The number of DCA- and TCA-promoted hepatocellular proliferative lesions examined are given in Table 3.2-2.

TABLE 3.2-2.	NUMBER OF DCA AND TCA-PROMOTED HEPATOCELLULAR PROLIFERATIVE
	LESIONS EXAMINED FOR BIOMARKERS

	Total									
Chloroacetate	Animals ^g	Lesion	Number of lesions examined							
			TGF-α	TGF-β	c-jun	c-fos	c-myc	2E1ª	4A1 ^b	$GST-\pi^{f}$
DCA	7									
		Foci [°]	12	13	12	12	14	15	14	38
		Ad^d	12	12	12	12	12	12	12	94
		Cae	0	0	0	0	0	0	0	6
		Total	24	25	24	24	26	27	26	138
TCA	6							×		
		Foci	0	0	0	0	0	0	0	3
		Ad	9	9	10	10	10	8	8	32
		Ca	4	4	3	3	4	4	3	64
		Total	13	13	13	13	14	12	11	101

^a Cytochrome oxidase CYP 2E1

^b Cytochrome oxidase CYP 4A1

^c Preneoplastic hepatocytes (altered hepatic foci)

^d Adenoma

^e Carcinoma

^f Glutathione S-transferase π

⁸Number of DCA- and TCA-treated mice examined for GST- π were 24 and 23, respectively.

Altered hepatic foci were small, usually less than 1 mm in diameter. There was either no compression of adjacent non-tumor hepatocytes or only minimal compression along the margin of a single quadrant of the lesion. Hepatocellular adenomas were usually greater than 1 mm in diameter, and compressed the adjacent parenchyma along the margin of 2 or more quadrants of the tumor and/or elevated the liver capsule. Hepatic cords were one-cell thick, and the hepatocytes were well-differentiated, resembling those of altered foci. Hepatocellular carcinomas examined were predominantly solid or trabecular with hepatic cords two or more cells thick. Neoplastic hepatocytes were poorly circumscribed along some margins in some tumors, and occasionally vascular invasion was present. Mitotic figures were observed in all types of proliferative lesions.

Foci of altered hepatocytes and neoplasms were eosinophilic, basophilic, or mixed. DCA exposure produced predominantly eosinophilic lesions, previously reported (Pereira, 1996; Pereria and Phelps, 1996). In general, the hepatocytes of DCA-promoted foci and tumors were less pleomorphic, uniformly larger, and had more distinctive cell borders than the hepatocytes in lesions caused by TCA. Parenchymal hepatocytes of DCA-treated mice were uniformly hypertrophied with prominent cell borders, and the cytoplasm was markedly vacuolated and morphologically consistent with glycogen deposition previously described (Bull et. al., 1990). In contrast, TCA-promoted proliferative lesions tended to be basophilic as previously reported (Pereira, 1996; Pereria and Phelps, 1996), comprised of hepatocytes with less distinct cell borders, slight cytoplasmic vacuolization, and considerably more variability in nuclear and cellular size.

The proportions of total proliferative hepatocellular lesions immunolabeled with different biomarkers are given in Tables 3.2-3 and 3.2-4 for DCA and TCA, respectively.

TABLE 3.2-3.COMPARISON OF THE PROPORTION OF TOTAL PROLIFERATIVE
HEPATOCELLULAR LESIONS IMMUNOLABELED WITH DIFFERENT
INTENSITY FOR BIOMARKERS IN DCA-TREATED FEMALE MICE²

		Lesions with <	50% cells stained	Lesions with > 50% cells stained		
Biomarker	Total no. lesions	Mi ^b	M to D	Mi	M to D	
TGF-α	24	2°	0	2	20	
TGF-β	25	25	0	0	0	
c-jun	24	10	4	7	3	
c-fos	24	24	0	0	0	
c- <i>myc</i>	26	11	1	11	3	
CYP 2E1	27	0	3	0	24	
CYP 4A1	26	0	0	5	21	
GST-π	138	0	0	0	138	

^a Total number of DCA-treated mice examined for biomarkers was 7 except for GST- π which was 24.

^b Cytoplasm of positively stained cells was classified either as Mi = minimally to mildly stained or M to D = moderately to densely stained.

^c Number of lesions.

The hepatocytes of altered foci and hepatocellular adenomas occurring in the female mice treated with DCA were positive immunohistochemically for TGF- α , c-*jun* and c-*myc*, CYP 2E1 and CYP 4A1, and GST- π . Approximately equal numbers of proliferative lesions with greater or less than 50% of the lesion hepatocytes immunopositive for c-*jun* and c-*myc* were observed; the intensity of cellular staining for these proteins was mostly minimal to mild. In general, the hepatocytes of tumors promoted by TCA stained more variably, usually greater than 50% (except for c-*jun*) of the hepatocytes of lesions being essentially negative or stained only minimally to mildly for the protein biomarkers studied. Some hepatocellular neoplasms, particularly TCA-promoted tumors, manifested regions of hepatocyte phenotypic variability, including immunoreactivity for c-*jun* and c-*myc* proteins consistent with clonal expansion or tumor progression. c-*Fos* was essentially negative for both DCA and TCA promoted lesions.

TABLE 3.2-4.COMPARISON OF THE PROPORTION OF TOTAL PROLIFERATIVE
HEPATOCELLULAR LESIONS IMMUNOLABELED WITH DIFFERENT
INTENSITY FOR BIOMARKERS IN TCA-TREATED FEMALE MICE^a

		Lesions with < 50% cells stained		Lesions with > 50% cells stained		
Biomarker	Total no. lesions	Mi ^b	M to D	Mi	M to D	
TGF-α	13	6°	7	0	0	
TGF-β	13	13	0	0	0	
c-jun	13	4	0	4	5	
c-fos	13	12	0	1	0	
c- <i>myc</i>	14	9	2	0	3	
CYP 2E1	12	6	4	0	2	
CYP 4A1	11	4	7	0	0	
GST-π	64	64	0	0	0	

^a Total number of TCA-treated mice examined for biomarkers was 6 except for GST- π which was 23.

^b Cytoplasm of positively stained cells was classified either as Mi = minimally to mildly stained or M to D = moderately to densely stained.

^c Number of lesions.

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The protein markers that elicited the most distinguishing differences between the two chloroacetates (Tables 3.2-3 and 3.2-4) were TGF- α , TGF- β , CYP 2E1, CYP 4A1, and GST- π . TGF- α positivity was moderate to dense in over 50% of the hepatocytes in nearly all lesions (20/24) promoted by DCA; non-lesion hepatocytes were negative for TGF- α . In contrast, in the TCA-promoted tumors, less than 50% of the neoplastic cells were immunopositive for TGF- α , and of these tumors, only 7/13 had moderately to densely staining cells. DCA-promoted proliferative lesions tended to express TGF- β at very low levels (minimally), while the non-lesion hepatocytes were consistently strongly positive. TGF- β also was only expressed at low levels in TCA-promoted lesions, but the immunoreactivity was more variable with many of the positive hepatocytes staining mildly. However, unlike in DCA-exposed mice, the non-tumor parenchymal hepatocytes were negative for TGF- β . Both of the metabolic enzymes, CYP 2E1 and CYP 4A1, were more intensely immunoreactive in the majority of hepatocytes of DCA-promoted lesions compared to those promoted by TCA; non-lesion parenchymal hepatocytes were mostly negative in DCA-treated mice. In contrast, their staining patterns in parenchymal non-lesion hepatocytes in TCA-treated mice were centrilobular for CYP 2E1 and panlobular for CYP 4A1. The cytoplasm of hepatocytes of proliferative lesions in DCA-treated mice were moderately to densely immunopositive for GST- π . Both proliferative lesions and non-tumor hepatocytes in TCA-treated mice were negative for GST- π .

DISCUSSION

Both growth factors, TGF- α and TGF- β , were expressed in distinctly different patterns in the liver of DCA- and TCA-treated female mice. Because hepatocytes in proliferative lesions from DCA-treated mice consistently over expressed TGF- α compared to parenchymal hepatocytes which were negative, one or more TGF- α signal transduction pathways (recently reviewed, Kumar et al., 1995) may be important in carcinogenesis promoted by DCA. Precursor TGF- α or TGF- α , binds epidermal growth factor receptor (EGF-R) on the surface of target cells. EGF-R has intrinsic tyrosine kinase activity, which triggers one or more potential signal transduction pathways that culminate in the activation of transcription factors; e.g., *jun, fos, egr*-1 (Kumar et al., 1995). These factors induce expression of genes that regulate cell proliferation and differentiation. In contrast, the highly variable immunoreactivity of hepatocytes in TCA-promoted tumors in our study, often with minimal if any increase in staining intensity compared to background staining of non-tumor parenchymal hepatocytes, suggests that TGF- α may not play a significant role in TCA-promoted tumorigenesis.

Tumor promoters enhance tumor development by clonal expansion of one or more sub-populations of responsive cells, directly modulating their growth or death, or indirectly, by down regulating proliferation and/or increasing apoptosis in non-tumor parenchymal cells (Goldsworthy et al., 1996). TGF- β inhibits hepatocyte proliferation. In our study, DCA-promoted TGF- β -immunonegative proliferative lesions together with TGF- β -immunopositive non-tumor parenchymal hepatocytes suggest that the potential effects of this negative growth factor on the two different

populations of hepatocytes may provide a significant growth advantage to these proliferative lesions compared to those of TCA-treated mice; tumor and non-tumor parenchymal hepatocytes of TCA-treated mice lacked TGF- β expression.

c-Myc is an immediate response gene induced very early after the addition of certain growth factors and positively regulates cell proliferation and differentiation (Moses, 1992). Recently, there have been several reports that concomitant over expression of c-myc and TGF- α in rodent liver has resulted in acceleration of neoplastic development (Sandgren et al., 1993; Murakami et al., 1993; Presnell et al., 1995). Both TGF- α and c-myc were co-expressed in most of the DCA-promoted proliferative lesions in our study. Synergism resulting from the over expression of these two proliferation-enhancing factors in initiated cells promoted by DCA might account for the increased number of proliferative lesions observed in mice promoted with DCA compared to TCA after 31 to 37 weeks exposure (Pereira and Phelps, 1996; Bull et al., 1990). Conversely, there did not appear to be strong positive correlative staining patterns for TGF- α and c-myc in the TCA-promoted proliferative lesions in our study.

The expression of cytochrome CYP 4A1 was strongly immunopositive in DCA- but not TCA-promoted proliferative lesions. CYP 4A1 is an important enzymatic marker for peroxisomal proliferation since it's expression precedes the peroxisomal response and is coordinated with the transcription of the peroxisomal β -oxidation enzymes (Green, 1992; Aldridge et al., 1995). Both DCA and TCA are peroxisome proliferators in the liver of mice (Elcombe, 1985; Elcombe et al., 1985; Goldsworthy and Popp, 1987). Proliferation of peroxisomes and increased β -oxidation putatively results in the generation of excess H₂O₂ and active oxygen species that can bind proteins and/or lipids mediating signal transduction, and thereby modulate gene transcription and the cell cycle to promote tumorigenesis (Green, 1992; Van Der Vliet and Bast, 1992; Janssen et al., 1993; Burdon, 1995; Byczkowski and Channel, 1996).

In the present study, we found GST- π to be an excellent biomarker for preneoplastic and neoplastic hepatocelluar lesions observed in DCA-exposed female mice initiated with MNU. These results are consistent with those previously reported in mice treated only with DCA (Pereira, 1996). GST- π is involved in the biotransformation of xenobiotics by catalyzing glutathione conjugation, and a number of them have been associated with cancer initiation and malignant transformation (Gajewska and Szczypka, 1992; Xu and Stambrook, 1994).

In summary, this study has demonstrated some striking differences in response of DCA and TCA to some molecular biomarkers of cell growth, differentiation, and metabolism, and further supports the hypothesis that the carcinogenic mechanisms of these two chloroacetes are different. These dissimilar characteristics provide a better molecular basis for the distinction in the mechanisms of the two chloracetates, and suggest some potential mechanisms that may contribute to their tumorigenesis. Moreover, the dissimilar characteristics of these two metabolites of TCE provide a rational basis for comparing TCE-promoted proliferative lesions. Such a comparison may give some

insight as to whether DCA, TCA, or both, are playing a significant role in the murine liver carcinogenesis of the parent compound, TCE.

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SECTION 4

HALON REPLACEMENT TOXICITY PROJECT

4.1 PHYSIOLOGICALLY BASED PHARMACOKINETIC (PBPK) MODELING OF SHORT TERM (0 TO 5 MINUTE) HUMAN INHALATION EXPOSURES TO HALOGENATED HYDROCARBONS

A. Vinegar, G.W. Jepson¹, and J.H. Overton²

ABSTRACT

Allowable human exposure to halons and halon replacement chemicals is often regulated on the basis of cardiac sensitization potential. Exposure guidelines are established using dose-response data obtained from animal testing. Results from animal tests are often summarized by No Observable Adverse Effect Level (NOAEL) and Lowest Observable Adverse Effect Level (LOAEL) values. This threshold 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 allows exposures to be assessed in terms of the chemical concentrations in blood during the exposure. A physiologically based pharmacokinetic (PBPK) model was modified to include a lung compartment containing a deadspace region and a pulmonary exchange area. Each of the subcompartments had its own airspace, tissue and capillary subregions. Refinement of the model to include a breath-by-breath description of respiratory tract uptake allowed successful simulation of exhaled breath concentrations during the first minute of exposure to the anesthetics halothane, isoflurane, and desflurane. The model has been applied successfully to an actual case of accidental exposure to halon 1211 during extinguishment of a fire in a military armored vehicle. This model ultimately will be useful in simulating various short-term exposure scenarios for better evaluation of safe use of halon replacement chemicals.

INTRODUCTION

Efforts to replace ozone-depleting substances (ODS) have necessitated the quantitative toxicological evaluation of halon replacement chemicals. Historically, volatile organic compounds with halogen substituents have been regulated on the basis of cardiac sensitization tests conducted in dogs (U.S. EPA, 1994). While not all cardiac sensitization protocols are identical, the most commonly used approaches have some common fundamental features. Dogs, the usual test species, are challenged simultaneously with epinephrine and the test chemical of interest, during which time cardiac electrical activity is monitored for cardiac arrhythmias. The test is repeated using several different concentrations of the test chemical in order to establish a No Observable

¹ Armstrong Laboratory, Toxicology Division, Wright-Patterson Air Force Base, OH.

² Toxicology Branch, U.S. Environmental Protection Agency, Research Triangle Park, NC.

Adverse Effect Level (NOAEL) and a Lowest Observable Adverse Effect Level (LOAEL). The NOAEL and LOAEL in dogs have been applied directly by federal regulators to establish allowable exposure limits such as Emergency Guidance Levels (EGL) for humans (Jarabek, et al., 1994).

The task of determining appropriate allowable human exposure limits for halon replacement chemicals requires consideration of both the exposure concentration and the duration of the exposure. Recent efforts supported by the EPA have focused on development of scientific approaches for establishing safe egress times for people occupying an area where halon replacement chemicals are discharged. This is a complex task and requires quantitative evaluation of the temporal aspects of biological response with respect to exposure level and duration. A mathematical tool with recognized utility in describing the temporal aspects of chemical distribution in biological systems is the physiologically based pharmacokinetic (PBPK) model. A procedure employing PBPK modeling to establish egress times for humans in a halon replacement chemical environment has recently been described by Vinegar and Jepson (1996). However, application of the procedure to accommodate very short-term exposures under a wide range of exposure concentrations requires modification of the lung compartment description commonly used in PBPK models. Routinely, the PBPK lung compartment is derived using a steady-state approach that results in an instantaneous arterial blood concentration determined by the blood-to-air partition coefficient. While the steady-state lung description produces no difficulty when describing inhalation exposures lasting several minutes to hours, it is a significant limitation when attempting to model the first seconds to minutes of an exposure.

Mathematical descriptions of the anatomical and physiological characteristics of the lung that address non steadystate conditions have been devised and published (Overton, 1990). Additionally, human data have been collected for volatile anesthetic agents that could be used to further develop and validate models designed to describe the pharmacokinetic behavior of similar halogenated compounds being considered as halon replacement chemicals. Short-term human data (including the first five breaths) applicable for use in modeling efforts were collected for the volatile anesthetics halothane, isoflurane, and desflurane (Yasuda, et al., 1991). The combination of a theoretical PBPK model and appropriate data for model validation provides the basic components needed to develop a scientifically based method for describing the pharmacokinetic behavior of volatile halogenated hydrocarbons in humans during and following short-term inhalation.

The purpose of this work was to develop and apply a PBPK model that could predict blood and expired breath chemical concentrations in humans during inhalation exposures of less than five minutes duration. An existing PBPK model (Vinegar et al., 1994; Vinegar and Jepson, 1996; Williams et al., 1996) was modified to include a breath-by-breath description of inhalation in humans using a physiologically based description of a lung compartment with both gas exchange and deadspace regions. Each region was further divided into gas, tissue,

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and blood subregions. With the appropriate model modifications defined and in place, the short-term inhalation data collected in humans were evaluated using the PBPK modeling approach. As a demonstration of the potential utility of the model and approach described in this work, a documented exposure to Halon 1211 by two individuals in the closed space of a military armored vehicle (Lerman et al., 1991) was evaluated using the human PBPK model. The PBPK modeling approach was shown to be useful in describing the human health outcomes of the accidental Halon 1211 exposure and for general use in predicting expired breath levels of chemicals in humans following exposure via inhalation.

MATERIALS AND METHODS

Data used for model development and validation were collected as described in detail by Yasuda et al. (1991). Briefly, eight healthy male volunteers (age 25 ± 5 years [mean \pm SD]; body weight 76 ± 7 kg; height 182 ± 4 cm) were exposed to a mixture of 2.0% desflurane, 0.4% isoflurane, and 0.2% halothane, balance 35% oxygen/65% nitrous oxide. Exposure occurred for 30 min using a controlled ventilation nonrebreathing system. End-tidal samples were collected from the first five breaths, and then from breaths at 0.75, 1, 1.5, 2, 3, 5, 7.5, 10, 12.5, 15, 20, 25, and 29 min. Minute ventilation was measured and inspired samples were collected at 5, 7.5, 10, 12.5, 15, 20, 25, and 29 min. Samples were analyzed for anesthetic concentrations using gas chromatography.

An existing PBPK model (Vinegar et al., 1994; Vinegar and Jepson, 1996; Williams et al., 1996) which had been used to simulate results of exposure to various halogenated hydrocarbons was modified to include more detailed descriptions of the lung and ventilation. The lung description (Figure 4.1-1) was a modification of one used by Overton (1990), where the lung was divided into upper respiratory tract, tracheobronchial region, and a gas exchange or pulmonary region. In the current model the upper respiratory tract and tracheobronchial regions are described as a single deadspace region. Each of these was then divided into airspace, tissue, and capillary subregions. These are assumed to be well mixed. The volumes are constant except for the pulmonary region airspace which expands and contracts during inhalation and exhalation, respectively. Time dependent changes in mass or concentration in each region were described by mass balance equations related by partition coefficients to the concentration in one subregion. For instance, the arterial concentration (CA) is related to the total mass of chemical in the pulmonary region (AP), as follows: CA = AP/(VOLP/PB+VTP(PR)+VCAPP), where VOLP, VTP, and VCAPP are the volumes of the airspace, tissue, and capillary subregions, and PB and PR are blood:air and richly perfused tissue: blood partition coefficients, respectively. The volume of the airspace (VOLP) consists of the fixed volume (VGP) plus the volume delivered with each breath which is expressed sinusoidally as a function of the breathing frequency. Thus, the volume delivered to the lung (DELVOL) at any time (T) is expressed as: DELVOL = (VT/2) (1.0-COS(2π FT)), where VT is the tidal volume and F is the frequency of breathing.



Figure 4.1-1. Physiologically based pharmacokinetic model. The section outside the dotted area represents the lung portion of the model. Abbreviations: VT is tidal volume; CA, CEX, CI, and CV are arterial, exhaled, inhaled, and venous concentrations, respectively; CVF, CVG, CVL, CVS, CVR, and CVCAPD are fat, gastrointestinal, liver, slowly perfused, richly perfused, and capillaries of deadspace region of lung venous concentrations, respectively; QC, cardiac output; QF, QG, QL, QS, QR, and QD are fat, gastrointestinal, liver, slowly perfused, rapidly perfused, and deadspace region of lung blood flows, respectively.

Human partition coefficients used were those reported by Eger (1990) and appear in Table 4.1-1. The gut:air partition coefficient and metabolic constants for halothane were taken from Williams et al. (1996) and partition coefficients for isoflurane and desflurane were extrapolated from the halothane value. V_{maxe} for isoflurane was set to one hundredth and for desflurane to one thousandth of that for halothane (Holaday, 1977; Holaday et al., 1975; Sutton et al., 1991). The effect of metabolism on kinetics for the first five minutes of exposure, in any case, is essentially nil.0

Anatomic and physiological parameters used in the model were those proposed by Dankovic and Bailer (1993) and used by Vinegar and Jepson (1996). Organ blood flows for resting individuals were used to simulate the data obtained from anesthetized subjects. Since frequency of ventilation was controlled and tidal volume was measured, the actual values were used for each subject. Cardiac output was assumed to be equal to alveolar ventilation.

	Chemical				
Parameter	Halothane	Isoflurane	Desflurane		
Molecular weight	197.39	184.5	168.0		
(g/mole)					
V _{maxc} , max. metabolic rate	7.4	.074	.0074		
(mg/h/kg)					
K_m , affinity constant	0.1	0.1	0.1		
(mg/L)					
Blood:air partition	2.4	1.4	0.42		
coefficient					
Liver:air partition	5.04	2.52	0.588		
coefficient	4				
Gut:air partition	3.1	1.5	0.362		
coefficient					
Fat:air partition	148.8	72.8	12.6		
coefficient					
Rapidly perfused:air	5.04	2.52	0.588		
partition coefficient					
Slowly perfused:air	9.6	4.76	0.966		
partition coefficient					

TABLE 4.1-1. CHEMICAL SPECIFIC MODEL PARAMETERS AND VALUES

RESULTS AND DISCUSSION

The usual steady-state solution used to describe ventilation in PBPK models resulted in the inability of the model to account for the short-term changes occurring with onset of exposure. Simulations of the eight subjects using the steady-state lung model are shown compared with the breath-by-breath lung model (Figure 4.1-2). The more detailed description of the lung and ventilation resulted in the model being able to simulate fluctuations in the concentration of the chemical in the exhaled breath. Examples of simulations for two individuals are shown for halothane, isoflurane, and desflurane (Figure 4.1-3). Of the eight individuals for which data were provided, the model fit the poorest with data from human volunteer identified as SPK. Other simulations were similar to those shown for volunteer RBZ. Differences in physiological parameter values between individuals and over time for each individual are the likely reason for differences in the fit between measured values and simulations.



Figure 4.1-2. End alveolar concentration/inhaled concentration during inhalation of halothane. The continuous lines represent simulations of 8 different inhalation exposures (2000 ppm) (Yasuda et al., 1991) using steady-state lung model (upper) and breath by breath lung model (lower). Individual points represent actual data from 8 individuals.



Figure 4.1-3. End alveolar concentration/inhaled concentration during inhalation of halothane, isoflurane, and desflurane by two male subjects (RBZ and SPK). The continuous line represents the simulation using a breath-by-breath description of the lung.

A case report of an accidental inhalation of Halon 1211 was used to illustrate the utility of a model that can successfully simulate short-term pharmacokinetic data. During an Israeli military exercise, a small fire was accidentally ignited in an armored vehicle and was extinguished by using two 1.36 kg cans of Halon 1211 (Lerman et al., 1991). The gunner, who was seated at the vehicle turret, escaped immediately and was symptomless within 60 seconds. Upon physical examination, findings were unremarkable.

The vehicle driver was in a more inward compartment which was isolated by closed hatches. He was not able to exit and was removed unconscious, pulseless, and not breathing a few minutes after the fire was extinguished. Upon taking an EKG 40 min later, ventricular fibrillation was evident which did not respond to DC cardioversion. He was pronounced dead 2 h later.

A reenactment was performed in an identical vehicle to determine the exposure to Halon 1211 received by the two men. Two cylinders of 1.36 kg Halon 1211 were sprayed in the vehicle and concentrations were measured at the driver's compartment and the turret. The published exposure profiles are shown in Figure 6.1-4. Both the driver and gunner potentially received exposures above the LOAEL for Halon 1211 (1.0%, 10000 ppm).



Figure 4.1-4. Concentration profiles of Halon 1211 in the driver's compartment and gunner's turret of an Israeli armored vehicle.

A PBPK model was used to generate 5-min simulations of exposure to Halon 1211 under rest and work conditions to determine the blood concentrations associated with exposure to the cardiac sensitization LOAEL of 1.0% (Figure 6.1-5). Rationale for this approach was presented by Vinegar and Jepson (1996). These concentrations were 7.1 and 4.1 mg/L for rest and work conditions, respectively. The simulated blood profile for the driver is shown in Figure 4.1-6, and assuming at least 2 to 3 min for removing him from the driver's compartment, his blood concentration greatly exceeded the LOAEL blood concentrations reaching levels as high as 15 to 25 mg/L. The simulated blood profile for the gunner (Figure 4.1-6) also exceeded the LOAEL blood concentrations reaching levels of 7 to 13 mg/L after 2 to 3 min of exposure. However, the gunner exited the vehicle in less than a minute, escaping well before his blood level would have reached the LOAEL blood concentration. Thus, in this particular case study, the correlation between exposures, blood concentrations, cardiac sensitization LOAEL, and outcome for the two individuals was consistent with modeling predictions.



Figure 4.1-5. Simulated blood concentration during 5-min exposure of a human to Halon 1211 at 1.0%, the LOAEL for cardiac sensitization in dogs, under conditions of rest or light work. Resulting blood concentrations are shown.



Figure 4.1-6. Simulated blood concentration during 5-min exposure of a human to Halon 1211 at concentrations measured in the driver's compartment and in the turret (gunner's position) of the Israeli armored vehicle.

The ability of the model to simulate short-term kinetics has been verified using two scenarios. First, the model successfully simulated exhaled breath data collected from volunteers who were exposed to three different anesthetic agents (halothane, isoflurane, and desflurane). Second, the model was used to predict target blood concentrations for the cardiac sensitization LOAEL for Halon 1211 and to simulate actual exposure scenarios for two individuals; one exceeded the target concentration, went into ventricular fibrillation and died, and the other escaped safely before the target concentration was reached. The eventual intended use of the model is to predict egress times for potential halon replacement chemicals using target blood concentrations for the cardiac sensitization LOAEL for each agent. In order to do this effectively, it will be necessary to consider individual-toindividual variability in physiological and biochemical parameters which will ultimately affect the kinetics of a given agent. The ability of the model to simulate exhaled breath varied among subjects. The model as used herein assumed single values of physiological and biochemical constants with physiological constants being scaled according to body weight. However, there is individual variability which is not accounted for and would be impractical, if not impossible, to measure for every individual that might potentially be exposed to a chemical agent. Continuing work will focus on ways to account for individual variability; e.g. Monte Carlo simulations. For this purpose, the variability of each physiological and biochemical parameter is taken into account by doing multiple simulations. Each simulation assigns a value to each parameter based on its statistical distribution. Using this statistical approach one can establish guidelines that can protect any percentage of the population (i.e., 95%, 99%, etc.) that regulators deem appropriate.

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4.2 ACUTE AND SUBCHRONIC TOXICITY EVALUATION OF THE HALON REPLACEMENT CANDIDATE PHOSPHORUS TRIBROMIDE - A PRELIMINARY REPORT

R.E. Wolfe, D.E. Dodd, M.L. Feldmann, D.H. Ellis, J.S. Eggers¹, C.D. Flemming, H.F. Leahy, and A. Vinegar

ABSTRACT

Environmental concern over the depletion of stratospheric ozone and global warming has led to an international treaty called the Montreal Protocol (1987) which calls for the phaseout of all halons by the year 2000. Presently, the U.S. Air Force is using Halon 1301 as a flooding agent for extinguishing inflight aircraft and electronic equipment fires. Because it is believed to have less ozone depleting activity, phosphorus tribromide (PBr₃) is being considered as a possible replacement for Halon 1301. To address environmental and health effects concerns with this compound, it is necessary to evaluate its short-term and long-term toxicity effects. This study was designed to determine the effects following short, high-concentration exposures which could occur in accidents, as well as long-term, low-concentration exposures which could occur on flight lines or where maintenance commonly occurs.

INTRODUCTION

The Department of Defense requires the development of a complete toxicity profile for replacement candidates of halons, which includes the results from acute and subacute toxicity testing. Many of these compounds, including phosphorus tribromide, have not been thoroughly investigated to determine their toxicological properties. The purpose of this study was to conduct acute and subchronic toxicity testing to provide acute toxicological hazard information to complete a risk assessment for phosphorus tribromide. This risk assessment will determine if phosphorus tribromide (PBr₃) can be used as a halon replacement compound. U.S. Air Force tests in a 500,000 BTU/h test burner demonstrated that PBr₃ is an effective fire extinguishant. The test burner was quenched by only 0.2 mL PBr₃. This volume is several thousand times less than the amounts required of other halon replacements to suppress fires. The new fire extinguishant systems based on PBr₃ usage will occupy less volume, weigh less, and require less mechanics than current systems used on U.S. Air Force aircraft and electronic equipment fires. Also, PBr₃ has no ozone depleting potential since it is rapidly hydrolyzed in the troposphere.

An accidental human exposure to bromine (Br_2) , PBr₃, and hydrogen bromide (HBr) was reported by Kraut and Lilis, 1988. While mixing PBr₃ and HBr, a female laboratory assistant was exposed to these compounds via splashing on the face, chest, and hair, and by inhalation of resulting vapors. She remained in the area of the

¹Army Medical Research Unit, Wright-Patterson Air Force Base, OH.

exposure for five to ten minutes. Immediate effects noted were complaint of dry cough, light-headedness, and slight congestion of the throat. Over the next two weeks, the subject experienced increasing shortness of breath. She was referred to the company physician. Chest x-rays revealed bilateral lobe infiltrates, and chemical pneumonitis was diagnosed.

In a study of rats exposed to 1300 ppm HBr for 30 min comparing nose-breathing effects to pseudo-mouthbreathing effects, tissue injury of the respiratory tract was observed (Stavert et al., 1991). Tissue damage following nose-breathing exposure to HBr was confined to the nasal region. Observations included epithelial and submucosal necrosis. Pseudo-mouth-breathing exposure to HBr caused higher mortality rates and major tissue disruption was noted in the trachea. Observations included epithelial, submucosal, glandular, and cartilage necrosis.

Phosphorus tribromide reacts with moisture and with oxygen to produce a number of chemical derivatives that are believed to include phosphonic acid and hydrobromic acid. There is limited toxicity information available in the literature for phosphorus tribromide and hydrogen bromide. No acute toxicity information is available for phosphonic acid or hydrobromic acid. The complete list of breakdown products is not known. The combined interaction of these derivatives to cause potential health hazards is also not known. The objectives of this study were to determine the acute and subchronic toxicity of PBr₃. The information gathered from these experiments will provide the necessary database for developing a risk assessment for PBr₃.

MATERIALS AND METHODS

Test Material

Phosphorus tribromide (PBr₃)

Source/Manufacturer:	Aldrich Chemical Company		
	Milwaukee, WI		
CAS No.:	7789-60-8		
Purity:	Certified by the manufacturer as 99.99+% pure		
Appearance:	Colorless to pale yellow liquid		
Specific Gravity:	2.850 g/mL		
Vapor pressure:	0.27 psi at 54 °C		

Animals

Male and female Fischer-344 (F-344) rats weighing between 100 and 125, and 75 and 100 g, respectively, were purchased from Charles River Breeding Labs, Raleigh, NC. Male New Zealand white (NZW) rabbits weighing

between 2 and 3 kg were purchased from Myrtle's Rabbitry, Inc., Thompson's Station, TN. All animals were subjected to a two-week quarantine period. The animals were housed in laminar-flow rooms during nonexposure periods. Rats were group housed (two per cage) in clear plastic cages with wood chip bedding. The rabbits were housed individually in wire-bottom, stainless-steel cages. Water and feed (Purina Rabbit Chow #5320, and Purina Formulab #5009 for rats) were available *ad libitum*, except during the inhalation exposure period, and for 12 h prior to sacrifice for the 5-day range-finding rats only. Animal room temperatures were maintained at 21° to 25° C and the light/dark cycle was set at 12-h intervals. Plexiglas restraining tubes used with the nose-only chambers were cleaned as needed to provide enough clean tubes for each day's exposure.

Acute Toxicity Tests

The approach for determining the acute toxicity of PBr_3 was to perform a battery of acute tests: Skin Irritation Screen in rabbits, and Acute Inhalation (Limit Test) in rats. Acute inhalation exposures were nose-only exposures. The information gathered in these tests will supply information necessary to establish limits for emergency conditions resulting from spills or leaks involving PBr₃.

Skin Irritation Screen

Due to the potential corrosiveness of PBr₃, a skin irritation screen was performed to establish if a minimally irritating volume of PBr₃ could be determined. Two male NZW rabbits had the hair shaved from their backs 24-h prior to the Skin Irritation Screen. The first rabbit was anesthetized with xylazine hydrochloride (9 mg/kg) plus atropine (0.16 mg) and ketamine hydrochloride (40 mg/kg) administered IM. Once the animal was anesthetized, a neat dose of 50 μ L (0.050 mL) test material was applied to a designated area of the shaved back. The test material was allowed to react with the skin for 10 min before observations were made. The skin was then observed and any signs of irritation recorded (Draize, 1959). If irritation occurred at the initial 50 μ L dose, the dose volume would be lowered to 10 μ L. This dose was applied to an area of the shaved back adjacent to the 50 μ L dose, and allowed to remain for 10 min before observations were made. Any signs of irritation were recorded. If irritation was apparent at the 10 μ L dose volume, the test material would be determined to be too irritating to the skin to perform a complete 72-h skin irritation test.

A second rabbit was also anesthetized, as above, and a neat dose of $50 \ \mu L$ (0.050 mL) test material was applied to a designated area of the shaved back. The treated skin was then washed with copious amounts of water 30 seconds after dosing. The skin was flushed with water for one minute. The skin was observed 10 min after flushing, and signs of irritation were recorded (Draize, 1959). The dose volume was then lowered following the regimen used for the first rabbit. All test material was again flushed from the skin 30 seconds after dosing, and observations were recorded 10 min after the treated skin was rinsed with water.

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Both rabbits utilized in this skin irritation screen were euthanatized, while still under anesthesia, with a percutaneous intravenous injection of Euthol euthanasia solution.

Acute Inhalation Toxicity

Five female F-344 rats were exposed to PBr₃ at the Limit Test value (5 mg/L). If deaths or signs of toxic stress were observed during the limit test, a series of acute inhalation exposures were to be performed to provide a measure of toxic potency that could be compared with other chemicals, including other halon replacement candidates. Four additional exposures were performed, spaced to produce a range of toxic effects. Only male F-344 rats were used at dose levels below the limit test. Male rats were also exposed to target PBr₃ concentrations of 2.5, 1.0, and 0.5 mg/L, five animals per exposure. All animals were weighed prior to exposure and on postexposure days 1, 2, 7, and 14. Exposed animals were observed for signs of toxic stress twice daily. All animals received a gross necropsy at sacrifice. Animals were sacrificed via CO_2 inhalation. Lungs from animals dying during exposure were taken for histopathologic examination. Exposures were performed using the nose-only Cannon exposure chambers (Cannon et al., 1983).

The required concentration of phosphorous tribromide was generated using Sage syringe pumps delivering the required mass of test material into the air supply for the Cannon-52 chambers. A minimum of 300 to 500 mL/min of dry (<20% RH) filtered house air was supplied per animal in the test system with a minimum of 10 L/min used for each of the test groups. The amount of test material delivered per unit time was calculated on the basis of air flow and desired concentration. PBr₃ is unstable in the presence of water vapor; therefore, the test material analysis required quantitating the sample prior to mixing with expired air. Analysis for bromide ion was performed to determine test material concentration. A grab sample technique was employed.

Subchronic Toxicity Tests

Five-Day Inhalation Range-Finding Study

This portion of the study was a one-week (5 exposure) pilot study to determine target concentration levels for a subchronic 28-day study. Three concentrations of PBr₃, 0.5, 0.1, and 0.05 mg/L, and a control group were used in this range-finding study. The highest concentration of PBr₃ was determined considering the results of the acute inhalation toxicity test. Five male F-344 rats were exposed via nose-only inhalation to air only or PBr₃ for 4 h/day 5 consecutive days. Exposures were performed using Cannon chambers. Animal body weights were measured pre-exposure and each exposure day. Animals were fasted 12 h prior to necropsy and terminal body weight were also recorded. A clinical examination was performed for each animal every study day. Animals were observed daily for visible signs of toxicity, and all observations were recorded. Animals were sacrificed via CO_2 inhalation. Blood was collected at necropsy via the posterior vena cava for complete clinical chemistry and hematology evaluations. At sacrifice, wet tissue weights were determined for heart, kidneys, testes, liver, lungs, brain, spleen, thymus, and

adrenal glands. A gross pathologic examination was performed on each rat. Tissues, including upper respiratory tract and gross lesions, were taken and held for future histopathologic examination for identification of target tissues or organs.

Subchronic Inhalation Toxicity Testing (28-days):

This 28-day study has not yet been performed. Results from this subchronic test will be included in the final technical report for this study. The concentrations of PBr₃ utilized in the 28-day inhalation study will be determined upon completion of analysis of data from the 5-day range-finding study. Any changes from control values noted for body weights, organ weights, clinical chemistry and hematology parameters, and histopathology evaluations will be considered when determining the PBr₃ concentrations for this phase of the study. A concentration of PBr₃ which produces toxic effects, but no mortality, will be employed as the highest PBr₃ level. A concentration which produces no evidence of toxicity will be utilized for the low level. An intermediate concentration will be targeted for Group III which should produce minimal observable toxic effects (OECD, 1993).

Three concentrations of PBr₃ will be used in this subchronic study and a control group will be included. Exposures will be performed using Cannon chambers. The chamber air flows will be maintained at approximately 10 L/min. This will result in an airflow of approximately 330 mL/min to each animal. Ten male and ten female F-344 rats will be exposed to PBr₃ or air only 4 h/day, excluding weekends, for 28 days (a total of 20 exposures). Animal body weights will be measured pre-exposure and weekly thereafter. A clinical examination will be made each study day and signs of toxicity will be recorded. Animals will be fasted 12 h prior to necropsy. A complete blood assay will be conducted on blood samples taken at sacrifice from all animals. The blood will be sampled via the posterior vena cava. Animals will be sacrificed via CO_2 inhalation. At sacrifice, wet tissue weights will be determined on adrenal glands, lungs, brain, ovaries/testes, heart, spleen, kidneys, thymus, and liver. A gross pathologic examination will be performed on each rat and tissues will be taken for histopathologic examination. Any animals dying during the study will have a gross necropsy performed and tissues collected, when possible.

Statistics

The following statistical analyses will be performed:

Body Weight: Repeated multivariate analysis of variance with Scheffe pairwise comparisons (Barcikowski, 1983).

Hematology, Clinical Chemistry, and Organ Weights: A two-factorial analysis of variance with multivariate comparisons (Barcikowski, 1983).

Histopathology: Fischer Exact Test, or, if not valid, Yates' Corrected Chi-square (Zar, 1974).

RESULTS

Acute Toxicity Tests

Skin Irritation Screen

Application of 50 μ L and 10 μ L neat PBr₃ to intact rabbit skin caused an immediate reaction, producing a white vapor. No change in the color of the rabbit skin occurred, although the treated areas were hardened when compared to adjacent untreated skin. Ten minutes after dosing, both rabbits (with either skin rinsed with water or not rinsed) had scores of 0 for erythema, 4 for edema, and 15 for necrosis (Draize, 1959) for each volume (50 or 10 μ L) of PBr₃ used. The affected skin was limited to the treated area only and was clearly demarcated from the adjacent untreated skin.

The animal tested dermally with no rinsing of test material had skin and muscle lesions noted at necropsy. These tissues were taken and examined histologically. Findings included focally extensive necrosis of the entire thickness of the haired skin, epidermis, and dermis. Multifocal, moderate hyalin degeneration and necrosis of the panniculus and skeletal muscle, and the underlying subcutis was also observed. The second rabbit tested dermally, but with the test material rinsed from the skin, had only skin lesions noted at necropsy. The skin lesion was taken and examined histologically. As in the first rabbit, histologic findings included extensive necrosis of the entire thickness of the haired skin, epidermis, and dermis. Multifocal moderate hyalin degeneration and necrosis of the panniculus and skeletal muscle were also noted as with the first rabbit. The appearance of these lesions were considered compatible with an acute chemical injury (burn). No further testing for skin irritation was performed.

Acute Inhalation Toxicity

During the period of performance for this annual report, the acute inhalation exposures were not initiated. All results from these inhalation exposures will be included in the final report for this study.

Subchronic Toxicity Tests

Five-Day Inhalation Range-Finding Study

The five-day range-finding study will be performed in December 1996. All data from this portion of the study will be reported in the final report.

Subchronic Inhalation Toxicity Testing (28-days)

The 28-day study will be performed in early 1997. Results from this subchronic test will be included in the final technical report for this study.

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SECTION 5

EXPLOSIVES, PROPELLANTS, FUELS, AND LUBRICANTS PROJECT

5.1 ACUTE, SUBCHRONIC, AND REPRODUCTIVE TOXICITY OF QUADRICYCLANE VAPOR ON SPRAGUE-DAWLEY RATS

R.E. Wolfe, E.R. Kinkead¹, M.L. Feldmann, H.F. Leahy, L. Narayanan, and J.S. Eggers²

ABSTRACT

The U.S. Air Force is currently developing High Energy Density Matter (HEDM) for use in advanced rocket propellants to improve their performance. An HEDM of immediate interest is quadricyclane (QC). Assessments were performed to determine the inhalation toxicity of QC. Acute, 4-h exposures of male Sprague-Dawley (SD) rats to QC vapor resulted in an LC_{50} value of 0.78 mg/L. Subchronic (two-week) inhalation exposure on groups of male and female SD rats at 0.0, 0.025, 0.075, and 0.25 mg QC/L resulted in no mortality. Body weights of exposed rats were significantly less than those of the controls after 10 exposures. No exposure-related gross lesions were noted at necropsy. A 90-day general toxicity/reproductive screen using concentrations of 0.0, 0.01, 0.025, and 0.10 mg QC/L produced no significant exposure-related differences in the reproductive or litter parameters measured. The only lesion noted at necropsy was minimal pulmonary inflammation in the lungs of the mid- and high-concentration male rats. Significant treatment-related increases in brain dopamine and 5-hydroxytryptamine levels were detected in all QC-exposed rats. The low-concentration level of 0.01 mg QC/L represents a no observable adverse effect level (NOAEL) based upon the female rat data, and the lack of consistent dose-response data for male rats.

INTRODUCTION

The Air Force is currently developing High Energy Density Matter (HEDM) for use as advanced rocket propellants (RP). The most near-term development effort is that of the strained-ring hydrocarbons. These compounds will be added to propellant RP-1 (kerosene) to improve performance. An HEDM of immediate interest is quadricyclane (QC) (CAS 278-06-9). Present plans are for producing a rocket fuel mixture of 70% QC and 30% RP-1.

Very little toxicologic data are available for QC. The acute oral and dermal toxicity of the compound and a mixture of QC in kerosene was reported by Kinkead et al. (1993). An oral dose of 3.5 g/kg body weight of the neat compound given to male F-344 rats resulted in 100% mortality within 24 h. An oral dose (1.7 g/kg) of the

¹ ManTech Environmental Technology, Inc. (Retired).

² Armstrong Laboratory, Toxicology Division, Wright-Patterson Air Force Base, OH.

70/30 (QC/RP-1) mixture produced prostration in all rats through 24 h posttreatment, but no mortality. Quadricyclane did not cause mortality when applied dermally to rabbits at a dose of 2 g/kg.

The approach for determining the inhalation toxicity of QC was divided into three areas: 1) Acute, (4-hour) inhalation exposures to identify signs of toxic stress. This information is necessary to establish limits for emergency conditions resulting from spills and accidents; 2) Subchronic exposures (repeated over 2 weeks) to determine potential cumulative toxic effects and for setting exposure concentrations in the definitive 90-day study; and 3) Long-term (90-day) general toxicity/reproduction screen exposures to provide information for normal daily operations or threshold limit values and on possible gonadal effects. These data can be applied by Air Force medical and safety authorities in specifying operational procedures, protection equipment, and control measures.

MATERIALS AND METHODS

A complete description of the materials and methods used in this study are reported in Wolfe et al., 1996.

Animals

Male and female Sprague-Dawley-derived outbred albino rats [Crl:CD®(BR)] were purchased from Charles River Breeding Laboratories, Raleigh, NC. Rats used in the acute and subchronic studies were 7 weeks of age and those in the reproductive screen were 9 weeks of age upon receipt. All rats were identified by tail tattoo and were acclimatized two weeks prior to use. During the acclimation period, quality control procedures were performed on selected rats as described in Kinkead et al. (1991).

Rats in the subchronic and reproductive screen studies were assigned to groups by means of a computer-generated randomization. The randomization was stratified by body weight such that the mean body weights of all groups were homogeneous by statistical analysis at study initiation. Water from a reverse-osmosis system and Purina Formulab #5002 feed were available *ad libitum*. Animal rooms were maintained on a 12-h light/dark cycle (fluorescent light) and targeted at a temperature of 23 ± 2 °C and a relative humidity of $55 \pm 15\%$.

Test Agent

The QC test compound was purchased from EniChem America, Inc., Houston, TX. The purity of the QC was determined by Gas Chromatography-Mass Spectrometry. Samples taken from each of the two containers received were analyzed for purity. The results for these samples indicated a purity of 94%.
Generation and Analysis of Exposure Atmospheres

Quadricyclane vapor was generated by continuously metering known amounts via syringe pump (Sage model 355, Sage Instrument Division, Cambridge, MA) into the chamber input air stream. The carrier air for the QC vapor was maintained at a flow rate between 9 and 10 L/min and entered the chamber input air stream in a counter current flow to aid in mixing. The atmospheres generated for the 4-h acute inhalation study were conducted in 30-L bell jar chambers and were analyzed throughout the exposure (every 6 to 7 min) using a Varian 3400 gas chromatograph (Varian Associates, Palo Alto, CA). The chamber atmospheres for the 2-week subchronic study were continuously analyzed using Miran 1A infrared analyzers (Foxboro Analytical, South Norwalk, CT) equipped with 20-M path gas cells. The low- and mid-concentration chamber atmospheres of the 90-day reproductive screen were analyzed continuously using Beckman 400 total hydrocarbon analyzers (Beckman Instruments, Inc., Fullerton, CA). The high-concentration chamber was analyzed using a Miran 1A equipped with 20-M path gas cells.

Acute Exposures

Groups of 6 male rats, approximately 10 weeks of age, were placed in 30-L bell jar chambers and exposed once for 4 hours. The initial exposure was at the limit test concentration of 5 mg/L. Dilutions of this concentration followed to achieve a no observable adverse effect level (NOAEL) concentration. All rats were maintained for a 14-day postexposure observation period. Records were maintained for body weights (Days 0, 7, and 14 postexposure), signs of toxic stress, and mortality. Gross pathology was performed at necropsy. Histopathology was performed on lungs and any lesions of animals that died on study.

Subchronic Inhalation Exposures/Repeated Two-Week

Exposure groups consisted of 10 male and 10 female rats approximately 9 weeks of age. The rats were exposed for 6 h daily for 2 weeks, excluding weekends (10 exposures over a 2-week period). Exposure concentrations were 0.0, 0.025, 0.075, and 0.25 mg QC/L. The rats were singly housed in wire-bottom cages during exposure and in plastic shoe-box cages with bedding during nonexposure periods.

Records were maintained for body weights (Days 0, 7, and 14) and a clinical examination was performed on each exposure day. At necropsy, blood samples were taken via the vena cava for standard hematology and clinical pathology analyses. Gross pathology was performed on all animals. Wet tissue weights were determined on brain, kidneys, liver, lungs, spleen, and thymus.

General Toxicity/Reproductive Toxicity Screen

Exposure Regimen

Twelve male and twelve 12 rats per group were placed in 690-L inhalation chambers and exposed for 6 h daily to air only, 0.01, 0.025, or 0.1 mg QC/L. Animals were exposed 5 days/week for 2 weeks prior to mating. Males and females were exposed 7 days/week during mating, gestation, and lactation. Following weaning of the last litter, the rats returned to a 5 day/week exposure regimen. Dams were excluded from exposure Gestation Day (GD) 21 through Postpartum Day (PD) 4. Pups were not exposed at any time during the study.

One male and one female were cohabited, selected randomly from within their respective exposure groups, starting on Study Day 14. The pairs remained cohabited during nonexposure hours for up to 14 days until either a copulation plug was observed or sperm was present in the vaginal wash. The day a copulation plug was present or sperm were noted in the vaginal wash was defined as GD 0.

Rats were observed twice daily for signs of toxic stress. Male rat body weights were measured weekly during the study. Female body weights were measured in the same manner until confirmation of mating. During gestation, females were weighed on GD 0, 7, 14, and 20. Dams producing litters were weighed on PD 0, 4, 7, 14, and 21, then weekly thereafter. All pups were counted and sexed on PD 0. Live pups were weighed 1, 4, 7, 14, and 21 days after birth. Standardization of litter sizes, 4 per sex when possible, occurred on PD 4. All pups were examined for external abnormalities during the lactation period and received a gross examination when necropsied at weaning (PD 21).

Clinical Measurement

At necropsy, blood samples were taken via the vena cava from fasted parental animals for complete hematology and clinical chemistry assays. Erythrocytes were enumerated on a Coulter counter (Coulter Electronics, Hialeah, FL) and sera for clinical chemistry evaluations were assayed on an Ektachem 700XR (Eastman Kodak, Rochester, NY). Selected hematological parameters and absolute leukocyte differentials were determined according to established procedures. Sera were processed according to the procedures in the Ektachem Operations manual.

Evaluations at Necropsy

Brain, liver, kidneys, spleen, thymus, testes, and epididymides were weighed at necropsy. Bouin's fixative was used to fix the testes and epididymides. The pituitary, spleen, liver, lungs, kidneys, bone with bone marrow, and reproductive organs were removed from parental animals of both sexes and fixed in 10% buffered formalin solution. After routine processing, the tissues were embedded in paraffin and stained with hematoxylin and eosin for histopathologic examination.

Neurotransmitter Analysis

Neurotransmitter analysis was performed on 6 male and 6 female rats per group from the study. At final sacrifice, the brains from these animals were surgically removed, weighed whole, and then nine regions of each brain were dissected, frozen on dry ice, and stored at -70 °C until analysis. The nine brain regions analyzed were the brain stem, frontal cortex, cerebral cortex, caudate nucleus, septum, hypothalamus, thalamus, hippocampus, and cerebellum. Each region was analyzed for four neurotransmitters, norepinephrine (NE), epinephrine (E), dopamine (DA), and 5-hydroxytryptamine (5-HT). Metabolites of DA, homovanillic acid (HVA), and 3,4-dihydroxyphenylacetic acid (Dopac), and a metabolite of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), were also analyzed. Analysis of the neurotransmitters was performed following the methods described in Kim et al., 1987.

Statistical Analysis

 LC_{50} values were calculated using the method of Weil (1952). Maternal body weights, pup weights, organ weights, organ-to-body weight ratios, serum chemistry, and hematology 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 paternal body weights (Barcikowski, 1983). Neurotransmitters and their metabolites were quantitated using linear regressions of their standards. Mating indices and histopathologic results were analyzed by a Chi-square test of proportions applied to the incidence data (Rosner, 1990). Tissue lesion severity data were analyzed using the Kruskal-Wallis analysis of variance (Rosner, 1990).

RESULTS

A complete description of the results from this study are reported in the technical report AL/OE-TR-1996-0128 (Wolfe et al., 1996).

Acute Exposures

Inhalation exposures to the limit test concentration (5 mg/L) of vaporized QC resulted in 100% mortality. Additional 4-h inhalation exposures were performed to determine an LC_{50} value. Exposure concentrations of 2.0, 0.5, 0.1, and 0.05 mg/L were selected to produce mortality rates between 0 and 100%. Gross pathology of animals that died from exposure revealed congested, bright-red colored lungs. Microscopic findings in these subjects included scattered aggregates of intravascular neutrophils, present in the lung, liver, and heart. Similar findings were present multifocally in the liver sinusoids of these animals. All rats that survived the 14-day observation period had normal lungs. All deaths occurred within 12 hours of exposure. Animals that survived the initial 12 hours postexposure appeared normal and survived through the 14-day observation period. These survivors gained weight during the second week of observation. Rats from the exposure groups producing no

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lethality gained weight throughout the 14-day observation period and no signs of toxicity were observed. The LC_{50} value (95% confidence limit) for male rats was 0.78 (0.29-1.65) mg/L.

Subchronic Exposures

During the two-week exposure period, daily mean concentrations were maintained close to the desired concentrations of 0.250, 0.075, and 0.025 mg QC/L. There were no mortalities during the study. The high-concentration animals, both male and females, became more aggressive with continued exposure. The rats displayed hyperesthesia and were aggressive towards each other. Mean body weights of the QC-exposed rats were significantly lower than controls at 7 and 14 days. At 14 days, the exposed male rat groups were 14, 9.5, and 8% less (p<0.01) than controls, while the female groups were 14, 15, and 11% less (p<0.01) than controls in the high-, mid-, and low-concentration levels, respectively. Alanine aminotransaminase (p<0.01), creatinine (p<0.05), and calcium (p<0.01) values were all decreased in the high-concentration male rats. Serum chemistry parameters in the exposed female rats were not different from controls. Hematology values were unaffected in both sexes.

Absolute liver weights were decreased in high-concentration male rats and absolute lung weights were decreased in mid-concentration male rats. Numerous differences in absolute organ weights were noted in all QC-exposed female rats. However, when these weights are corrected for body weights (ratio), the only differences noted are relative brain weights in all QC-exposed female rats and the high-concentration male rats. No exposure-related gross lesions were noted at necropsy.

General Toxicity/Reproductive Screen

During the 90-day inhalation exposure period, daily mean concentrations were maintained near the target concentrations of 0.10, 0.025, and 0.01 mg QC/L. There were a total of 84 exposures during the study. No exposure-related mortalities occurred in any of the parental rats during the study. The high-concentration animals began to demonstrate hyperesthesia during the second week of exposure and became more aggressive with continued exposure. The mid-concentration female rats became aggressive and sensitive to touch during the gestation period. The low-concentration animals did not demonstrate signs of hyperesthesia or aggressiveness.

A concentration-related effect was noted in the mean body weights of both male and female rats. Mean body weights of the high-concentration male rats were significantly less than controls after one week of exposure and continued in that manner throughout the study. The mid-concentration male rats showed a decrease beginning at 34 days and the low-concentration males at 41 days which also continued throughout the study. Mean body

weights of the high-concentration and mid-concentration female rats were significantly less than controls beginning on Day 13. Mean body weights low-concentration female rats were not different from control values during the study.

No exposure-related differences were noted in male or female hematologic parameters at necropsy. A number of serum chemistry values were statistically different than control values in the male rats. However, the only differences that appear concentration- and/or exposure-related are a decrease in potassium and creatinine and an increase in sodium. Potassium was also decreased in female rats at the high-concentration levels. Total protein was decreased in the high- and mid-concentration female rats.

Absolute liver weights of the high-concentration male and female rats were statistically less (p<0.01) than controls. Relative testis weights were increased in all QC-exposed male rat groups. The decrease in liver weights appears to be a result of the difference in mean body weights of the groups. The liver to body weight ratios indicate no differences. Relative testes weights did not change proportionally to mean body weight. At necropsy, all rats utilized in this study were in good general health. No exposure-related gross lesions were noted during necropsy.

Histopathology

Statistical analyses revealed a significant incidence of minimal pulmonary inflammation in the lungs of the high-(p<0.05) and the mid-concentration (p<0.01) male rats. This inflammation was determined to be either perivascular or interstitial (Table 5.1-1). Perivascular inflammation consisted of various combinations of mononuclear cells which multifocally formed cuffs around small veins and arterioles. Interstitial inflammation was often present in animals with perivascular inflammation and consisted of similar inflammatory cells extending out into the surrounding parenchyma, occasionally associated with type II cell hyperplasia, fibrosis, and intra-alveolar accumulations of foamy macrophages. Lesions observed in the remaining tissues examined were common for rats during subchronic studies and did not occur at statistically significant higher incidence in any treatment group compared to the controls.

TABLE 5.1-1. INCIDENCE SUMMARY OF SELECTED MICROSCOPIC LESIONS OF MALE RATS FOLLOWING TREATMENT WITH QUADRICYCLANE DURING THE GENERAL TOXICITY/REPRODUCTIVE SCREEN

Organ/Lesion	Control	Low	Medium	High
Lung (N)	11	12	12	12
Perivascular inflammation (N)	0	0.0	11ª	6 ^b
(severity)°	0.0		0.9	0.5
Interstitial inflammation (N)	0	0	8ª	5 ^b
(severity)°	0.0		0.8	0.7

*Statistically different from control at p<0.01.

^bStatistically different from control at p<0.05.

^cMean grades of severity based on: 0 = Normal; 1 = Minimal; 2 = Mild; 3 = Moderate; 4 = Marked; 5 = Severe.

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Reproductive Indices

The exposures showed no adverse effects on mating. The mating index was 100% for the high-, low-, and control groups mated (Table 5.1-2). No significant exposure-related differences were noted in length of gestation, sex ratio, gestation index, or mean number of offspring per litter. During the 21-day lactation phase, mean pup weights were statistically significantly lower between the high-concentration group and control group. Mid-concentration mean pup weights were only statistically different at LD 1. Gross necropsy at weaning of all pups revealed no gross lesions or external abnormalities.

Dose Level:	Control	Low	Medium	High	
Number of Mated Pairs	12	12	12	12	
Number of Copulated Pairs	12	12	10	12	
Number of Dams with Pups Born	12	12	9	12	
Number of Dams with Pups Alive	12	12	9	12	
Mean Number of Pups per Litter	15.3	15.6	13.9	13.5	
Average Length of Gestation (days)	22.5	22.5	22.3	22.4	
Gestation Index (%) ^a	100.0	100.0	90.0	100.0	
Live Birth Index (%) ^b	98.9	98.4	98.4	95.7	
4-Day Survival Index (%)	99.5	98.4	98.4	95.7	
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	99.0	
Lactation Index (%)	100.0	100.0	100.0	99.0	

TABLE 5.1-2. REPRODUCTIVE DATA FOR RATS TREATED WITH QUADRICYCLANE

^a <u>Number of females with live litters</u> Number of females pregnant

^b <u>Number of live pups at birth</u> Total number of pups born

^c <u>Number of pups surviving 21 days</u> Number of pups surviving 4 days

Neurotransmitter Analysis

Statistically significant treatment-related increases in DA were detected in QC-exposed male and female rats in all brain regions analyzed. In male rats, the increase in DA concentration was greatest in the brain stem, while in female rats, the largest increase of DA occurred in the cerebellum. Elevated levels of the metabolites of DA were also found in brain regions of QC-exposed rats. Statistically significant increases in 5-HT levels were observed in all nine brain regions of high-concentration males rats. Mid-concentration males had significant increases in 5-HT levels were increased over control in five of the nine brain regions. Quadricyclane-exposed female rats had significant (p<0.01) increases in 5-HT levels in all brain regions except the cerebral cortex.

DISCUSSION

Inhalation of QC at the limit test concentration resulted in total mortality. The lack of delayed deaths (none after the first day posttreatment) and evidence of pulmonary congestion at necropsy suggest that death was caused by acute irritation of the respiratory system. Repeat exposure of male and female rats to QC vapor at concentrations of 0.25, 0.075, 0.025, and 0.0 mg/L resulted in no mortality. Treatment-related decreases in body weights were observed over the two-week exposure period. The only organ-to-body weight difference noted was for the brain in all exposed female rats and the high-concentration male rats. In general, the brain does not increase or decrease in size with body weight changes and, therefore, the absolute weight which was not different from controls, is the better indicator of effect. Isolated differences in serum creatinine, alanine aminotransaminase, and calcium were noted only in high-concentration male rats, but were not considered biologically significant.

Inhalation exposure of male and female rats to QC vapors at concentrations of 0.10, 0.025, 0.01, and 0.0 mg/L produced no adverse effects on reproductive performance or litter parameters. Treatment-related decreases in mean body weight were noted in both male and female rats. High-concentration animals began to demonstrate hyperesthesia during the second week of exposure, and these animals became more aggressive as the study progressed. Mid-concentration female rats became aggressive and sensitive to touch only during the gestation period. The low-concentration animals did not demonstrate this behavior. At necropsy, all animals were in good general condition. No exposure-related gross lesions were noted at necropsy and clinical pathology data revealed no significant alterations in hematology values in any group of rats. Isolated significant differences in serum chemistry were noted in male and female rats. The decrease in potassium was the only consistent finding in both sexes of the high-concentration group. This may be related to the significant body weight loss in these animals. A decrease in food consumption may also have been a contributing factor, but food intake was not measured during the study.

The only statistically significant histologic finding in rats following QC exposure was minimal pulmonary inflammation observed in 6 of 12 high- and 11 of 12 mid-concentration male rats. Similar lesions were not

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observed in the lungs of female rats. This type of lesion most likely represents an immunologic response and is compatible with lesions described in rats in the aftermath of a viral respiratory infection (Brownstein, 1985). The possibility that this lesion was a male-rat specific inflammatory reaction to inhalation of QC in male rats seems unlikely since the incidence did not occur in a concentration-related manner. The lesion was not thought to be biologically significant as the severity of the lung lesions was never more than mild, and no clinical signs of respiratory distress were reported in affected animals during the in-life portion of the study.

The most significant finding from the neurotransmitter analyses of brain tissue from QC-exposed male and female rats was an increase in dopamine levels in all brain regions. An increase in dopamine level was detected even in the low-concentration animals which did not display hyperesthesia or aggressive behavior. The role of 5-HT in aggressive behavior has been documented (Brown and Linnoila, 1990; Brunner et al., 1993). Alterations in brain 5-HT levels in male and female QC-exposed rats may be one of the reasons for the aggressive behavior observed during the studies. Hyperesthesia observed in QC-exposed rats may be due to some alterations in the filtering mechanism in the reticular formation where afferent impulses from different sensory modalities are processed before they reach the sensory cortex in the cerebrum (Spence, 1992). Increased sensitivity to environmental stimuli such as sound, light, temperature, and touch can make an animal highly irritable, which may progress into aggressive behavior.

The results of this study indicate effects were seen at all of the exposure concentrations. The effects in female rats at the low-concentration level were limited to neurotransmitter changes. The increase in neurotransmitter levels is of unknown significance and may represent a reversible, physiological response to QC. The low-concentration level of 0.01 mg/L QC probably represents a NOAEL based on the female rat data and a lack of consistent dose-response data for male rats. In addition, the body weight depression for male rats in the low-concentration group was less than 10%.

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5.2 ACUTE TOXICITY EVALUATION OF JP-8 JET FUEL AND JP-8 JET FUEL CONTAINING ADDITIVES

R.E. Wolfe, E.R. Kinkead¹, M.L. Feldmann, H.F. Leahy, W.W. Jederberg², K.R. Still², and D.R. Mattie³

ABSTRACT

To reduce fuel fouling in current U.S. Navy and Air Force aircraft systems and to provide additional heat sink and thermal stability for future systems, the Air Force is developing an improved JP-8 jet fuel (JP-8 + 100). Two companies (Betz and Mobil) have developed additive packages that are currently being tested in aircraft systems. To determine if the additive packages will produce health effects for flightline personnel, acute testing was performed on JP-8 and the two JP-8 + 100 jet fuels. A single oral dose of 5 mg jet fuel/kg body weight to five male and five female Fischer-344 rats, and a single dermal application of 2 g jet fuel/kg body weight applied to five male and five female New Zealand White rabbits resulted in no deaths. No signs of toxic stress were observed, and all animals gained weight over the 14-day observation periods. Single treatment of 0.5 mL neat jet fuel to rabbit skin produced negative results for skin irritation. Guinea pigs failed to elicit a sensitization response following repeated applications of the jet fuels. Inhalation vapor exposure to JP-8, JP-8 + 100 (Betz), and JP-8 + 100 (Mobil) resulted in LC₅₀s >3.43, >3.52, and >3.57 mg/L, respectively. LC₅₀ values for aerosol exposure to JP-8, JP-8 + 100 (Betz), and JP-8 + 100 (Mobil) were >4.44, >4.39, and >4.54 mg/L, respectively. Under the conditions of these tests, the additive packages did not potentiate the acute effects normally associated with JP-8 jet fuel exposure.

INTRODUCTION

The U.S. Navy and Air Force aircraft subsystems and engine heat loads are increasing rapidly. Fuel, within the aircraft, is used in thermal management systems to cool aircraft subsystems and the engine lubricating oil. The current thermal stresses are pushing JP-8 fuel to its thermal stability limits, resulting in fouling (coking) in engine fuel nozzles, afterburner spray assemblies, and manifolds. In some instances fuel degradation changes the spray pattern in the combustor or afterburner leading to damage to engine components. Certain aircraft are experiencing such severe problems with afterburner spraybar plugging that operational readiness is being adversely impacted. The Air Force has invested millions of dollars in advanced cleaning facilities to handle the resulting maintenance load. Some of these facilities generate hazardous waste, an additional cost factor.

¹ ManTech Environmental Technology, Inc. (Retired).

² Naval Medical Research Institute/Toxicology Detachment, Wright-Patterson Air Force Base, OH.

³ Armstrong Laboratory, Toxicology Division, Wright-Patterson Air Force Base, OH.

To reduce fuel fouling in current systems and to provide additional heat sink and thermal stability for future systems, the U.S. Air Force is developing an improved JP-8 fuel (JP-8 + 100) that offers a 100 °F improvement in thermal stability and a 50% increase in fuel heat sink capability. Two companies (Betz and Mobil) have developed additive packages that are currently being tested in aircraft systems. Other companies (Exxon, DuPont, and Texaco) are in the process of developing additive packages. The JP-8 + 100 with the Betz package is currently being used in F-16s at Kingsley Air National Guard base and at Sheppard Air Force Base. The JP-8 + 100 with the Mobil package has been tested on a more limited basis.

In order to accurately evaluate the numerous additive packages, a number of physical, clinical, and health effect evaluations are required. Of particular concern to the Navy and Air Force industrial hygienists is whether the additive packages will potentiate the health effects on flightline personnel. Previous acute testing of JP-8 fuel determined that it is nonirritating to eyes, slightly irritating to the skin, and has weak sensitizing potential (Kinkead et al., 1992). Jet Fuel A, a commercial jet fuel similar to JP-8 but without additives, produced no dermal sensitization, minimal irritation to eyes, and a mild skin irritation potential (Vernot et al., 1990). Genetic toxicity tests revealed no mutagenicity and no evidence of genetic risk associated with JP-8 jet fuel (Brusick and Matheson, 1978).

Rats and mice exposed continuously over a 90-day period to JP-8 vapors showed limited toxic effects and no tumor formation (Mattie et al., 1991). The toxicity reported was either not treatment-related, or it was due to male rat specific nephropathy. A developmental toxicity study determined that JP-8 fuel is not a teratogen in the rat (Cooper and Mattie, 1996).

The approach for determining the acute toxicity of JP-8 + 100 was to perform a battery of acute tests as follows: oral, dermal, inhalation (vapor and aerosol), dermal irritation, and dermal sensitization. The acute toxicity battery was performed on the JP-8 + 100 with the Betz additive package, the JP-8 + 100 with the Mobil additive package, and on the JP-8 jet fuel as a comparison group. A JP-8 jet fuel group was necessary due to the variability that exists between fuels from different geographical locations and different refineries. The group tested with JP-8 alone permitted a direct comparison of a sample of jet fuel currently in use with samples containing the new additives. In addition, it has been a number of years since JP-8 was originally tested for acute toxicity. Acute inhalation values, for both vapor and aerosol are not readily available in the literature as are irritation and sensitization data (Kinkead et al., 1992). A negative control group (no JP-8 treatment) was included in the testing battery for oral toxicity.

MATERIALS

Test Agent

The JP-8 jet fuel and the two JP-8 + 100 fuel packages were supplied by the U.S. Air Force. In addition to the standard additives, JP-8 + 100 (Betz) contained Spec-Aid 8Q405 (100 ppm), Dupont Metal Deactivator (2 ppm), and BHT as an antioxidant (25 ppm). The JP-8 + 100 (Mobil) package contained MCP147B (150 ppm), DuPont Metal Deactivator (2 ppm), and BHT as an antioxidant (25 ppm). A sample of each test material will be maintained in the chemical archives located in Building 429. Detailed chemical analysis of the JP-8 +100 samples was not performed because analysis was done by other military groups as part of the fuel evaluation study.

Test Animals

Male and female Fischer-344 (F-344) rats weighing between 100 and 125, and 75 and 100 g, respectively, were purchased from Charles River Breeding Labs, Raleigh, NC. Male Hartley guinea pigs weighing between 200 and 250 g were purchased from Charles River Breeding Labs, Kingston, NY. Male and female New Zealand white (NZW) rabbits weighing between 2 and 3 kg were purchased from Myrtle's Rabbitry, Inc., Thompson's Station, TN. All animals were subjected to a two-week quarantine period. Rats were group housed (two per cage) in clear plastic cages with wood chip bedding. The guinea pigs and rabbits were housed individually; the guinea pigs in plastic cages with wood chip bedding, and the rabbits in wire-bottom, stainless-steel cages. Water and feed (Purina Rabbit Chow #5320, Purina Formulab #5008 for rats, and Purina Formulab #5025 for guinea pigs) were available ad libitum, except during the inhalation exposure period and for 16 h prior to oral dosing. Animal room temperatures were maintained at 21 to 25 °C and the light/dark cycle was set at 12-h intervals.

EXPERIMENTAL APPROACH

A complete description of all methods used in this study are reported in the technical report AL/OE-TR-1996-0136 (Wolfe et al., 1996).

Skin Irritation

Six NZW female rabbits per test material were clipped on the back and sides 24 h prior to dosing to allow for recovery of the skin from any abrasion resulting from the clipping. The test agents (0.5 mL) were applied to designated patch areas and covered by a 3-cm square of surgical gauze two single layers thick. The patches remained in place for 4 h, then all wrappings were removed and the residual test agent wiped from the skin. Test areas were evaluated for irritation using the Draize Table (Draize, 1959) as a reference standard at 4, 24, 48, and 72 h. Total scores of the four observations for all rabbits were divided by 24 to yield a primary irritation rating which was interpreted using the National Institute for Occupational Safety and Health skin test rating.

Sensitization

Prior to the start of the study, 10 male guinea pigs per test material (30 animals total) were treated on the clipped left flank with 0.1 mL of the undiluted test material to determine the baseline irritation response. This site was clipped with an Oster® animal clipper and depilated with a commercial depilatory (Surgex Hair Remover Cream, Sparta Instrument Corp., Hayward, CA) 4 h prior to treatment. One-tenth of a mL of the test material was topically applied to the test area and covered with gauze, dental dam, and adhesive tape. This was repeated until a total of four sensitizing treatments were applied and evaluated. At the time of the third sensitizing treatment, 0.2 mL of a 25% aqueous dilution of TiterMax® adjuvant (Bacto Adjuvant Complete, Freund, Difco Laboratories, Detroit, MI) per animal was injected intradermally using two or three sites next to the test site. Following the fourth sensitizing treatment, the animals were rested for two weeks. Both flanks were then clipped and challenged on one flank with 0.1 mL of the test material. The challenge application was not occluded. The skin response at these sites was recorded at 4, 24, and 48 h after application. Any animal eliciting a score of two or more at the test solution challenge site at the 48-h scoring interval was rated a positive responder. The percentage of animals responding was the important factor in determining sensitization potential.

Oral Toxicity

Five male and five female F-344 rats per test material (15 male and 15 female total) were fasted 16 h prior to the administration of the oral dose. Each rat was weighed prior to oral gavage dosing and 5 g/kg of neat compound was administered. Surviving rats were weighed at 1, 2, 4, 7, 10, and 14 days postexposure. Signs of toxicity were recorded twice daily on symptomatology data sheets. On the 14th day postexposure, rats were sacrificed and gross pathology was performed for each animal.

Dermal Toxicity

Twenty-four hours prior to dosing, the back and sides of 5 male and five female NZW rabbits per test material (15 male and 15 female total) were clipped with an Oster® animal clipper. The undiluted dose of 2 g/kg was applied to the back of the rabbits and spread evenly to both sides. The dose was kept in place by applying an eight-ply gauze patch over the liquid. A clear plastic wrap was then applied over the entire midsection and was held in place with Vetrap® and elastoplast tape. The dose was kept in contact with the rabbit skin for 24 h. The tape, plastic wrap, and gauze were then removed and the residual test material was wiped from the animal. Animal body weights were recorded on days 1, 2, 4, 7, 10, and 14 posttreatment. Signs of toxicity and mortality were monitored and gross pathology was performed at the termination of the study.

Inhalation Exposure

The limit test exposures were conducted in a stainless steel, 690-L Toxic Hazards Research Unit exposure chamber. The exposure chamber was operated in a dynamic (continuous flow) mode. The airflow through the chamber for the vapor exposures was maintained at a rate that provided 4 to 6 volumes of air per hour; the airflow for the vapor plus aerosol exposures provided 8 to 10 volumes of air per hour. The aerosol/vapor generation system consisted of two 250-mL round-bottom flasks, each containing a six-jet Collison (BGI, Inc., Waltham, MA) compressed air nebulizer operated at a pressure of 62 psi. The flask was kept in a 34 °C water bath.

The jet fuel vapor exposure concentrations were analyzed using a Model 400 hydrocarbon analyzer (Beckman Instrument Corp., Fullerton, CA) and quantified hexane vapor was used as a calibration standard. The vapor-only exposures were monitored for presence of aerosol with a Ram-S aerosol mass analyzer (GCA Corp., Bedford, MA) operated in the 200 μ g/L mode. No aerosol was observed by Ram-S or by weighing the hydrocarbon analyzer filters.

The jet fuel aerosol concentrations were monitored using a filter in-line before the hydrocarbon analyzer (Extra Thick Glass Fiber Filter, 25 mm, Gelman Sciences, Ind., Ann Arbor, MI). An airflow of 3 L/min was maintained to the hydrocarbon analyzer. The filtered vapor sample was analyzed using the hydrocarbon analyzer, and three 20-second filter samples were taken for mass analysis of the aerosol. The hydrocarbon analyzer filters were replaced every 15 min to maintain the 3-L/min air flow. The aerosol concentration exceeded the Ram-S maximum analytical level.

Five male and five female F-344 rats per test material (15 male and 15 female total) were placed in a 690-L chamber and exposed for 4 h to a target 5 mg/L (Limit Test) concentration of vaporized and aerosolized test material. Records were maintained for body weights (Day 0, 7, 10, and 14 postexposure), signs of toxicity, and mortality. At sacrifice, gross pathology was performed and lungs were removed for histopathologic evaluation.

Statistical Analysis

Mean body weights and body weight gains of the inhalation rats were compared using a three factorial repeated measures analysis of variance (Johnson and Wichern, 1988). The factors were treatment, sex, and type of exposure (vapor or aerosol). The repeated measure was the difference between body weights which were measured an Days 0, 1, 2, 7, 10, and 14. If the overall F was significant (p<0.05), Bonferroni multiple comparisons were done to find pairwise comparisons (Johnson and Wichern, 1988). A probability of 0.05 or less inferred a significant change between groups.

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RESULTS

Complete results of this study, including body weight and other tables, are reported in the technical report AL/OE-TR-1996-0136 (Wolfe et al., 1996).

Skin Irritation

Six rabbits per test material were treated dermally with 0.5 mL of either JP-8, JP-8 + Mobil additives, or JP-8 + Betz additives. Slight erythema was noted in two of the six JP-8-treated animals immediately following 4-hdermal contact with the test material. No erythema, edema, or necrosis was observed in any of the JP-8 + 100 (Betz)- or the JP-8 + 100 (Mobil)-treated rabbits upon examination following 4-h dermal contact. Subsequent irritation observations at 24, 48, and 72 h were all negative for the JP-8 + 100 (Betz)-treated animals. Three JP-8-treated animals displayed mild erythema over the 72-h observation period, and three JP-8 + 100 (Mobil)-treated animals displayed mild erythema beginning 48-h posttreatment. Primary skin indices for JP-8, JP-8 + 100 (Mobil), and JP-8 + 100 (Betz) were determined to be 0.5, 0.25, and 0.0, respectively.

Sensitization

No test animals exhibited edema following the baseline response treatment of 0.1 mL test material to the shaved flank. All three test materials caused very slight to slight erythema by the 24-h posttreatment observations (4/10, 5/10, and 3/10 for JP-8, JP-8 + 100 (Betz), and JP-8 + 100 (Mobil), respectively). Very slight to slight erythema continued through the 48-h observations; 4/10, 6/10, and 4/10 for JP-8, JP-8 + 100 (Betz), and JP-8 + 100 (Mobil), respectively. Following 10 days of sensitization dosing and two weeks of rest, the test animals were challenged with 0.1 mL of the test material. All three test materials produced no edema at 24 and 48 h after this challenge treatment. All three test materials did produce very slight to slight erythema. There were no differences between sensitizing and challenge dose effects.

Oral Toxicity

Five male and five female rats per test material were orally dosed at 5 g/kg body weight with either JP-8, JP-8 + 100 (Mobil), or JP-8 + 100 (Betz). No deaths resulted from the oral administration of the test agents. Clinical observations noted after oral gavage included lethargy and shallow breathing for all fuel-treated groups. By 24-h posttreatment, all animals appeared normal; however, all fuel-treated male rats and the JP-8 + 100 (Mobil)-treated female rats had slight depression (but statistically significant) in body weight gains compared to controls.

Dermal Toxicity

Five male and five female rabbits per test material were treated at 2 g/kg body weight with either JP-8, JP-8 + 100 (Mobil), or JP-8 + 100 (Betz). No mortality occurred due to treatment with the test agents. Clinical signs of toxicity noted during the 14-day observation period included mild erythema and coriaceous skin at the site of

treatment. These observations were noted for animals of all three test groups. One female rabbit from the JP-8 treatment group died of accidental injury immediately after being dermally treated. All rabbits in the JP-8 and JP-8 + 100 (Mobil) groups gained weight during the 14-day observation period. Seven of 10 JP-8 + 100 (Betz) animals gained weight over the observation period; two animals maintained their initial weights, and one animal lost weight during the observation period.

Inhalation Toxicity

Vapor Exposures

The limit test concentration of 5 mg/L was not obtainable in the vapor-only exposures. The system limit of when a condensate aerosol was detected with a Ram-S was between 3.7 and 4.0 mg/L, so the maximum concentration for vapor-only exposures was 3.7 mg/L. No aerosol was detected during the vapor-only exposures. Chamber air flow was limited to 2 cfm of saturated vapor.

All rats survived the 4-h inhalation exposures to vapors of JP-8, JP-8 + 100 (Mobil), and JP-8 + 100 (Betz). During exposure, test animals exposed to JP-8 and JP-8 + 100 (Mobil) vapors demonstrated signs of eye or upper respiratory irritation. All treated animals gained weight over the 14-day observation period. Combined weight differences from Study Days 0 to 1 for the JP-8 animals were statistically significantly lower (p<0.01) than both the JP-8 + 100 (Betz) and the JP-8 + 100 (Mobil) groups. Weight gains from Day 2 to Day 7, and from Day 7 to Day 17 for the JP-8 and JP-8 + 100 (Mobil) groups differed (p<0.01) from the weights of the JP-8 + 100 (Betz) group. Gross observations at sacrifice failed to reveal any treatment-related lesions.

Aerosol Exposures

The vapor plus aerosol exposures were performed using the same generation system as the vapor-only exposures, except the HEPA filter was removed to allow the aerosol to pass through into the exposure chamber. Additional air was supplied to move the vapor and aerosol through the system. The total air flow for the vapor and aerosol exposures averaged 3.5 cfm. One impactor sample (30 seconds at 20 L/min flow) was taken approximately 2 h into each exposure. The ratio of the mass of vapor to the mass of aerosol was approximately 3:2 during exposure.

All rats survived 4-h inhalation exposure to vapor and aerosol of JP-8, JP-8 +100 (Mobil), and JP-8 + 100 (Betz). All treated animals gained weight during the 14-day observation period. Statistical analyses of the difference between group body weights over the 14-day observation period determined there were no differences among the treatment levels. Gross observations at sacrifice failed to reveal any treatment-related lesions.

DISCUSSION

In the oral and dermal toxicity studies, no deaths or toxic signs were observed in any of the animals, and body weight gains during the subsequent 14-day observation periods appeared to be unaffected by treatment. Remarkable irritating effects were not observed as a result of exposure to intact skin of rabbits. The results of animal irritation studies do not predict irritation of human skin reported by military personnel (Bell et al., 1996). Acute inhalation of the vapors or aerosols of the test materials near limit concentrations produced no mortality in male and female F-344 rats. The acute toxicity results reported in this report for the JP-8 and JP-8 + 100 jet fuels are similar to those reported for Jet Fuel A (Vernot et al., 1990). The results differ slightly from those in Kinkead et al. (1992) which reported JP-8 jet fuel had a weak sensitizing potential. No sensitization reaction in guinea pigs occurred in this study. Again, the sensitization results in animal studies are not a good predictor of sensitization of human skin reported by military personnel (Gould, 1996). Differences between the results of this study and previously reported data may be due to variation in the JP-8 fuel samples. Fuel differences are due to location of their source and the refinery from which they are produced.

Tables 5.2-1 and 5.2-2 are summaries of these acute test results with JP-8 jet fuel and JP-8 jet fuel containing Mobil and Betz additives. Under the conditions of these tests, the additive packages did not potentiate the acute effects normally associated with JP-8 jet fuel exposure.

· · · · · · · · · · · · · · · · · · ·	Skin Irritation	Sensitization	Oral LD ₅₀ (g/kg)	Dermal LD ₅₀ (g/kg)
JP-8	Negative	Negative	> 5.0	> 2.0
JP-8 + 100 (Betz)	Negative	Negative	> 5.0	> 2.0
JP-8 + 100 (Mobil)	Negative	Negative	> 5.0	> 2.0

TABLE 5.2-1. ACUTE TEST	RESULTS FOR JP-8.	JP-8 + 100 (Betz)	AND JP-8 + 100 (Mobil)
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Test Material	Inhalation LC ₅₀ (mg/L)	
VAPOR		
JP-8	>3.43	
JP-8 + 100 (Betz)	>3.52	
JP-8 + 100 (Mobil)	>3.57	
VAPOR + AI	EROSOL	
JP-8	>4.44	
JP-8 + 100 (Betz)	>4.39	
JP-8 + 100 (Mobil)	>4.54	

TABLE 5.2-2.SUMMARY OF ACUTE INHALATION RESULTS FOR JP-8, JP-8 + 100 (BETZ),
AND JP-8 + 100 (MOBIL)

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5.3 SEVEN-DAY ORAL RANGE-FINDING STUDIES FOR LONG CHAIN PETROLEUM HYDROCARBON SURROGATE CANDIDATES n-NONANE AND HEXADECANE, WITH NEUROBEHAVIORAL TESTING BATTERY VALIDATION

D.H. Ellis, R.E. Wolfe, D.E. Dodd, and W.H. Weisman¹

ABSTRACT

Contamination of soil and groundwater with petroleum products is a common environmental problem at Department of Defense (DoD) installations. Remediation of contaminated sites is currently based on risk assessments derived from a single numerical standard for total petroleum hydrocarbons. Since various petroleum products have different toxicity and leaching characteristics, and weathered sites primarily contain long chain petroleum hydrocarbons (LCPH), an initiative is under way to establish toxicity parameters for surrogate LCPH. The toxicity parameters can then be used to more accurately assess the risk associated with weathered petroleum-contaminated sites. Seven-day range-finding studies were performed as the initial step in establishing no observed adverse effect levels (NOAEL), which will be used to calculate toxicity parameters. Female Ficher 344 rats and male C57BL/6 mice were dosed orally (gavage) with a surrogate LCPH, neurobehavior-positive control, soil, or control water for seven days. Neurobehavior parameters tested were open-field activity, forelimb grip strength, and auditory startle response. Cage-side observations, body weights, food consumption, gross necropsy, and organ weights were evaluated for toxic effects. The dose range tested for n-nonane was recommended for a long-term study to establish the n-Nonane NOAEL, while further study of n-hexadecane will require use of a lower dose. Neurobehavior testing results are currently being analyzed and will be presented in a technical report. 2,5-Hexanedione and acrylamide are known to produce neurobehavioral deficites and were used to validate the ability of the neurobehavior testing battery to detect dose-related neurobehavior deficits. An oral (gavage) study of a water and sterile soil slurry revealed the difficulties involved with orally administering contaminated soil.

INTRODUCTION

Contamination of soil and groundwater with petroleum products is a common environmental problem at Air Force (AF) bases and other DoD installations. At over 4000 groundwater contamination sites belonging to the AF, 60 percent involve some type of petroleum product such as gasoline, diesel fuel, and jet propulsion (JP) fuel (Staats, 1994). Millions of dollars are spent each year at petroleum contaminated sites for remediation which may not be necessary. Though petroleum products are a complex mixture of hydrocarbons, cleanup of these

¹ Armstrong Laboratory, Toxicology Division, Wright-Patterson Air Force Base, OH.

contaminants is often regulated by a single numerical standard for total petroleum hydrocarbons (TPH) which varies greatly between states, ranging from 10 ppm to 10,000 ppm (Staats, 1994). The various types of petroleum products have different chemical compositions, each chemical having different toxicity, mobility, and degradation characteristics (Staats, 1994). Therefore, the components of most importance for remediation differ according to what product is involved and the weathered state of the contaminated site. More precise, site-specific evaluation of human health risk from petroleum contamination could save millions of dollars in unnecessary cleanup costs.

Petroleum hydrocarbons are commonly divided into four major groups: alkanes, alkenes, cycloalkanes and aromatics. One approach to determining risk based corrective action at petroleum contaminated sites is to characterize the risk for a "surrogate compound" from each subgroup of petroleum hydrocarbons quantified at a site. The long chain petroleum hydrocarbon (LCPH) subgroup comprises a large portion of most petroleum products. To perform a quantitative risk assessment for exposure to LCPH in soil, a chemical-specific risk factor must be available. A risk factor for LCPH has not been developed, primarily due to the limited amount of toxicological data for these compounds. There are many compounds in the LCPH group, thus, more than one risk factor may be necessary for a site assessment. However, it is impractical to develop a risk factor for every LCPH compound due to high costs. Moreover, many of the compounds have not yet been isolated and identified. The purpose of this dose range-finding study is to determine and evaluate the toxic effects associated with short-term repeated exposure to select LCPH, or mixtures thereof, to assist in selecting candidates and dose concentrations for long-term studies. The results will provide information on health hazards likely to arise from repeated exposure to a LCPH by the oral route.

Sufficient analytical data are not available to describe the subgroups of petroleum hydrocarbons that exist at a weathered JP-4 spill site, but the carbon range of neat JP-4 fuel is approximately C5 to C15 (Leahy, personal communication). Although soils differ in their ability to retain petroleum products, higher carbon range constituents are expected to remain in the soil longer (Jarsjö et al., 1994). n-Nonane has been proposed as a surrogate compound for the LCPH subgroup C9 through C18 (Massachusetts Department of Environmental Protection, 1994). Due to limited information on the health effects of n-Nonane, a reference dose (RfD) was derived with considerable conservatism and a large amount of uncertainty (Staats, 1994). Further, inhalation toxicity was the only data available to derive an oral RfD. EPA defines the RfD as an estimate of a daily exposure to the human population that is likely to be without an appreciable risk of deleterious effects during a lifetime, and considers a single, well-conducted, subchronic, mammalian bioassay, by the appropriate route, a minimum database for estimating RfD (Dourson, 1994).

Toxicity data on LCPH and petroleum mixtures are limited, but the results of the toxicological investigations with C9 to C13 hydrocarbon alkanes (Clark et al., 1989; Cooper and Mattie, 1993; Kinkead et al., 1993; MacEwen and Vernot, 1977; Mullin et al., 1990) indicate a low order of toxicity in mammalian species. Yet, the database for this

subgroup of compounds is minimal and inadequate for the purpose of establishing RfD. To develop accurate human risk factors, the database needs to be expanded with a single, well-conducted, subchronic, oral, mammalian bioassay for each proposed reference compound.

MATERIALS AND METHODS

n-Nonane and n-hexadecane were the first surrogates chosen for possible long-term study. Acrylamide and 2,5-hexanedione were chosen as known neurobehavior altering chemicals to validate the neurobehavior testing battery. Uncontaminated soil, in a slurry with water, was used to test the feasability of orally administering soil contaminated with individual or a mixture of LCPH. Animals were observed twice daily for signs of toxic stress. Body weights were measured before treatment initiation and prior to sacrifice. Upon sacrifice, a gross necropsy was performed and brain, liver, kidney, spleen, adrenal, lung, and testes or ovary weights were recorded. Neurobehavioral tests including open-field activity, auditory startle response, and forelimb grip strength, were conducted before study initiation and on the last day of study, prior to dose adminisration. A more detailed description of the methods and experimental evaluations performed for this study will be provided in the technical report in preparation.

RESULTS

Perianal irritation was observed in all high dose rats and mice recieving either n-Nonane or n-hexadecane. Neither chemical altered mean body weights in mice, while both chemicals supressed rat body weights in the high dose groups. Absolute and relative organ weights were also different between dose groups. The alterations observed in body weights, organ weights, and relative organ weights are displayed in Tables 5.3-1, 5.3-2, and 5.3-3.

Dosing trauma resulting from difficulty in orally administering the water and soil slurry caused death in both rats and mice. Uncontaminated soil also suppressed mean body weights and relative brain weights in rats (Table 5.3-5). 2,5-Hexanedione caused death in the high dose rat group, while both 2,5-hexanedione and acrylamide altered absolute and relative organ weights as summarized in Tables 5.3-4, 5.3-5, and 5.3-6. Pending completion of neurobehavioral data analysis, all findings for n-Nonane, n-hexadecane, acrylamide, and 2,5-hexanedione will be provided in the technical report in preparation.

TABLE 5.3-1.BODY WEIGHTS^A AND ABSOLUTE AND RELATIVE^B ORGAN WEIGHTS^A OF MALEMICE ORALLY TREATED WITH n-NONANE FOR 7 DAYS

	CONTROL	1.0 mL/kg/day	2.5 mL/kg/day	5.0 mL/kg/day
Liver	0.94 ± 0.05	0.97 ± 0.05	1.02 ± 0.08	$1.12 \pm 0.10^{c,d}$
Relative	4.39 ± 0.08	4.49 ± 0.27	$4.80 \pm 0.17^{e,f}$	$5.14 \pm 0.10^{c,g,h}$
Spleen	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
Relative	0.18 ± 0.05	0.21 ± 0.02	0.22 ± 0.02	0.24 ± 0.02^{i}

^a Grams; Mean \pm SD, N = 5.

^bRelative= organ weight/body weight x 100.

^eSignificantly different than Control at p<0.01.

^d Significantly different than 1.0 mL/kg/day at p<0.05.

Significantly different than Control at p<0.05.

^f Significantly different than 1.0 mL/kg/day at p<0.10. ^g Significantly different than 1.0 mL/kg/day at p<0.01. ^h Significantly different than 2.5 mL/kg/day at p<0.05.

ⁱSignificantly different than Control at p<0.10.

TABLE 5.3-2.BODY WEIGHTS^A AND ABSOLUTE AND RELATIVE^B ORGAN WEIGHTS^A OF
FEMALE RATS ORALLY TREATED WITH n-NONANE FOR 7 DAYS

	CONTROL	1.0 mL/kg/day	2.5 mL/kg/day	5.0 mL/kg/day
Spleen	0.31 ± 0.01	0.30 ± 0.02	0.30 ± 0.01	$0.30 \pm 0.02^{\circ}$
Relative	0.27 ± 0.01	0.26 ± 0.01	0.26 ± 0.01	0.27 ± 0.01
Body Weight	118.6 ± 5.93	115.7 ± 2.30	112.8 ± 5.63	$109.4 \pm 4.47^{\circ}$

^a Grams; Mean ± SD; N=5 for control and 2.5 mL/kg/day; N= 3 for 1.0 mL/kg/day; N= 4 for 5.0 mL/kg/day. ^bRelative= organ weight/body weight x 100. ^cSignificantly different than Control at p<0.10.

TABLE 5.3-3. BODY WEIGHTS^A AND ABSOLUTE AND RELATIVE^B ORGAN WEIGHTS^A OF
FEMALE RATS ORALLY TREATED WITH N-HEXADECANE FOR 7 DAYS

	CONTROL	1.0 mL/kg/day	2.5 mL/kg/day	5.0 mL/kg/day
Brain	1.57 ± 0.03	1.54 ± 0.05	1.55 ± 0.06	1.56 ± 0.05
Relative	1.20 ± 0.05	1.28 ± 0.13	1.25 ± 0.03	$1.36 \pm 0.05^{\circ}$
Liver	3.95 ± 0.23	3.99 ± 0.32	3.94 ± 0.07	4.03 ± 0.33
Relative	3.01 ± 0.10	$3.30 \pm 0.20^{\circ}$	3.18 ± 0.11	$3.51 \pm 0.15^{d,e}$
Kidneys	1.05 ± 0.03	1.02 ± 0.08	1.04 ± 0.04	1.02 ± 0.04
Relative	0.80 ± 0.02	0.84 ± 0.06	0.84 ± 0.03	$0.88 \pm 0.03^{\circ}$
Spleen	0.31 ± 0.02	0.32 ± 0.02	0.33 ± 0.02	0.31 ± 0.03
Relative	0.24 ± 0.01	0.26 ± 0.03	0.26 ± 0.01	0.27 ± 0.01^{f}
Adrenal Gland	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	$0.06 \pm < 0.01$
Relative	0.04 ± 0.01	0.04 ± 0.01	0.05 ± <0.01	$0.05 \pm < 0.01^{\rm f}$
Body Weight	131.5 ± 3.98	121.5 ± 12.4	124.1 ± 3.98	$114.9 \pm 6.08^{\circ}$

^a Grams; Mean \pm SD, N = 5.

^bRelative= organ weight/body weight x 100.

Significantly different than Control at p<0.05.

^d Significantly different than Control at p<0.01.

^e Significantly different than 2.5 mL/kg/day at p<0.05.

^fSignificantly different than Control at p<0.10.

TABLE 5.3-4.BODY WEIGHTS^A AND ABSOLUTE AND RELATIVE^B ORGAN
WEIGHTS^A OF MALE MICE ORALLY TREATED WITH
2,5-HEXANEDIONE OR SOIL FOR 7 DAYS

	CONTROL	0.6 g/kg/day	1.2 g/kg/day
Testes	0.16 ± 0.01	0.16 ± 0.03	0.17 ± 0.02
Relative	0.84 ± 0.09	0.79 ± 0.09	0.96 ± 0.06^{c}

^a Grams; Mean ± SD; N=4 for control and 0.6 g/kg/day; N=3 for soil and 1.2 g/kg/day.

^bRelative= organ weight/body weight x 100.

^cSignificantly different than 0.6 g/kg/day at p<0.10.

TABLE 5.3-5.BODY WEIGHTS^A AND ABSOLUTE AND RELATIVE^B ORGAN WEIGHTS^A OF
FEMALE RATS ORALLY TREATED WITH 2,5-HEXANEDIONE OR SOIL FOR
7 DAYS

	CONTROL	SOIL	0.6 g/kg/day	1.2 g/kg/day
Brain	1.52 ± 0.05	1.54 ± 0.01	1.53 ± 0.05	1.52
Relative	1.21 ± 0.07	$1.36 \pm 0.10^{\circ}$	1.34 ± 0.04^{d}	1.56 ^e
Liver	4.00 ± 0.30	3.57 ± 0.19	4.18 ± 0.55	4.19
Relative	3.17 ± 0.09	3.15 ± 0.32	3.66 ± 0.42	4.30 ^c
Kidneys	1.03 ± 0.07	0.99 ± 0.04	1.09 ± 0.07	1.09
Relative	0.81 ± 0.01	0.87 ± 0.08	0.95 ± 0.06^{e}	1.11 ^{e,f}
Spleen	0.32 ± 0.02	0.30 ± 0.04	0.22 ± 0.02^{e}	0.07 ^{e,g}
Relative	0.25 ± 0.01	0.26 ± 0.04	$0.20 \pm 0.02^{\circ}$	0.08 ^{e,g}
Adrenal Gland	0.05 ± 0.01	0.04 ± 0.01	0.06 ± 0.01	0.06
Relative	$0.04 \pm < 0.01$	0.04 ± 0.01	0.05 ± 0.01	0.07 ^d
Body Weight	126.3 ± 8.20	113.7 ± 7.96^{d}	113.9 ± 2.82^{d}	97.6°

^a Grams; Mean ± SD, N=5 except 1.2 g/kg/day N=1.

^bRelative= organ weight/body weight x 100.

^c Significantly different than Control at p<0.05.

^d Significantly different than Control at p<0.10.

^e Significantly different than Control at p<0.01.

^f Significantly different than 0.6 g/kg/day at p<0.10.

^g Significantly different than 0.6 g/kg/day at p<0.01.

TABLE 5.3-6.BODY WEIGHTS^A AND ABSOLUTE AND RELATIVE^B ORGAN WEIGHTS^A OF
FEMALE RATS ORALLY TREATED WITH ACRYLAMIDE FOR 7 DAYS

	CONTROL	5.0 mg/kg/day	25 mg/kg/day	50 mg/kg/day
Kidneys	1.04 ± 0.06	1.08 ± 0.08	1.09 ± 0.04	1.08 ± 0.04
Relative	0.88 ± 0.05	0.90 ± 0.05	0.93 ± 0.04	$0.98 \pm 0.03^{c,d}$
Spleen	0.30 ± 0.02	0.30 ± 0.02	0.27 ± 0.02	$0.25 \pm 0.02^{c,e}$
Relative	0.25 ± 0.01	0.25 ± 0.01	0.23 ± 0.01	$0.22 \pm 0.02^{f,d}$
Body Weight	118.8 ± 4.42	120.7 ± 4.85	117.2 ± 3.74	$110.5 \pm 2.75^{f,e,g}$

^a Grams; Mean \pm SD, N = 5.

^bRelative= organ weight/body weight x 100.

^cSignificantly different than Control at p<0.01.

* Significantly different than 5.0 mg/kg/day at p<0.01.

^fSignificantly different than Control at p<0.05.

^g Significantly different than 25 mg/kg/day at p<0.10.

^d Significantly different than 5.0 mg/kg/day at p<0.05.

DISCUSSION

Signs of adverse effects occurred in rats receiving 5.0 mL/kg/day of n-Nonane, and that dose affected organ weights in mice. The lower doses of 2.5 mL/kg/day and 1.0 mL/kg/day did not produce toxic effects. The range of doses used in this study is recommended for 90-day studies intended to produce an oral NOAEL for n-Nonane.

Attributing pulmonary effects to aspiration, which is common in oral hydrocarbon studies (Scharf et al., 1981; Algren et al., 1992), n-hexadecane did not cause adverse effects at 1.0 mL/kg/day. However, increased mean liver weight occurred even at 1.0 mL/kg/day in rats. Although the range of doses used in this study are recommended for 90-day mouse studies intended to define an oral RfD, 1.0 mL/kg/day will be higher than the 90-day NOAEL in rats.

Uncontaminated soil's effect on survival, mean body weight, and relative brain weight was likely related to dosing trauma, due to the difficulty of oral dosing a water and soil slurry. Eighteen gauge, standard bore needles were used for rats, while 21 gauge was used for mice. A larger bore gavage is recommended for studies involving contaminated soil.

Validation of neurobehavioral testing battery is pending completion of data analysis. However, preliminary data review suggests that while open-field and forelimb grip strength assessment may reveal dose-related neurobehavior disfunction, the auditory startle response system employed did not provide accurate, reproducible information, and did not reveal any dose-related deficits.

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5.4 NINETY-DAY ORAL TOXICITY STUDY ON n-NONANE IN FEMALE FISCHER 344 RATS AND MALE C57BL/6 MICE - A PRELIMINARY REPORT

D.E. Dodd, R.E. Wolfe, J.H. English¹, and W.H. Weisman²

ABSTRACT

Nonane is a long chain petroleum hydrocarbon that is common in Air Force jet petroleum fuels, such as JP-4. Contamination of soil and groundwater with petroleum products is a common environmental problem at Department of Defense installations. To determine and evaluate the potential toxic effects of nonane following repeated oral (gavage) treatment, four groups of female Fischer 344 rats and male C57BL/6 mice were administered n-nonane (neat) at daily doses of 5, 1.0, 0.1, and 0 (control) g/kg, 7 days/week for 90 days. Biological endpoints included clinical observations, neurobehavioral assessments, body weights, food consumption, hematology, serum chemistry, organ weights, gross pathology, and histopathology. In this preliminary report, results to date are presented without interpretation or discussion. A detailed technical report is in progress.

INTRODUCTION

Contamination of soil and groundwater with petroleum products is a common environmental problem at Air Force bases and other Department of Defense (DoD) installations. Millions of dollars are spent each year to assess and remediate petroleum contamination. However, much of this remediation may not be necessary. Site-specific evaluation of the risk to human health from petroleum contamination could save millions of dollars in unnecessary cleanup costs.

The ultimate goal of this research effort is to provide toxicity data for long chain petroleum hydrocarbons (LCPH) that will be used to establish a reference dose (RfD) for LCPH, and ultimately an RfD for weathered JP-4 in soil. EPA defines the RfD as an estimate of a daily exposure to the human population that is likely to be without an appreciable risk of deleterious effects during a lifetime. The EPA considers a single, well-conducted, subchronic mammalian bioassay by the appropriate route as a minimum database for estimating a reference dose (Dourson, 1994). The work to be performed in this study is a thorough 90-day general toxicity bioassay in rodents. Oral administration of the test substance was selected due to the concern of contaminated soil and groundwater.

¹Army Medical Research Unit, Wright-Patterson Air Force Base, OH.

²Armstrong Laboratory, Toxicology Division, Wright-Patterson Air Force Base, OH.

Long chain petroleum hydrocarbons are a category of petroleum hydrocarbons that comprise between 50 and 98 percent of most petroleum products. Petroleum hydrocarbons are commonly divided into four major groups:

alkanes, alkenes, cycloalkanes, and aromatics. A qualitative analysis of neat JP-4 fuel indicates that its hydrocarbon components lie in a carbon range of approximately C_5 to C_{15} . Establishing health-based criteria for each of the components of JP-4 fuel is unreasonable. Similarly, assigning a single concentration value for a cleanup standard for the entire range of petroleum hydrocarbons as one mass is unfounded. One approach to determining risk -based corrective action at petroleum contaminated sites is to identify a "reference compound" for each subgroup of petroleum hydrocarbons quantified at the site. Sufficient analytical data are not available to describe the subgroups of petroleum hydrocarbons that exist at a weathered JP-4 spill site, but the long chain alkanes with a carbon range of C_9 through C_{15} are suspected to represent a large portion of the total petroleum hydrocarbons measured in soil at spill sites. Reference compounds for the LCPH subgroups C_5 through C_{8} , C_9 through C_{18} and C_{19} through C_{32} have been proposed (Massachusetts Department of Environmental Protection, 1994). They are n-hexane, n-Nonane, and eicosane, respectively. Due to limited information on the health effects of the proposed reference compounds, RfDs were derived with considerable conservatism and a large amount of uncertainty. Further, except for n-hexane, inhalation data were the only data available to derive oral RfDs. Thus, this study was designed to provide mammalian toxicity information on one of the proposed reference compounds for the LCPH subgroups for the LCPH subgroups, n-Nonane.

Oral toxicity studies with nonane are not reported in the open literature. Acute 7-day and 90-day studies in rats with nonane vapor (Carpenter et al., 1978; Nilsen et al., 1988) indicated central nervous system or peripheral nervous system abnormalities (tremors, convulsions, coordination loss, limb paralysis) and irritation (lacrimation, salivation), including microscopic lesions in the liver (fatty changes), lungs (edema), and brain (loss of Purkinje cells) at high (approximately 2400 ppm) concentrations; mild irritation, tremors, body weight depression with no microscopic lesions at intermediate (approximately 1600 ppm) concentrations; and no observable effects at low (approximately 600 ppm) concentrations.

Clearly, the database for nonane is minimal and inadequate for the purpose of establishing RfDs. To develop accurate human risk factors, the database needs to be expanded with a well-conducted, subchronic oral mammalian bioassay. The purpose of this study is to determine the no observable adverse effect level (NOAEL) and toxic effects associated with repeated exposure to nonane for a period of 90 days. The results will provide information on health hazards likely to arise from repeated exposure to nonane by the oral route. This information can be of value for establishing safety criteria for human exposure.

MATERIALS AND METHODS

n-Nonane (CAS No. 111-84-2, 99%, Lot No. 5921EL) was obtained from Aldrich Chemical Co., Milwaukee, WI. Gas chromatographic separation and mass spectrographic analysis of the test substance indicated there were no impurities. Neat n-Nonane was administered orally (via gavage) on a daily basis throughout the study. Dosages were administered on the basis of weight of test substance per animal body weight (not to exceed a volume of 1.0 mL/100 g body weight).

Control animals received an equivalent volume (1 mL/100 g body weight) of distilled water. Animals were not fasted prior to dosing, but dosing was scheduled towards midday (approximately 1100) since rodents eat, in general, during the night.

Rodents are the preferred species for general toxicity testing. Members of the total petroleum hydrocarbon (TPH) criteria working group (the DoD participates in and supports this group) evaluated the "data gaps" on animal toxicity of LCPH and suggested that data from two species (rats and mice) would be of greater value than data from a single species for establishing a test substance reference dose. However, toxicity data from male rats may be difficult to interpret due to the development of α -2 μ -globulin nephropathy, induced by many hydrocarbons. To keep animal numbers to a minimum, one sex of each species was considered appropriate for meeting the objectives of this study. Thus, female rats and male mice were selected. Both the Fischer 344 (F-344) rat and the C57BL/6 mouse were used extensively in the Toxic Hazards Research Unit laboratory (AL/OET) for the toxicity testing of jet fuels from 1973 to 1983. Except for the development of α -2 μ -globulin nephropathy in male rats, differences between the sexes were not observed in the biological endpoints monitored. To strengthen the current database, this study used the identical species and strains that the jet fuel studies used.

Forty-four female F-344 [CDF (F-344)/CrlBR, Lot T68] rats (6 weeks of age) and 44 male C57BL/6 [C57BL/6NCrlBR, Lot E42] mice (6 weeks of age) were supplied by Charles River Breeding Laboratories, Raleigh, NC. Details on animal husbandry will be provided in the technical report (in progress).

Results from a 7-day dose range-finding study (Ellis et al., 1997) were evaluated to assist in selecting the target doses for the 90-day study. The number of groups, group size, and target dose levels follow.

	Number	of Animals	Dose Level
	Males Nales	Females	of Test Substance
Group	(mice)	(rats)	(g/kg body wt/day)
Control	10	10	0.0
Low	10	10	0.1
Middle	10	10	1.0
High	10	$10 + 2^*$	5.0

^{*}Due to unanticipated mortality during the first four days of the study, two additional rats were assigned to this group.

It was anticipated that at the highest dosage level, some toxicological or pharmacological effect(s) may be observed, and that at the lowest dosage level no treatment-related effects would be seen. The highest dose level is EPA's (1990) "limit test" value for acute studies.

Details of the experimental evaluations (cage-side observation of animals, neurobehavioral tests, body weights, food consumption, hematology, serum chemistry, blood and tissue sampling for test substance analysis, necropsy, organ weights, histopathology, and statistics) will be provided in the detailed technical report (in progress).

RESULTS (To-Date)

Clinical Observations

Results of pre-study quality control procedures were negative, indicating that the animals were healthy upon initiation of the study. Deaths, attributed to oral dosing procedures, were observed in both mice and rats (Table 5.4-1). Rats dying during the first four days of the study were replaced with rats that had not been randomly assigned to a study group, but were healthy and part of the original shipment of animals received for this investigation.

		Number Dead/	Time of Death	
Species	Dose (g/kg/day)	Number on Study	Week (Number dead)	
	0.0	0/10		
Rats	0.1	0/10	-	
	1.0	1/10	13 (1)	
			1 (2)	
	5.0	6/12	2 (3)	
			11 (1)	
	0.0	1/10	8 (1)	
Mice	0.1	0/10	-	
	1.0	2/10	12 (1)	
			13 (1)	
	5.0	2/10	3 (1)	
			12 (1)	

TABLE 5.4-1. MORTALITY IN THE 90-DAY ORAL STUDY WITH n-NONANE

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Except for an occasional incidence of a dry red material around the eyes of rats in the 0.0 (control), 0.1 and, 1.0 g/kg groups, clinical signs of irritancy and/or toxicity were observed throughout the study in all or nearly all rats of the 5.0 g/kg group. The clinical findings included wet urogenital/perianal areas, matted fur in the anal area, perianal alopecia, perianal/hindlimb erythema, dark-colored urine, diarrhea, erythema/excreta at base of tail, hunched posture, dry red material around the eyes and nose, lower jaw alopecia, and matted "rough" body fur. Mice of the 5.0 g/kg group had similar clinical signs with occasional redness and swelling of the penis and scrotal area. Mice of the 0.0, 0.1, and 1.0 g/kg groups were normal in appearance.

Neurobehavioral Tests

For grip strength, there were no statistically significant differences between control and treated animals (rats or mice) throughout the study. Results of locomotor activity and auditory startle reflex tests will be provided in the detailed technical report (in progress).

Body Weights and Food Consumption

Weekly mean body weight values are reported for rats and mice in Tables 5.4-2 and 5.4-3, respectively. Weekly mean food consumption values are reported for rats and mice in Tables 5.4-4 and 5.4-5, respectively. For both rats and mice, there were no statistically significant differences in mean body weights between control and treated groups throughout the study. Food consumption values for female rats of the 5.0 and 1.0 g/kg groups were lower than the control values for the first two weeks on study. However, no further decreases from control means were observed, except for the 1.0 g/kg group on study Days 43 and 50. For male mice, there were no statistically significant differences in mean food consumption throughout the 90-day study.

	Dose (g/kg/day)						
Study Day		0.1*	1.0 ^a	5.0 ^b			
1	131.3 ± 1.4	131.2 ± 1.6	130.9 ± 1.6	130.5 ± 2.2			
6	133.9 ± 1.3	135.1 ± 1.2	132.6 ± 2.1	126.2 ± 2.1			
13	140.3 ± 1.6	142.5 ± 1.3	137.5 ± 2.1	136.0 ± 2.4			
20	149.6 ± 1.8	149.5 ± 1.1	146.2 ±1.8	144.4 ± 2.0			
27	151.4 ± 2.1	150.5 ± 1.8	148.7 ± 2.3	150.0 ± 2.5			
34	156.1 ± 2.1	154.3 ± 1.4	151.2 ± 2.5	155.0 ± 2.7			
41	158.4 ± 2.3	157.5 ± 1.6	153.9 ± 3.1	159.5 ± 2.4			
48	162.6 ± 2.4	160.7 ± 1.8	156.9 ± 2.8	164.0 ± 2.6			
55	166.7 ± 2.4	164.7 ± 1.7	160.0 ± 2.6	166.7 ± 2.6			
62	168.1 ± 2.4	166.6 ± 1.9	162.2 ± 2.8	169.3 ± 2.8			
69	170.8 ± 2.6	166.9 ± 2.1	160.5 ± 3.7	167.9 ± 4.2			
76	171.9 ± 2.6	168.7 ± 2.0	162.6 ± 2.5	166.7 ± 5.5			
83	172.8 ± 2.7	168.8 ± 1.4	163.7 ± 2.7	170.6 ± 4.5			

TABLE 5.4-2.MEAN (±SE) BODY WEIGHTS (g) OF FEMALE RATS IN THE 90-DAY ORAL
STUDY WITH n-NONANE

^a Group size = 10 (Days 1-83).

^b Group size = 10 (Days 1-6), 7 (Days 13-69), 6 (Days 76-83).

	Dose (g/kg/day)						
Study Day	0.0ª	0.1 ^b	1.0°	5.0 ^d			
1	24.0 ± 0.3	24.1 ± 0.3	24.0 ± 0.3	24.0 ± 0.3			
6	24.6 ± 0.4	24.2 ± 0.2	24.6 ± 0.4	23.4 ± 0.4			
13	25.0 ± 0.4	24.7 ± 0.3	25.1 ± 0.4	24.5 ± 0.5			
20	25.9 ± 0.3	25.6 ± 0.3	26.0 ± 0.5	26.1 ±0.5			
27	26.2 ± 0.3	25.6 ± 0.4	25.7 ± 0.4	26.3 ± 0.4			
34	26.9 ± 0.3	26.5 ± 0.4	26.6 ± 0.5	26.8 ± 0.4			
41	27.4 ± 0.4	26.6 ± 0.4	27.0 ± 0.4	27.3 ± 0.5			
48	28.1 ± 0.4	27.0 ± 0.3	27.4 ± 0.6	27.5 ± 0.5			
55	28.4 ± 0.4	27.9 ± 0.5	27.9 ± 0.8	27.7 ± 0.5			
62	29.4 ± 0.5	28.1 ± 0.4	27.8 ± 0.6	28.5 ± 0.6			
69	29.9 ± 0.6	28.5 ± 0.6	28.5 ± 0.6	28.6 ± 0.5			
76	30.6 ± 0.6	29.2 ± 0.6	28.9 ± 0.6	28.4 ± 0.5			
83	30.5 ± 0.5	29.2 ± 0.5	28.7 ± 0.6	29.3 ± 0.5			

TABLE 5.4-3. MEAN (±SE) BODY WEIGHTS (g) OF MALE MICE IN THE 90-DAY ORAL STUDYWITH n-NONANE

^a Group size = 10 (Days 1-48), 9 (Days 55-83).

^b Group size = 10 (Days 1-83).

^c Group size = 10 (Days 1-76), 9 (Day 83).

^D Group size = 10 (Days 1-13), 9 (Days 20-55, 69-76), 8 (Days 62 and 83).

Dose (g/kg/day) and Group Size (N)								
Study Day	0.0	N	0.1	N	1.0	N	5.0	N
1	10.8 ± 0.2	10	10.1 ± 0.2	9	$9.5^{a} \pm 0.3$	10	$7.9^{a} \pm 0.4$	10
8	11.5 ± 0.2	10	11.4 ± 0.4	10	11.3 ± 0.3	10	$9.4^{a}\pm0.4$	7
15	11.8 ± 0.2	10	11.3 ± 0.3	10	$9.6^{a} \pm 0.4$	10	$9.8^{b} \pm 0.6$	4
22	11.3 ± 0.3	10	10.7 ± 0.2	10	10.5 ± 0.3	10	10.8 ± 0.3	7
29	10.3 ± 0.3	10	11.2 ± 0.3	10	10.4 ± 0.5	10	9.9 ± 0.6	7
38°	10.6 ± 0.2	10	10.3 ± 0.3	8	9.6 ± 0.4	10	10.5 ± 0.7	7
43	10.6 ± 0.2	10	10.2 ± 0.3	10	$8.3^{a} \pm 0.4$	10	10.5 ± 0.4	7
50	10.7 ± 0.3	10	10.5 ± 0.2	10	$9.2^{a} \pm 0.3$	10	11.3 ± 0.6	7
57	10.9 ± 0.5	10	10.8 ± 0.3	10	9.7 ± 0.3	10	10.8 ± 1.0	7
64	10.2 ± 0.3	10	9.9 ± 0.4	10	9.7 ± 0.5	10	11.5 ± 0.4	7
71	10.6 ± 0.3	10	10.3 ± 0.3	10	9.3 ± 0.5	10	11.5 ± 0.5	7
78	10.4 ± 0.3	10	10.4 ± 0.6	10	9.3 ± 0.3	10	11.4 ± 0.4	6
85	10.6 ± 0.3	10	10.4 ± 0.3	10	10.0 ± 0.4	10	11.2 ± 0.4	6
90	10.6 ± 0.4	10	10.8 ± 0.9	10	8.7 ± 0.3	10	9.8 ± 0.8	6

TABLE 5.4-4.MEAN (±SE) FOOD CONSUMPTION (g) OF FEMALE RATS IN THE 90-DAY ORAL
STUDY WITH n-NONANE

a = p<0.01 compared to control.

b = p<0.05 compared to control.

c = Day 38 was reported due to problems in data collection on Day 36.

Dose (g/kg/day) and Group Size (N)								
Study Day	0.0	N	0.1	N	1.0	N	5.0	N
1	7.2 ± 0.5	8	7.1 ± 0.7	7	7.0 ± 0.7	7	7.9 ± 0.6	7
8	4.9 ± 0.2	10	5.0 ± 0.4	10	5.8 ± 0.4	10	5.2 ± 0.5	10
15	4.4 ± 0.4	10	4.1 ± 0.3	10	4.5 ± 0.4	10	5.4 ± 0.3	10
22	5.2 ± 0.4	10	4.3 ± 0.5	10	4.8 ± 0.6	9	5.7 ± 0.5	9
29	4.5 ± 0.6	10	$\textbf{4.8} \pm \textbf{0.3}$	10	4.3 ± 0.6	10	4.4 ± 0.5	9
36	4.2 ± 0.2	10	4.3 ± 0.3	10	3.7 ± 0.3	10	3.7 ± 0.5	9
43	4.7 ± 0.2	10	4.8 ± 0.5	10	4.9 ± 0.4	10	5.0 ± 0.3	9
50	5.0 ± 0.4	10	5.3 ± 0.3	10	4.4 ± 0.3	9	4.4 ± 0.4	8
57	2.6 ± 0.3	8	3.4 ± 0.5	10	4.0 ± 0.7	10	4.4 ± 0.3	9
64	5.3 ± 0.3	9	5.5 ± 0.7	10	5.0 ± 0.5	9	5.1 ± 0.2	9
71	4.7 ± 0.4	9	4.7 ± 0.2	10	5.2 ± 0.4	10	5.5 ± 0.3	9
78	4.2 ± 0.2	9	4.3 ± 0.2	10	4.9 ± 0.5	10	4.9 ± 0.3	9
85	4.6 ± 0.2	9	4.8 ± 0.4	10	4.9 ± 0.3	9	5.6 ± 0.3	8
88	5.4 ± 0.5	8	4.8 ± 0.3	10	5.9 ± 0.5	8	6.4 ± 0.4	8

TABLE 5.4-5. MEAN (±SE) FOOD CONSUMPTION (g) OF MALE MICE IN THE 90-DAY ORALSTUDY WITH n-NONANE

Hematology and Serum Chemistry (results not shown)

In rats of the 5.0 g/kg group, the white blood cell count was increased compared to the control value. The mean percentages of neutrophils and basophils were also increased in the 5.0 g/kg group, but the lymphocyte percentage was decreased. The only other statistically significant difference compared to control values was an increase in the WBC count for the 0.1 g/kg group. In mice, decreases in red blood cell count, hemoglobin concentration, hematocrit percentage, and percent lymphocytes were observed in the 5.0 g/kg group. Neutrophil percentage was increased compared to control in the 0.1, 1.0, and 5.0 g/kg groups.

In rats of the 5.0 g/kg group, there were decreases in mean values of cholesterol, triglycerides, and albumin and an increase in alanine aminotransferase. Rats of the 1.0 g/kg group had lower albumin and total protein concentrations compared to the control group. No additional statistically significant difference was observed in serum chemistry values in rats. Decreases in the mean values of chloride, aspartate aminotransferase, alkaline phosphatase, total bilirubin, and albumin were observed in mice of the 5.0 g/kg group. Mice of the 1.0 g/kg group had lower
chloride, alkaline phoshatase and albumin values compared to control mice. Alkaline phosphatase was also lower in the 0.1 g/kg group. No other statistically significant difference between treated and control groups was observed.

Test Substance Analysis in Blood and Tissues - Rats only (results not shown)

Blood concentrations increased with dose and were considerably lower in value prior to dosing compared to post dosing. Further, blood concentrations were consistent between study weeks at each dose level. At the conclusion of the study, concentrations of n-Nonane were the highest in fat tissue compared to muscle or liver. Though interanimal variability was large, tissue concentrations increased with dose.

Organ Weights

Mean organ weight values for female rats and male mice are presented in Tables 5.4-6 through 5.4-9. Since the mean values between control and treated groups were similar for final body weights and absolute brain weights in both rats and mice, statistically significant differences in absolute organ weight values agreed, in the majority of cases, with statistically significant differences in relative organ weight values. Female rats of the 5.0 g/kg group had increased liver, lung, and adrenal weights, but decreased spleen and ovary weights. The increase in adrenal weights and decrease in ovary weights were also observed in the 1.0 g/kg female rats, but there were no differences in organ weights between the control and 0.1 g/kg groups.

In male mice, liver weights were increased and kidney weights were decreased in the 5.0 and 1.0 g/kg groups. There were no statistically significant differences in mean organ weights between the control and 0.1 g/kg groups.

	Dose (g/kg/day)				
Organ	0.0 ^a	0.1*	1.0 ^b	5.0°	
Liver	4.19 ± 0.10	4.10 ± 0.11	4.03 ± 0.09	$4.82^{d} \pm 0.18$	
Kidneys	1.17 ± 0.02	1.14 ± 0.02	1.13 ± 0.02	1.21 ± 0.04	
Lungs	1.32 ± 0.12	1.25 ± 0.07	1.21 ± 0.09	$2.28^{d} \pm 0.44$	
Spleen	0.39 ± 0.01	0.38 ± 0.01	0.35 ± 0.01	$0.34^{d} \pm 0.02$	
Adrenals	0.054 ± 0.003	0.053 ± 0.002	0.060 ± 0.002	0.066 ± 0.003	
Ovaries	0.091 ± 0.002	0.088 ± 0.002	$0.076^{d} \pm 0.003$	$0.067^{\text{d}}\pm0.008$	
Brain	1.69 ± 0.02	1.67 ± 0.01	1.64 ± 0.03	1.67 ± 0.02	
Body weight	163.3 ± 2.5	161.9 ± 1.5	154.0 ± 2.5	160.8 ± 4.4	

TABLE 5.4-6. MEAN (±SE) ORGAN WEIGHTS (g) AND FINAL BODY WEIGHT (g) OF FEMALERATS IN THE 90-DAY ORAL STUDY WITH n-NONANE

^a Group size = 10.

^b Group size = 9.

^c Group size = 6.

^D Significantly different compared to control, p<0.05.

TABLE 5.4-7. MEAN (±SE) RELATIVE (TO BODY WEIGHT) ORGAN WEIGHTS (%) OF FEMALERATS IN THE 90-DAY ORAL STUDY WITH n-NONANE

	Dose (g/kg/day)			
Organ	0.0ª	0.1ª	1.0 ^b	5.0°
Liver	2.56 ± 0.04	2.53 ± 0.06	2.62 ± 0.04	$3.00^{d} \pm 0.06$
Kidneys	0.72 ± 0.01	0.71 ± 0.01	0.74 ± 0.01	0.75 ± 0.01
Lungs	0.80 ± 0.07	$\textbf{0.77} \pm \textbf{0.04}$	0.79 ± 0.05	$1.43^{d} \pm 0.30$
Spleen	$0.24 \pm < 0.01$	0.23 ± 0.01	0.23 ± <0.01	$0.21^{d} \pm 0.01$
Adrenals	0.033 ± 0.002	0.033 ± 0.001	$0.039^{\text{d}} \pm 0.001$	$0.041^{d} \pm 0.001$
Ovaries	0.056 ± 0.001	0.055 ± 0.001	0.050 ± 0.002	$0.041^{d} \pm 0.004$
Brain	1.04 ± 0.01	1.03 ± 0.01	1.07 ± 0.01	1.04 ± 0.03

^a Group size = 10.

^b Group size = 9.

^c Group size = 6.

^D Significantly different compared to control, p<0.05.

	Dose (g/kg/day)				
Organ	0.0 ^a	0.1 ^b	1.0 ^c	5.0°	
Liver	1.14 ± 0.04	1.20 ± 0.03	1.27 ± 0.06	$1.40^{d} \pm 0.08$	
Kidneys	0.45 ± 0.01	0.41 ± 0.01	$0.40^d\pm0.01$	$0.40^d \pm 0.02$	
Lungs	0.34 ± 0.01	0.31 ± 0.02	0.29 ± 0.02	0.28 ± 0.02	
Spleen	0.05 ± <0.01	$0.06 \pm < 0.01$	$0.07 \pm < 0.01$	0.12 ± 0.04	
Adrenals	0.006 ± 0.001	0.007 ± 0.001	0.005 ± 0.001	0.005 ± 0.001	
Testes	0.22 ± <0.01	0.20 ± 0.01	0.21 ± 0.01	0.20 ± 0.01	
Brain	0.43 ± <0.01	$0.42 \pm < 0.01$	$0.42 \pm < 0.01$	0.41 ± 0.01	
Body weight	28.5 ± 0.5	27.6 ± 0.5	27.6 ± 0.9	26.9 ± 0.7	

TABLE 5.4-8. MEAN (±SE) ORGAN WEIGHTS (g) AND FINAL BODY WEIGHT (g) OF MALEMICE IN THE 90-DAY ORAL STUDY WITH n-NONANE

^a Group size = 9.

^b Group size = 10.

 $^{\circ}$ Group size = 8.

^d Significantly different compared to control, p<0.05.

	Dose (g/kg/day)			
Organ	0.0ª	0.1 ^b	1.0°	5.0°
Liver	4.01 ± 0.13	4.35 ± 0.08	$4.60^{d} \pm 0.11$	$5.18^{d} \pm 0.20$
Kidneys	1.58 ± 0.02	1.50 ± 0.03	$1.46^{d} \pm 0.02$	1.47 ± 0.05
Lungs	1.21 ± 0.03	1.13 ± 0.07	1.04 ± 0.06	1.04 ± 0.04
Spleen	0.19 ± 0.01	0.23 ± 0.01	0.24 ± 0.01	0.46 ± 0.14
Adrenals	0.023 ± 0.004	0.025 ± 0.004	0.018 ± 0.003	0.020 ± 0.003
Testes	0.78 ± 0.02	0.74 ± 0.03	0.77 ± 0.03	0.76 ± 0.03
Brain	1.51 ± 0.03	1.54 ± 0.02	1.53 ± 0.06	1.54 ± 0.04

TABLE 5.4-9.MEAN (±SE) RELATIVE (TO BODY WEIGHT) ORGAN WEIGHTS (%) OFMALE MICE IN THE 90-DAY ORAL STUDY WITH n-NONANE

^a Group size = 9.

^b Group size = 10.

^c Group size = 8.

^D Significantly different compared to control, p<0.05.

Results of histopathologic evaluation, a discussion, and study conclusions will be provided in the detailed technical report (in progress).

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5.5 DEVELOPMENTAL TOXICITY SCREENS OF LIQUID PROPELLANT XM46, AMMONIUM DINITRAMIDE, TRINITROBENZENE, AND AMMONIUM PERCHLORATE USING *HYDRA ATTENUATA*

R.E. Wolfe, E.R. Kinkead, and P.D. Confer

ABSTRACT

The developmental toxicity screen using *Hydra attenuata* was developed to evaluate substances for possible developmental toxicity in a relatively short time when compared to conduction of a vertebrate animal developmental toxicity study. Four military compounds were tested using the *in vitro* hydra assay: liquid propellant XM46 (LP), ammonium dinitramide (ADN), trinitrobenzene (TNB), and ammonium perchlorate (AP). The toxicity screens were performed to determine the developmental hazard index (A/D ratio) for these compounds. The hydra assay employs exposing both adult hydra and "artificial embryos" composed of disassociated hydra cells to test compounds to investigate developmental toxicity. A/D ratios produced from the hydra assay of LP, ADN, TNB, and AP of 1.25, 2.14, 2.11, and 1.71, respectively, indicate that these military compounds should not be considered primary developmental toxins.

INTRODUCTION

This study was performed to determine the developmental hazard indices (A/D ratios) for liquid propellant XM46 (LP), ammonium dinitramide (ADN), trinitrobenzene (TNB), and ammonium perchlorate (AP) through use of the *in vitro* hydra assay developmental toxicity screen. LP is being considered by the U.S. Army as a replacement for solid propellants, both as part of a regenerative injection gun system and as a working fluid in an electrothermal gun system. Evaluation of the reproductive toxicity of LP through use of a modified Screening Information Data Set (SIDS) study using Sprague-Dawley (SD) rats did not produce any adverse effects on reproduction or litter parameters (Kinkead et al., 1995a). ADN is being considered as a clean burning replacement for ammonium perchlorate in a formulation that also gains in performance. Currently, Air Force personnel are working with ADN during field testing trials. Reproductive toxicity evaluation of ADN through use of a SIDS study using SD rats produced adverse effects on litter parameters of treated animals (Kinkead et al., 1995b). TNB is a dimorphic crystalline solid that is produced during the nitration step of trinitrotoluene synthesis as a result of methyl group oxidation (Budavari et al., 1989). It is primarily used as an explosive, but has also had limited use in the vulcanization of rubber (Barnhart, 1981). TNB is used to produce plastics, herbicides, and paints and can enter domestic drinking water reservoirs via domestic effluent (Ryon et al., 1984; U.S. EPA, 1989). A SIDS study of TNB did not produce adverse effects on reproduction or litter parameters evaluated (Kinkead et al., 1995c). TNB is

not readily biodegradable and has a tendency to leach out into the groundwater near production or disposal sites. AP is a class 1.1 oxidizer that is used as a component in solid rocket propellants, munitions, and fireworks (CPIA, 1989; TERA, 1996). The production and storage of AP has resulted in contaminated soils and groundwaters. No reproductive or developmental toxicity data are available for AP.

The Hydra Assay is performed using the fresh water coelenterate *Hydra attenuata* as the test species. *Hydra attenuata* is the most primitive invertebrate composed of complex tissues and organs, and it is the highest form that has the capability for whole body regeneration. The assay employs the use of both adult hydra and artificial embryos to investigate the potential toxicity of test compounds. Artificial embryos (also called pellets) are prepared by disassociating adult hydra into their component cells (Gierer et al., 1972). These cells can be reaggregated randomly into the artificial embryo (Johnson, 1980) which will regenerate into adult hydra within a few days under normal conditions. In order for the pellets to regenerate into new adult hydra, the embryo must accomplish most, if not all, of the developmental events required during true embryogenesis (Johnson, 1990).

A concentration of a test chemical which causes abnormal development in the embryo may or may not cause an effect in the adult hydra. For this reason, both adult hydra and the artificial embryos are tested concurrently. The lowest concentration of test chemical that causes death in the intact adult hydra is compared to the lowest concentration that produces death in the developing artificial embryo. The adult toxic (A) to developmentally toxic (D) ratio (A/D ratio) is calculated using these concentrations. The A/D ratio, also referred to as the developmental hazard index, is predictive of a chemical's hazard potential in standard laboratory animals and man (Johnson and Gabel, 1982). A low A/D ratio (<3) predicts a test chemical being toxic to an embryo only at levels which will also cause toxic signs in the adult animal. A high A/D ratio (\geq 3) reveals a chemical's teratogenic hazard, displaying a toxic effect in the developing embryo while causing little to no toxicity in the adult (Johnson et al., 1988).

MATERIALS AND METHODS

Test Materials

Liquid Propellant XM46 (LP)

Source/Supplier:	U.S. Army
Synonym:	LP 1846
Appearance:	Clear, colorless liquid
CAS No.:	None

Components:	Hydroxylammmonium nitrate (61%)		
	Water (20%)		
	Triethanolammonium nitrate (19%)		
Density:	1.42 g/mL at 20 °C		

Ammonium Dinitramide (ADN)

Source/Supplier:	SRI International		
	Menlo Park, CA		
Common Name:	SRI-12		
Appearance:	Solid, pale yellow to white crystals		
Empirical formula:	$NH_4N(NO_2)_2$		
CAS No.:	None		
Density:	\approx 1.8 g/mL at 20 °C		

The ADN sample, a water-soluble powder, was known to be contaminated with 1-2% ammonium nitrate and was maintained in an enclosed cabinet due to light sensitivity (Koppes, 1993). Stability of ADN in water was determined during the reproductive toxicity screen of ADN (Kinkead et al., 1995b).

1,3,5-Trinitrobenzene (TNB)

Source/Supplier:	U.S. Army
Synonyms:	Trinitrobenzene, Benzite
CAS No.:	99-35-4
Empirical Formula:	$C_6H_3N_3O_6$
Formula Weight:	213.11
Vapor Pressure	3.2 x 10 ⁻⁶ mmHg at 20 °C

Ammonium Perchlorate (AP)

Source/Supplier:	Aldrich Chemical Company,		
	Milwaukee, Wisconsin		
Appearance:	Solid, white powder		
CAS No.:	7790-98-9		
Purity:	99.8%		
Density:	1.4 g/mL at 20 °C		

Test Species

Hydra attenuata are the only hydroids with which a successful hydra assay may be performed. Polyps of the species *Hydra attenuata* live in fresh water, they are not complicated by algae associations, and they derive all nutrition by feeding (Johnson et al., 1988). The hydra colony was produced and housed in shallow Plexiglas aquaria which provided controlled aeration (referred to as farms). The hydra farms and test hydra were maintained within a Plexiglas enclosure. The temperature within the laboratory and the enclosure was maintained near 18 ± 2 °C using an attached cooling system. Hydra were maintained in a water-based medium (hydra medium). Ultrapurified water was used in the preparation of all solutions used throughout the study. The pH of the medium was adjusted to 6.90-6.95 using 0.5 N NaOH. The hydra were fed regularly and allowed to propagate naturally by budding. *Artemia nauplii* (brine shrimp) were provided as food for the hydra.

Prior to testing, hydra were group housed within the hydra farms. Test hydra were fed daily for 3 days prior to testing, but were not fed while on study. Test hydra were maintained in separate aquaria during the 3 days prior to testing. During exposure, test hydra were placed in glass test dishes.

Experimental Design

The preparation of regenerating artificial hydra embryos and the performance of the hydra assays were accomplished following the methods in The Hydra Assay Manual (Johnson et al., 1992). A complete description of the methods used in the assays of LP, ADN, and AP can be found in Wolfe et al., 1996a,b, and Confer et al., 1996. The final report for TNB is currently being written. The methods used during this assay were the same as those included in the listed reports.

Artificial embryos (also called pellets) were created by disassociating adult hydra into their component cells (Gierer et al., 1972). These cells were reaggregated randomly into the artificial embryo (Johnson, 1980) which can regenerate into adult hydra under normal conditions and within a few days. In order for the pellets to regenerate into new adult hydras, the "embryo" must accomplish most, if not all, of the developmental events required during true embryogenesis (Johnson, 1990).

Test chemicals may affect one or more of these developmental events, causing abnormal development and/or death of the artificial embryo. The concentration of test chemical which causes abnormal development in the embryo may or may not cause an effect in the adult hydra. For this reason, both adult hydra and the artificial embryos were tested concurrently with each test agent.

Determination of A/D Ratios

The lowest concentration of test chemical that caused death in the intact adult hydra was compared to the lowest

concentration that produced death in the developing artificial embryo. The adult toxic (A) to developmentally toxic (D) ratio (A/D ratio) was calculated using these concentrations. The A/D ratio for each test material was calculated using the average minimal affective concentrations (MAC) from the final experiments for each test material using the following equation:

MAC adult hydra ÷ MAC developmental hydra (or artificial embryo) = A/D ratio

STATISTICS

Standard statistical analyses are not applicable for results from the hydra assay due to the small number of invertebrates used per concentration. The endpoints for the hydra assay are specific and conclusive for both the adult and the developing artificial embryo, and statistical analysis would be unreasonable (Newman et al., 1990).

RESULTS

Table 5.5-1. lists the developmental MAC, the adult MAC, and the developmental hazard indices (A/D ratios) determined for LP, ADN, TNB, and AP through performance of the hydra assay developmental toxicity screen.

TABLE 5.5-1. ADULT AND DEVELOPMENTAL MINIMAL AFFECTIVE CONCENTRATIONS
(MAC) AND A/D RATIOS DETERMINED FOR LP, ADN, TNB, AND AP USING THE
HYDRA ASSAY DEVELOPMENTAL TOXICITY SCREEN

Test Agent	Adult MAC	Developmental MAC	A/D Ratio
Liquid Propellant XM46 (LP)	0.0025 mL LP/L	0.002 mL LP/L	1.25
Ammonium Dinitramide (ADN)	750 mg ADN/L	350 mg ADN/L	2.14
Trinitrobenzene (TNB)	3.8 µg TNB/L	l.8 μg TNB/L	2.11
Ammonium Perchlorate (AP)	600 mg AP/L	350 mg AP/L	1.71

DISCUSSION

A/D ratios calculated from hydra assays have been compared to the A/D ratios from standard Segment II-type studies (Johnson et al., 1988). A comparison was made of A/D ratios obtained for 61 chemicals in which both hydra assays and Segment II-type studies were performed. Of the 61 chemicals, 57 of them had A/D ratios for hydra and Segment II-type studies which were in agreement. The hydra assay is 90% accurate and has a low incidence of false negatives (Johnson et al., 1988).

The A/D of 1.25 determined for LP would indicate that LP would only be toxic to a developing embryo at levels which would also cause maternal toxicity. This is comparable to the results obtained for LP in the teratology study (Cooper and Caldwell, 1994) and the 90-day SIDS study (Kinkead et al., 1995a). No adverse effects were detected for LP in fetal sizes or development, or in reproductive, litter, and pup parameters measured.

The A/D ratio of 2.14 determined for ADN would predict that ADN would adversely affect a developing embryo only at maternally toxic levels. This result is comparable to the results obtained when pregnant SD rats were treated with ADN during the pre- and postimplantation periods (Kinkead et al., 1996). Female SD rats were orally dosed with 2.0 g ADN/L in the drinking water during the preimplantation period (Gestation Days [GD] 1-3) or during the postimplantation period (GD 4-8). Complete blockade of implantation occurred in animals receiving ADN during the preimplantation period. Animals treated during the postimplantation period had a mean of 16.4 implantations per dam, similar to the mean of 15.2 implantations per dam in the control group. The preimplantation exposure to ADN is the probable cause for these adverse effects. The postimplantation dosing regimen reported in Kinkead et al., 1996, would be comparable to that used in developmental toxicity studies, where pregnant animals are dosed during the period of organogenesis, GD 6-15 for mice and rats (OECD, 1993). Therefore, the A/D ratio determined in this developmental toxicity screen of ADN using *Hydra attenuata* should be analogous to results obtained from ADN exposure using a vertebrate developmental toxicity dosing regimen.

The A/D of 2.11 determined for TNB would predict that TNB would not adversely affect an embryo at dose levels below the maternal toxic exposure level. This result corresponds to the data obtained during the 90-day SIDS study of TNB (Kinkead et al., 1995c). During this study, the reproductive findings were limited to treatment-related decreases in mean pup body weights observed during the 21-day postpartum period, and a reduction in sperm concentration in the TNB-treated rats. Fertility of the males appeared to be unaffected since no adverse effects were found for reproductive or litter parameters measured in this study. A developmental toxicity study of TNB was performed, and a report of the morphological evaluation of fetal specimens from this study by WIL Research Laboratories, Inc. (1995) concluded that developmental toxicity occurred only at TNB dose levels in which there was also maternal toxicity. No treatment-related malformations were observed in fetuses in this study.

The A/D ratio of 1.71 determined for AP would predict that AP would produce coaffective toxicity in the adult and developing embryo. Although no data are available on human developmental toxicity of AP, Crooks and Wayne (1960) reported twelve infants of mothers who were treated with 600 to 1000 mg perchlorate/day for Graves' Disease during gestation were born with no abnormalities (with the exception of one infant born with a very slightly enlarged thyroid which returned to normal size in 6 weeks).

Under the conditions of the hydra assay performed in this laboratory, LP, ADN, TNB, and AP should not be considered primary developmental toxins.

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5.6 EVALUATION OF THE EFFECTS OF 14-DAY SIMULATED PERSIAN GULF WAR EXPOSURE ON MALE SPRAGUE-DAWLEY RATS

R.E. Wolfe, K.L. MacMahon¹, D.H. Ellis, M.L. Feldmann, H.F. Leahy, L. Narayanan, and J. Rossi, III²

ABSTRACT

Eight groups of 16 male Sprague-Dawley rats were exposed for 6 h/day for 14 consecutive days to combinations of simulated stress and low doses of chemicals that military personnel were exposed to during the Persian Gulf War. Exposures consisted of combinations of JP-4 jet fuel vapor (2 mg/L via inhalation), pyridostigmine bromide (PB, 1 mg/kg oral gavage), N-N-diethyl-m-toluamide (DEET, 33% solution in alcohol, 300 µL/day dermally), and stress (intermittent mild foot shock). Half of the animals were rested for two weeks followed by behavioral testing. These animals were then sacrificed 30-days posttreatment. The remaining animals were rested for two months, and then behaviorally tested. These animals were sacrificed 60-days posttreatment. Body weights were recorded weekly during exposure and postexposure. Organ weights were recorded at necropsy. Blood, brain, testes, liver, and kidney samples were taken from animals in each group at necropsy for clinical examinations. Tissues were also taken for histopathologic examination. No clinical signs of toxic stress were noted during the study or during the postexposure observation periods. No statistically significant differences were found for body weight, clinical chemistry or hematology parameters, or organ weights between the 8 groups at the 30- and 60-day posttreatment sacrifices. Statistically significant differences were found in chemically-exposed animals when compared to aironly controls for some neurobehavioral parameters measured, and brain and serum neurotransmitter analyses in the animals sacrificed 30-days posttreatment. Tissue samples and neurobehavioral data for the animals sacrificed 60days posttreatment are still undergoing evaluation.

INTRODUCTION

The investigation into the health problems of Persian Gulf veterans is a major program within the Department of Defense (DoD) and other federal agencies. Of the 600,000 American soldiers sent to the Middle East during the Persian Gulf War, more than 54,000 have since qualified for disability compensation, and more than 1,600 have

¹Armstrong Laboratory, Toxicology Division, Wright-Patterson Air Force Base, OH.

²Naval Medical Research Institute/Toxicology Detachment, Wright-Patterson Air Force Base, OH.

died (Rhein, 1993). The study described in this report was performed to evaluate the potential of a simulated Persian Gulf War exposure consisting of multiple chemical exposures, alone or combined with an imposed psychological stressor, to induce toxic effects in male Sprague-Dawley rats similar to those reported by Persian Gulf War veterans. The interaction of physiological stress responses, exposure to fuel vapors, insecticides and insect repellents, the neurotoxic war gas prophylactic drug pyridostigmine bromide, and other environmental factors may account for some of the reported effects. This study investigated in a rodent model these factors which may have contributed to the development of the symptomatologies reported by Persian Gulf War veterans.

MATERIALS AND METHODS

A complete description of the materials and methods used during this study will be included in the final technical report MacMahon et al., 1997a.

Animals

Male Sprague-Dawley-derived outbred albino rats [Crl:CD®(BR)] were purchased from Charles River Breeding Laboratories, Raleigh, NC. Rats were 50 days of age upon receipt. All rats were identified by tail tattoo and were acclimatized two weeks prior to use. During the acclimation period, quality control procedures were performed on selected rats as described in Kinkead et al. (1991). Rats were assigned to groups by means of a computer-generated randomization. The randomization was stratified by body weight such that the mean body weights of all groups were homogeneous by statistical analysis at study initiation. Water from a reverse-osmosis system and Purina Formulab #5002 feed were available *ad libitum*, except during the 6-h inhalation exposures. Animal rooms were maintained on a 12-h light/dark cycle (fluorescent light) and targeted at a temperature of 23 ± 2 °C and a relative humidity of $55 \pm 15\%$.

Test Agents

JP-4 Jet Fuel

JP-4 jet fuel was chosen for vapor exposure of the animals since it was one of the fuels used in the Persian Gulf War to which military personnel may have been exposed. The concentration of JP-4 which the fuel-exposed animals received was 2 mg/L vapor via the inhalation route. The JP-4 jet fuel was supplied by Wright Laboratory, Wright-Patterson Air Force Base, Ohio. Pertinent physical and chemical properties are listed below.

JP-4 Jet Fuel

Source: Appearance: Flash Point Specific gravity: Vapor Pressure: U.S. Air Force Clear, colorless liquid 100 °F 0.760 g/mL 72 mmHg @ 70 °F

Generation and Analysis of Exposure Atmospheres

JP-4 jet fuel vapor was generated by metering approximately 1.8 mL fuel/min via a Buchler multistaltic pump (Model 426-2000, Buchler Instruments, A Labconco Co., Lexena, KA) into the top of a counter-flow (3 cfm), heated (45 °C) evaporator tower. The output was divided between the two fuel exposure chambers and combined with chamber input air for a total flow of 6 cfm per chamber. The fuel mass concentration in each chamber during the 14-day study was continuously quantified using the IR absorbance band between 3.4 and 3.5 microns (Miran 1A, Foxboro Analytical, Wilks Infrared Center, South Norwalk, CT) calibrated with known mass concentrations of hexane. Less than 2% of the input fuel was recovered as flow-by or in-line condensate. No aerosol was detected when chamber atmospheres were sampled with Gelman 25-mm, extra thick glass fiber filters (Gelman Sciences, Ann Arbor, MI). Gas chromatographic analysis of the fuel, fuel vapor, and spent fuel were also performed. The chamber vapor chromatograms resembled closely those of the original jet fuel, with only a slight shift to the lighter fractions of JP-4 jet fuel components. The spent fuel was essentially devoid of jet fuel components lighter than C11 (Undecane) fraction.

N-N-Diethyl-m-toluamide (DEET)

Persian Gulf War personnel are known to have been exposed (dermally, through inhalation, or through ingestion) to insecticides/pesticides and repellents, one of which was DEET (DoD CCEP, 1995). DEET has been available since 1957, and is normally applied to and absorbed through the skin. DEET can cause CNS disturbances in adults and experimental animals (Sax and Lewis, 1989). The DEET-exposed animals were dosed with a 33% DEET solution (in ethyl alcohol), with each rat receiving a daily dermal treatment of 300 μ L 33% DEET mixture per day of exposure applied to the shaved skin of the back. Pertinent physical and chemical properties of DEET are listed below.

Source: Purity: Appearance: Density: DEET Sigma Chemical Co., St. Louis, MO 97% Clear, colorless liquid 1.00 g/mL

Pyridostigmine bromide (PB)

In anticipation that U.S. Troops might be exposed to neurotoxic gases such as soman or sarin, many troops were routinely issued prophylactic drugs, including PB, a potent cholinesterase inhibitor. Troops were instructed to self-administer one pyridostigmine tablet (10 mg) every eight hours. The animals receiving PB in this study were orally gavaged with 1 mg PB/kg body weight each exposure day. This dosage has been shown to inhibit cholinesterase activity by 40% in rodents, the target inhibition rate in military troops (Kerenyi et al., 1988). Pertinent information on the PB as purchased is listed below.

	PB
Source:	ICN Pharmaceuticals, Inc., Costa Mesa, CA
Trade Name:	Mestinon, syrup
Appearance:	Purple liquid, raspberry flavor and smell

The PB syrup consisted of 60 mg PB per 5 mL liquid in a vehicle containing 5% alcohol, glycerin, lactic acid, sodium benzoate, sorbitol, sucrose, FD&C Red No. 40, FD&C Blue No. 1, flavoring, and water.

Psychological Stress Simulation

Throughout the 6-h exposures, half of the animals were exposed to low levels of randomly generated, unpredictable mild electrical shock to simulate psychological stress conditions. Foot shock was delivered through Lafayette Electronics Units (controlled by a central computer) to the gridbar flooring of the exposure cages. The shock occurred randomly six times per hour (total 36 shocks per exposure day), and was no more than 0.5 ma for 300 msec. The shock delivery was scrambled so that alternating gridbars became positive or grounded many times per second. The computer simultaneously delivered shock to each rat within a given exposure chamber so that the rats could not anticipate impending shock based upon the reaction of other rats receiving shock. The shock level selected was barely painful when applied to the finger of a human subject (Ritchie and Binole, 1996) and was self-tested each day by an experimenter prior to animal exposures.

14-Day Repeat Exposure Regimen

Eight groups of 16 male rats (Table 5.6-1) were placed in 690-L inhalation chambers and exposed for 6 h daily to either air only (Groups 5-8) or JP-4 jet fuel vapor (Groups 1-4). Two groups from each inhalation regimen were subjected to a mild shock to stress the animals (Groups 1, 2, 5, and 6). Two groups from each inhalation regimen were orally gavaged with PB and dermally treated with DEET (Groups 1, 3, 5, and 7). Group 4 received JP-4 vapor only, and Group 8 received air only. The animals were exposed to the combination of test agents and treatments for 14 consecutive days. One animal from the A/OD group (Group 7) was euthanatized during the two-week exposure due to respiratory tract trauma caused during oral gavage.

Each exposure chamber held four cages. Each of these cages had eight separate sections to contain rats during exposure. The animals were housed individually and randomly assigned to specific exposure cage locations within the appropriate group chamber. The exposure cages were rotated clockwise (moving one position) within each inhalation chamber every exposure day. The rats were observed prior to each day's exposure, during exposure, and again postexposure for signs of toxic stress. Rat body weights were measured prior to study initiation, and then weekly throughout the study.

Neurobehavioral Evaluations

Neurobehavioral evaluations were performed by U.S. Navy personnel. A complete description of this testing and the analyzed results will be reported in the final report for this study (MacMahon et al., 1997a). Following the 2-week exposure, all 128 animals were rested for two weeks. Half of the animals (64) were then evaluated on a battery of 7 neurobehavioral tests on postexposure days 14-32, while the remaining 64 animals were held and then evaluated on postexposure days 60-78. Tests were selected from the Navy Neurobehavioral Toxicity Assessment Battery to identify category deficits in the rats that may model symptoms reported by Persian Gulf War veterans. These tests included Forelimb Grip Strength, Photosensitivity Avoidance, Generalized Olfactory Sensitization, Acoustic Startle, Prepulse Inhibition and Habituation, Total Locomotor Activity, Tail Flick Analgesia, and the Treadmill Test of Physical Fatigue (Rossi et al., 1997).

Clinical Measurements.

At each necropsy, blood samples were taken via the vena cava from fasted animals for complete hematology and clinical chemistry assays. Erythrocytes were enumerated on a Coulter counter (Coulter Electronics, Hialeah, FL) and sera for clinical chemistry evaluations were assayed on an Ektachem 700XR (Eastman Kodak, Rochester, NY). Selected hematological parameters and absolute leukocyte differentials were determined according to established procedures. Sera were processed according to the procedures in the Ektachem Operations manual.

Plasma, testes, liver, and kidney samples were also taken for protein analysis during each animal sacrifice. A complete description of the protein analysis methods will be included in the final report.

Group	Group	Inhalation	Stress	Dermal	Oral
Number	Name	Exposure	Treatment	Treatment	Treatment
1	JP4/S/OD	JP-4	Shock	DEET	PB
2	JP4/S	JP-4	Shock		
3	JP4/OD	JP-4		DEET	PB
4	JP4	JP-4			
5	A/S/OD	Air Only	Shock	DEET	PB
6	A/S	Air Only	Shock		
7	A/OD	Air Only		DEET	PB
8	Α	Air Only			<u>-</u>

 TABLE 5.6-1.
 SIMULATED PERSIAN GULF WAR EXPOSURE TREATMENT GROUPS

Evaluations at Necropsy

Brain, liver, kidneys, spleen, adrenal glands, testes, and lungs were weighed at necropsy. Bouin's fixative was used to fix the testes and epididymides. Nasal turbinates, pituitary, spleen, liver, lungs, stomach, duodenum, jejunum, ileum, colon, kidneys, thymus, peripheral nerve, skeletal muscle, cervical lymph nodes, thyroids, parathyroids, adrenal glands, bone with bone marrow, and cervical and lumbar spinal cord sections were removed from the animals and fixed in 10% buffered formalin solution. After routine processing, the tissues were embedded in paraffin and stained with hematoxylin and eosin for histopathologic examination. All tissues from four air-only control animals and four JP4/S/OD animals from each sacrifice (16 animals) are undergoing histopathological examination. Tissues from the remaining animals will be held for possible future histopathologic examination.

Neurotransmitter Analysis

Neurotransmitter analysis was performed on 6 male rats per group, per postexposure sacrifice, from the 2-week exposure. At each sacrifice, the brains from these animals were surgically removed, weighed whole, and then nine regions of each brain were dissected, frozen on dry ice, and stored at -70 °C until analysis. The five brain regions analyzed were the brain stem, cerebral cortex, caudate nucleus, hippocampus, and cerebellum. Serum was also taken from these animals at necropsy, frozen, and then analyzed for neurotransmitter levels. Analysis of the neurotransmitters was performed following the methods described in Kim et al., 1987.

Statistical Analysis

Organ weights, organ-to-body weight ratios, neurotransmitter analyses, serum chemistry, and hematology 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 weights (Barcikowski, 1983). Tissue lesion severity data were analyzed using the Kruskal-Wallis analysis of variance (Rosner, 1990). Statistics for the neurobehavioral evaluations were performed by U.S. Navy personnel, and these methods will be listed in the final report for this study.

RESULTS

Many parameters measured in this study are still undergoing analyses at the time of this report. Complete results of this study will be reported in MacMahon et al., 1997a.

During the two-week inhalation exposure period, daily mean concentration of JP-4 jet fuel was maintained at the target concentration of 2 mg/L. No clinical signs of toxic stress were noted during exposure or during the postexposure period for any animals. No statistically significant differences were noted in the mean body weights of the male rats during the exposure or posttreatment (Figure 5.6-1).



Figure 5.6-1. Mean Body Weights of Male Sprague-Dawley Rats, Control and Treated, during the Simulated Persian Gulf War Exposures

Neurobehavioral Evaluation

Neurobehavioral evaluation of the animals sacrificed 30-days postexposure have been completed. Significant deficits (p<0.05 or 0.01) compared to the air-only control group were detected in the JP4 and JP4/S groups for Forelimb Grip Strength, in the JP4 group for Generalized Olfactory Sensitization, and in the A/S, JP4/S, A/OD, JP4/OD, and A/S/OD groups for Tail Flick Analgesia, and in the A/S, JP4, JP4/S, A/S/OD, JP4/OD, and JP4/S/OD groups for Acoustic Startle or Habituation (Rossi et al., 1997).

Clinical Measurements and Necropsy Evaluations

At each necropsy, all male rats utilized in this study were in good general health. No exposure-related gross lesions were noted during necropsy. No exposure-related differences were noted in clinical chemistry or hematologic parameters measured at both the 30-day and 60-day postexposure sacrifices. Protein analyses of tissues are currently in progress. Absolute organ weights and organ-to-body weight ratios were also not statistically significantly different between all eight groups of male rats at each postexposure sacrifice.

Histopathology

No significant differences between air-only control and JP4/S/OD animals have been noted for tissues which have received complete histopathological examination. Many tissues are still undergoing histopathological evaluation.

Neurotransmitter Analysis

Significant increases in serum 5-hydroxyindoleacetic acid and 5-hydroxytryptamine levels were present in the A/S, JP4, JP4/S, and JP4/S/OD animals sacrificed 30-days postexposure (MacMahon et al., 1997b). Brain neurotransmitter level analyses for all animals and serum neurotransmitter analyses for the animals sacrificed 60-days posttreatment are currently being evaluated.

DISCUSSION

Repeat exposure of male rats to air only, or JP-4 jet fuel vapor, with or without the combination of stress, oral PB, and dermal DEET, resulted in no mortality. No clinical signs of toxic stress were noted during exposure or postexposure. No treatment-related decreases in animal body weights were observed over the 2-week exposure period, nor during the 30- or 60-day postexposure periods. No absolute organ or organ-to-body weight differences were noted for any of the eight treatment groups. No significant histopathology findings have been noted for tissues with completed evaluations.

The statistically significant findings from the neurotransmitter analyses of serum from Simulated Persian Gulf War exposure male rats sacrificed 30-days postexposure were increases in 5-hydroxyindoleacetic acid and 5-hydroxytryptamine levels in four groups of rats. Neurobehavioral deficits have been found for some groups of animals sacrificed at 30-days postexposure.

Many parameters measured during this study are still undergoing evaluation and analysis. A complete report of the findings from this study will be published as an Armstrong Laboratory Technical report, MacMahon et al., 1997a.

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SECTION 6

ENVIRONMENTAL INITIATIVE PROJECT

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6.1 EXPERIMENTAL CALIBRATION OF BIOLOGICALLY BASED DOSE-RESPONSE MODEL FOR LIPID PEROXIDATION INDUCED BY TRICHLOROETHYLENE

J.Z. Byczkowski, C.R. Miller¹, M.A. Curran², W.J. Schmidt², and S.R. Channel²

ABSTRACT

A biologically based pharmacodynamic (BBPD) dose-response model was developed to simulate chemically induced lipid peroxidation in precision-cut mouse liver slices *in vitro*. The model was written in Advanced Continuous Simulation Language (ACSL) and simulations were performed using SIMUSOLV software on a VAX/VMS mainframe computer. The BBPD model simulated formation of lipid hydroperoxides and thiobarbituric acid reactive substances (TBARS) over time as a function of the remaining amount of cytochrome P450 (CYP)-activated chemical inducer and the remaining amount of antioxidants. The rate of peroxidation was controlled by lipid peroxidizability, destruction of CYP, autoxidation, and activity of glutathione peroxidase. The BBPD model was initially calibrated with the literature data for TBARS formed during lipid peroxidation in rat liver slices and in precision-cut mouse liver slices treated with tert-butyl hydroperoxide. Then, the biochemical parameters were optimized to reflect physiology of the mouse liver and the BBPD model was used to simulate TBARS formation during trichloroethylene (TCE)-induced lipid peroxidation in mouse liver slices. Two basic algorithms for production of free radicals from trichloroethylene were tested, namely, linear and square root. Predictions by the BBPD model which related free radical concentration to the square root of the initial TCE concentration were in agreement with the experimental data employing TBARS as an end point.

INTRODUCTION

Many chemicals entering metabolic pathways of the living cell generate free radicals (Byczkowski and Gessner, 1988) and are known to cause oxidative stress (Byczkowski and Channel, 1996). Several enzymatic and nonenzymatic systems may be involved in this process (Manson, 1992). For instance, transition metals may catalyze production of primary peroxyl and alkoxyl radicals and reactive oxygen species (O_2 , H_2O_2 , OH, ' ΔgO_2) from simple inorganic and organic hydroperoxides (Kulkarni and Byczkowski, 1994); NADPH:cytochrome P450 reductase may catalyze redox cycling of several quinones, aromatic imines, etc., yielding the respective semiquinones and reactive oxygen species (Byczkowski and Gessner, 1988); cytochrome P450 (CYP) may

¹Army Medical Research Unit, Wright-Patterson Air Force Base, OH.

²Armstrong Laboratory, Toxicology Division, Wright-Patterson Air Force Base, OH.

convert many organic compounds, including some chlorinated hydrocarbons, to carbon-centered free radicals whose interaction with oxygen can generate, in turn, secondary peroxyl and alkoxyl radicals (Roberfroid and Calderon, 1995). Also, several chemicals, including heavy metal compounds, may inactivate cellular antioxidants and free radical scavengers, leaving the cell vulnerable to the substantial amounts of free radicals produced during normal, physiological, aerobic function (Poulsen and Loft, 1995). When the antioxidant defense system becomes overwhelmed, the primary and secondary free radicals may interact with cellular macromolecules causing enzyme inhibitions, derepression of genes, DNA damage, stimulation of DNA repair, and initiation of lipid peroxidation. This last process may lead to production of tertiary free radicals, derived from polyunsaturated fatty acids (peroxyl, alkoxyl, and alkenyl radicals). Lipid peroxidation may further propagate as a self-sustaining chain reaction. Aldehydes (e.g., malondialdehyde), alkanes (e.g., ethane), and lipid hydroperoxides are produced as stable end products, and a measurement of the rate of their production may serve as an index of lipid peroxidation (Weber, 1990). Colorimetric or fluorimetric determination of malondialdehyde and other material similar to malondialdehyde (thiobarbituric acid reactive substances - TBARS) is the most popular method for measurement of lipid peroxidation (Roberfroid and Calderon, 1995; Janero, 1990).

Several reports have been aimed at aquantitative description or mathematical modeling of lipid peroxidation and production of TBARS in tissues (Tappel et al., 1989; Babbs and Steiner, 1990; Antunes et al., 1994; Suzuki and Ford, 1994; Vroegop et al., 1995), but to date, only the mathematical description by Tappel et al. (1989) has been successfully incorporated into a computerized, biologically based pharmacodynamic (BBPD) model (Byczkowski et al., 1995). Initially, the BBPD model was experimentally calibrated using our data from mouse liver slices treated with tert-butyl hydroperoxide (Byczkowski et al., 1996). In this report, a further validation of the BBPD model is described using precision-cut mouse liver slices induced with trichloroethylene (TCE).

MATERIALS AND METHODS

The model was written in Advanced Continuous Simulation Language (ACSL; Mitchell and Gauthier, 1993) and simulations were performed using SIMUSOLV software with optimization capabilities (DOW Chemical Co., Midland, MI) on a VAX/VMS mainframe computer.

Precision-cut slices were prepared from livers of $B_6C_3F_1$ male mice (Charles River Breeding Laboratories, Kingston, NY) and maintained using the dynamic roller culture method (Sipes et al., 1987; Brendel et al., 1993), and also described by Byczkowski et al. (1995). The mice were provided with Purina Formulab #5008 and *Pseudomonas*-free softened water *ad libitum*. The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Academy Press, 1996, and the Animal Welfare Act of 1966, as amended.

The mice were euthanized with CO₂, their livers were removed and placed in ice-cold Sacks buffer (containing $KH_2PO_4 0.75 g/L$, $K_2HPO_4 9.5 g/L$, NaHCO₃ 1.2 g/L, KHCO₃ 0.6 g/L, mannitol 37.5 g/L, and MgCl₂; pH 7.4). Liver cores, 8-mm diameter, were prepared and sliced in ice-cold Sacks buffer using a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL) (Brendel et al., 1993; Krumdieck et al., 1980). The slices were loaded on rollers (two slices per roller) in ice-cold Sacks buffer. The rollers were then placed in scintillation vials containing 1.7 mL of Waymouths MB 752/1 media at 37 °C (Formula #78-5107EC, without phenol red, pH 7.4, Gibco BRL, Grand Island, NY) supplemented with NaHCO₃ 1.3 g/L, HEPES 2.38 g/L, NaCl 0.292 g/L, l-glutamine 0.35 g/L, gentamycin sulfate 50 mg/L, and 10% fetal bovine serum (Hyclone, Logan, UT), and capped with a scintillation vial cap with ¼ in hole for gas exchange. The vials were placed in a Dynamic Roller Culture Incubator (Vitron, Tucson, AZ) and gassed with 95% O₂/5% CO₂ for a 2-h preincubation period.

After a 2-h preincubation period, the rollers were removed from the vials, placed into prewarmed, sealed vials containing fresh media (pH 7.4), and dosed through the septa with TCE vapors at the desired final concentration. The dosed vials containing rollers were then returned to the roller culture incubator. Final concentrations of TCE in the media were calculated using a partition coefficient of $1.94 (\pm 0.17, n=18)$ determined for the equilibrated medium/air system at 37 °C. Zero time controls were processed immediately. Then, the vials were removed at intervals over a 2-h incubation. Slices were weighed and sonicated in their own media. Aliquots of each sample were removed for TBARS assay and protein content measurements. Samples for TBARS assay were added to ice-cold D-PBS/GSH/EDTA buffer (pH 7.4) containing 20 mg reduced GSH and 48 mg EDTA in 100 mL D-PBS (Dulbeco's buffer; Gibco BRL, Grand Island, NY).

Lipid peroxidation was measured by the formation of TBARS, employing the fluorescence spectrophotometry of solvent tissue extracts (Janero, 1990). Essentially, in this assay the aldehyde products generated by splitting the endoperoxide alkoxyl radicals (mostly malondialdehyde, MDA, formed during the peroxidation of unsaturated fatty acids) react with thiobarbituric acid (TBA) to yield a 1:2 MDA:TBA red, fluorescent, complex (Janero, 1990). Incubation of liver slices without chemical inducer did not increase significantly the fluorescence for up to 2 h. Since the control values for liver slices at time zero were subtracted from the results, under conditions of the assay, the determined amount of MDA:TBA complex reflected the extra amount of lipid hydroperoxides produced in addition to the normal physiological level.

At 1 h and 2 h of incubation, the samples of liver slices (control and treated) were removed for viability analysis. The viability was assessed from lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) leakage, and intracellular potassium content. The enzyme leakage was determined using a Kodak Ektachem Analyzer (model 700XR) for aminotransferase activities and DuPont acaV for dehydrogenase activity. An acceptable enzyme leakage level for precision-cut liver slices was assumed to be less than 20% of the total content of enzymatic activity. Potassium content in sonicated tissue samples was determined using an AVL

982-S Electrolyte Analyzer (Roswell, GA). The acceptable level of intracellular potassium content in precision-cut liver slices was assumed to be greater than 35 mM K⁺/g wet weight. If the average viability tests of either control or treated liver samples did not meet the above acceptable levels, the experimental results were discarded. All chemicals used in this study were of analytical grade.

RESULTS AND DISCUSSION

Based on the mathematical description by Tappel et al. (1989), a BBPD model was developed to simulate chemically induced lipid peroxidation in precision-cut mouse liver slices *in vitro* (Byczkowski et al., 1995; 1996). The model described lipid peroxidation and production of TBARS over time and allowed for dose-dependent simulations of TBARS versus concentration of chemical inducer. According to the time-dependent description of lipid peroxidation by Tappel et al. (1989), a chemical inducer is being activated by CYP in a way directly proportional to the concentration of the remaining inducer. Although this description gave quite realistic time-dependent simulations of TBARS production in BrCCl₃-induced rat liver slices, the dose-dependent predictions for tert-butyl hydroperoxide in mouse liver slices were less accurate (Byczkowski et al., 1996). Because the activated chemical inducer capable of initiating lipid peroxidation is probably a free radical (Roberfroid and Calderon, 1995; Gardner, 1989), two algorithms describing the relationship between steady-state concentration of free radicals and local TCE concentration were tested:

(i) Linear algorithm

 $k_i \qquad k_t$ TCE \rightarrow FRAD \rightarrow Nonradical products assumption: dFR/dt = $k_i * C_L - k_t * FRAD = 0$

$$FRAD = k_i * C_L/k_t$$

where: FRAD is a steady state concentration of TCE-derived free radicals [mM]; C_L is local TCE concentration [mM]; k_i is a rate constant of free radical formation from TCE [1/mM/h]; k_t is the lumped rate constant of free radical recombination and quenching by the biosystem [1/mM/h].

(ii) Square root algorithm

 $\begin{array}{ccc} k_{i} & k_{t} \\ TCE \rightarrow FRAD + FRAD \rightarrow Nonradical \ products \\ assumption: \ dFR/dt = k_{i} * C_{L} - k_{t} * FRAD * FRAD = 0 \end{array}$

 $FRAD = \sqrt{k_i * C_L/k_t}$

Quantitative measurements of FRAD *in vitro* using an EPR-spin trapping method failed to confirm either algorithm (Figure 6.1-1).



Figure 6.1-1. Calibration of the algorithm describing concentration of free radicals under steady-state conditions (FRAD; Byczkowski and Flemming, in press) with experimental data of Steel-Goodwin et al. (1995) for free radical generation by different concentrations of TCE [μmole/g] in mouse liver slices using an EPR/spin-trapping method. FRAD - concentration of PBN-reactive free radicals [μmole/g liver]. The squares represent actual average experimental data points (after subtraction of physiological background level of free radicals produced in the absence of TCE; Steel-Goodwin *et al.*, 1995). The continuous lines are computer-generated simulations (with sub-model described by Byczkowski and Flemming, 1995) involving: A - linear algorithm; B - square root algorithm.

However, an attempt to calibrate the relationship between concentration of TCE (the chemical inducer) and free radicals generated in mouse liver slices, measured by Steel-Goodwin et al. (1995) using an electron paramagnetic resonance (EPR) spectroscopy, turned out to be inconclusive. Both algorithms, linear and a square root, gave equally poor fits to the experimental data (Figure 6.1-1).

It was demonstrated that TCE and its metabolites generate free radicals *in vitro* (Gonthier and Barret, 1989; Ni et al., 1994) and are capable of inducing lipid peroxidation both *in vitro* (Tse et al., 1990) and *in vivo* (Larson and Bull, 1992). An EPR/spin-trapping study in precision-cut mouse liver slices incubated with TCE revealed the formation of carbon-centered free radicals (Steel-Goodwin et al., 1995). While the quantity of free radicals increased with increasing concentration of TCE (Figure 6.1-1), their identity remained unknown (Steel-Goodwin et al., 1995). Thus, lipid peroxidation caused by an accompanying TCE metabolic activation to free radicals amplified but also obliterated the original free radical signal with the lipid-derived signal. The spin trap used (PBN) interacts to some degree with any lipid-derived free radical, alkoxyl, peroxyl, etc., no matter whether primary, secondary, or tertiary, making the EPR/spin trapping method of questionable value for quantitative verification of the relationship between concentration of TCE and free radicals generated in liver slices.

The result of time-dependent simulation by the BBPD model of TBARS production in precision-cut mouse liver slices induced with TCE is shown in Figure 6.1-2.



BBPD: Time-Dependent Production of TBARS After TCE

Figure 6.1-2. Results of time-dependent simulation of lipid peroxidation in mouse liver slices, induced by 1 mM TCE. TBARS - thiobarbituric acid reactive substance x 10^3 [µmole/0.1 g liver]. Small squares depict average experimental data from our laboratory (n=4), as described by Byczkowski et al. (1996). The continuous line depicts computer simulations with BBPD model involving the linear algorithm and parameters optimized by SIMUSOLV software (ANOX1 = 0.0037; PTIND1=3.62, INDLF=0.0001, ACTDGF=0.0014), amount of TBARS at time=0 was subtracted from the data.

The linear algorithm was used to link the concentration of inducer with its active free radical form. As the same concentration of the chemical inducer (1 mM TCE) was used at different timepoints, the computer-generated predictions (Figure 6.1-2, continuous line) reasonably followed the experimental data (Figure 6.1-2, small squares), despite the uncertain stoichiometry of the inducer activation process. However, the same linear algorithm gave a poor fit when used to simulate the dose-dependent effects of TCE on TBARS generation (Figure 6.1-3a). Increasing the "potency of inducer" parameter (PTIND1) in the BBPD model did not solve the problem, as the shape of the dose-dependent curve was too flat. The fit was improved dramatically when this algorithm in the BBPD model was substituted with a square root algorithm (Figure 6.1-3b). It seems, thus, that



BBPD: Dose-Dependent Simulations of TBARS Production

Figure 6.1-3. The results of dose-dependent simulations of lipid peroxidation in mouse liver slices for 0.5 h, induced by different concentrations of TCE [μ mole/g liver]. TBARS - thiobarbituric acid reactive substance x 10⁻³ [μ mole/0.1 g liver]. Small squares depict average experimental data from our laboratory (n=4). Lines depict computer simulations with BBPD model involving: A - linear algorithm (PTIND1=6.9); B - square root algorithm (PTIND1=250). The other parameters were optimized by SIMUSOLV software as described earlier by Byczkowski et al. (1996). Amounts of TBARS in untreated controls were subtracted from the data.

activation of TCE to its free radical derivatives in mouse liver follows the stoichiometry in which free radical concentration is proportional to the square root of the initial concentration of TCE, as discussed Byczkowski and Flemming (in press). This algorithm seems to be justified, as two unpaired electrons with opposite spins eventually must be formed from each split pair of electrons, and this should yield two free radicals per one molecule of the chemical inducer (Vroegop et al., 1995). The improved BBPD model also required an increase in the PTIND1 parameter, under square root, to 250 mmole per 0.1 g liver. Predictions by this improved BBPD model, which related free radical concentration to the square root of the initial TCE concentration, were in agreement with both time- (Figure 6.1-2) and dose-dependent (Figure 6.1-3) experimental data employing TBARS as an end point.

Quantitative measurements of TBARS *in vitro* confirmed an adequate description of the relationship between concentration of free radicals and local TCE concentration by the square root algorithm (Figure 6.1-3).

Availability

The source codes of original *.CSL and *.CMD files are archived in PBPK-L Public Domain Source Library and are accessible through the World Wide Web at the following URL: http://www.navy.al.wpafb. af.mil/new.htm. Copies of the computer program may be obtained for non-commercial use by sending an e-mail request to the following Internet address: JBYCZKOWSKI@AL.WPAFB.AF.MIL.

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6.2 DOSE-RESPONSE CHARACTERISTICS OF LIPID PEROXIDATION INDUCED BY BROMOTRICHLOROMETHANE IN $B_6C_3F_1$ MICE

J.Z. Byczkowski, M.A. Curran¹, C.R. Miller², and W.J. Schmidt¹

ABSTRACT

In this report, the relationship between dose of bromotrichloromethane (BrCCl₃) and its effect on lipid peroxidation was studied *in vitro* and *in vivo*, using the generation of thiobarbituric acid reactive substances (TBARS) in liver slices *in vitro* and the exhalation of ethane by B6C3F1 mice *in vivo*. BrCCl₃ enhanced lipid peroxidation both *in vitro* and *in vivo*. Lipid peroxidation products increased non-linearly with increasing concentration of BrCCl₃ and increasing time of incubation, and the dose-response curves had a characteristic sigmoidal shape. The results suggest that the extent of chemically induced oxidative stress and effects of lipid peroxidation are linked with both time and dose of BrCCl₃ by a non-linear function. Therefore, considering quantitatively biological effects or modeling *in silico* (in a silicone chip - computer simulated) health effects of bromochloromethanes, both variables (time and dose) must be taken into account for realistic predictions and risk characterization.

INTRODUCTION

Chemically induced oxidative stress causes derangement of antioxidant mechanisms in tissues, may lead to lipid peroxidation, and may result in cell injury. Lipid peroxidation is a common result of oxidative stress, induced in biological systems by several pro-oxidant chemicals, including chlorinated hydrocarbons and their metabolites (Byczkowski and Channel, 1996). Bromotrichloromethane (BrCCl₃) is one of these chlorinated hydrocarbon solvents which was extensively investigated for its pro-oxidant properties as well as for its ability to induce lipid peroxidation *in vitro* and *in vivo* (Burk and Lane, 1979; Fraga et al., 1988). Bromochloromethanes are ubiquitous environmental pollutants, detected in groundwater through the U.S. Air Force installation restoration program (IRPIMS 1991), and thus a dose-response characterization of their biological effects is essential for the Air Force operations.

In this report, the relationship between dose of $BrCCl_3$ and its effect on lipid peroxidation was studied *in vitro* and *in vivo*, using the generation of thiobarbituric acid reactive substances (TBARS) in liver slices *in vitro* and the exhalation of ethane by B6C3F1 mice *in vivo* as measured end points, specific to lipid peroxidation (Figure 6.2-1).

¹Armstrong Laboratory, Toxicology Division, Wright-Patterson Air Force Base, OH. ²Army Medical Research Unit, Wright-Patterson Air Force Base, OH.



Figure 6.2-1. A simplified scheme of lipid peroxidation reactions which lead to production of TBARS and to generation of ethane (according to Sagai and Ichinose, 1980).

MATERIALS AND METHODS

Precision-cut liver slices were prepared from $B_6C_3F_1$ male mice (Charles River Breeding Laboratories, Kingston, NY) and maintained using the dynamic roller culture method (Brendel et al., 1987; 1993), as described in detail by Byczkowski et al. (1995). After a 2-h preincubation period, the rollers were removed from the vials and placed into pre-warmed vials containing the media (pH 7.4) dosed with BrCCl₃ at the desired final concentration. The dosed vials containing rollers were then returned to the roller culture incubator. Zero time controls were processed immediately. Then, the vials were removed at 20-min intervals over an incubation (up to 2 h). Slices were weighed and sonicated in their own media. Aliquots of each sample were removed for TBARS assay and protein content measurements. Samples for TBARS assay were added to ice-cold D-PBS/GSH/EDTA buffer (pH 7.4) containing: 20 mg reduced GSH and 48 mg EDTA in 100 mL D-PBS (Dulbeco's buffer; Gibco BRL, Grand Island, NY).

Lipid peroxidation *in vitro* was measured by the formation of TBARS, employing the fluorescence spectrophotometry of solvent tissue extracts (Janero, 1990) as described by Byczkowski et al. (1996). Under conditions of the assay, the amounts of MDA:TBA complex, and thus color and fluorescence intensity, are stoichiometrically dependent on lipid hydroperoxides and the rate of their production corresponds to the rate of lipid peroxidation (Valenzuela, 1991).

At 1 h and 2 h of incubation, the samples of liver slices (control and treated) were removed for viability analysis. The extent of necrosis was estimated from lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT) leakage, and intracellular potassium content. The enzyme leakage was determined using a Kodak Ektachem Analyzer (model 700XR) for aminotransferase activities and DuPont acaV for the dehydrogenase activity. Acceptable enzyme leakage level for precision -cut liver slices was assumed to be less than 20% of the total content of enzymatic activity. Potassium content in sonicated tissue samples was determined using an AVL 982-S Electrolyte Analyzer (Roswell, GA). The acceptable level of intracellular potassium content in precision-cut liver slices was assumed to be greater than 35 mM K⁺/g wet weight. If the average viability tests of liver samples did not meet the above acceptable levels, the experimental results were discarded.

Ethane exhalation was measured *in vivo* as described earlier by Byczkowski and Seckel (1996). Briefly, five $B_6C_3F_1$ male mice were placed in a 0.75 L closed gas chamber containing 50 grams of soda lime that absorbs CO_2 . Chamber oxygen concentration was monitored (MDA oxygen analyzer, MDA Scientific, Lincolnshire, IL) and kept at a range of 20-21.5% throughout the experiment. For each group of mice, a baseline ethane exhalation was measured for 2 h (physiological control level). Then, the animals were treated intraperitoneally (i.p.) with an appropriate dose of BrCCl₃ (Sigma Chemical, St. Louis, MO) dissolved in 0.2 mL of mineral oil. Immediately after treatment, ethane exhalation was monitored using a closed gas uptake system. (Gargas et al., 1986).

Samples of chamber atmosphere were collected using an automatic sampling valve (1 mL sample loop) connected to a Hewlett Packard 5890 gas chromatograph (GC), equipped with an HP 3396A integrator and connected to the
computer network with a PE Nelson 900 series interface. Ethane was separated from other respiratory gases by a 6 ft. x 1/8 in. stainless steel column packed with Chromosorb 102, 80-100 mesh (Alltech, Deerfield, IL). The column temperature was 50 °C, and the injector and flame ionization detector temperatures were 125 °C and 200 °C, respectively. A flow rate of 20 mL/min was set for the nitrogen carrier and the air *plus* hydrogen flow rate was 405 mL/min. A Turbochrom® software was used to estimate area under the ethane peak [counts, μ V*s], and ethane concentrations were calculated using a calibration curve prepared with ethane standards.

The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Academy Press, 1996, and the Animal Welfare Act of 1966, as amended.

All chemicals used in this study were of analytical grade.

RESULTS

Bromotrichloromethane stimulated TBARS generation by precision-cut mouse liver slices *in vitro* (Figure 6.2-2). The dose-response curves had a characteristic sigmoidal shape with plateau reached at about 1 mM BrCCl₃ when incubated for 0.5 h, and at about 0.5 mM BrCCl₃ when incubated for 1 h, respectively. A prolonged incubation (above 1 h) with higher than 0.5 mM concentrations of BrCCl₃ failed to further increase the rate of TBARS generation by liver slices (results not shown here).

Figure 6.2-3 shows the results of measurement of ethane exhalation by mice in a closed gas chamber. The treatment with $BrCCl_3$ at a dose above 0.025 g/kg resulted in increased ethane exhalation measured 1 h after the exposure. A dose twice as high was required to produce a significant increase in ethane exhalation after 0.5 h from the exposure. Again, the dose-response curves had a sigmoidal shape.

DISCUSSION

Riely et al. (1974) have demonstrated that chlorinated hydrocarbons, such as carbon tetrachloride (CCl₄), increase ethane exhalation by mice due to lipid peroxidation *in vivo*. Ethane exhalation by animals treated with CCl₄ was enhanced by pretreatment with phenobarbital and suppressed by α -tocopherol (vitamin E), and it was suggested that the formation of carbon trichloromethyl free radical (CCl₃) by cytochrome P450 (CYP) in the liver was responsible for initiation of lipid peroxidation (Riely et al., 1974). Bromotrichloromethane, another pro-oxidant chemical from which CYP generates CCl₃, increased ethane



Figure 6.2-2. Effects of different doses of BrCCl₃ (concentration in the medium [mM]) on production of TBARS (µmole/g liver) by mouse liver slices incubated for either 0.5 or 1 h in the presence of inducer. The respective background values were subtracted from data points. Background production of TBARS in controls were respectively: 0.0226 µmole/g liver (± 0.0025 S.D., n=4) at 0.5 h, and 0.0303 µmole/g liver (±0.0035 S.D., n=4) at 1 h. Data points were significantly different from the corresponding controls (at p≤0.05, n=4) by Student's t-test.

exhalation in rats, especially in vitamin E- and selenium-deficient group (Burk and Lane, 1979). However, the same authors postulated also that CCl_4 - and $BrCCl_3$ -induced lipid peroxidation does not necessarily correlate with liver necrosis (Burk and Lane, 1979). Since then, the ethane exhalation test was proven to be a reliable, non-invasive index of oxidative stress *in vivo* (Jeejeebhoy, 1991; Kneepkens et al., 1994). On the other hand, despite causing lipid peroxidation as a relatively early event, oxidative stress may actually stimulate cellular proliferation, induce apoptosis, and, at a very high dose of pro-oxidant chemical only, it may cause necrosis (Byczkowski and Kulkarni, 1996). Because chemically induced oxidative stress depends on a dose of pro-oxidant chemical, it seemed essential in our study to follow the effects of a range of doses of BrCCl₃ on lipid peroxidation rather than to check only a time-course at one effective dose, as other authors have done (Riely et al., 1974; Burk and Lane, 1979).



Figure 6.2-3. Effects of different doses of BrCCl₃ (injected i.p. [g/kg]) on ethane exhalation (concentration in the chamber [p.p.m.]) by five mice during either 0.5 or 1 h from the exposure to inducer. The respective background values from the same group of animals before treatment were subtracted from the data points. Physiological ethane exhalation by groups of five mice (controls) were respectively 0.0435 p.p.m. (±0.022 S.D., n=5) at 0.5 h, and 0.0447 p.p.m. (±0.020 S.D., n=4) at 1 h. The ethane exhalation time-courses after exposure to 0.05 (after 1 h but not 0.5 h), 0.1, and 1.0 g BrCCl₃/kg were significantly different from the corresponding control curves before treatment (at p≤0.05) by one-way Anova.

From our *in vitro* experiments, presented in Figure 6.2-2, it appeared that lipid peroxidation is both a time- and dose-dependent phenomenon; TBARS generation increased non-linearly with both increasing concentration of BrCCl₃ and increasing time of incubation. However, liver slices incubated with the highest BrCCl₃ concentration (1.5 mM) for 1 h generated slightly less TBARS than with the lower, 1 mM concentration of BrCCl₃. This decrease is unlikely to be caused by the necrotic action of a high dose of BrCCl₃ acting for a prolonged time, since the liver slices were screened for signs of necrosis after the incubation. More probable is a mechanism of CYP suicide-inhibition, caused by the accumulated damage to the enzyme by CCl_3 .

Similarly, $BrCCl_3$ administered *in vivo* showed non-linear time- and dose-dependent characteristics of lipid peroxidation stimulation, measured by ethane exhalation. This appeared from the data presented in Figure 6.2-3 that the maximum no-effect dose of $BrCCl_3$ of about 0.05 g/kg, when measured half an hour after the exposure, dropped to almost 0.025 g/kg after one hour from the exposure. On the other hand, the maximum stimulation of ethane exhalation, measured 1 h after the exposure, was reached at 0.1 g/kg of $BrCCl_3$, while ten times as high a dose of $BrCCl_3$ was still not saturable when measured half an hour earlier (Figure 6.2-3). Even more dramatic differences were noted 2 h after the exposure (results not shown here); at 1g /kg of $BrCCl_3$, the ethane exhalation was still at the plateau level (0.33 p.p.m.), while a dose ten times lower (0.1 g/kg of $BrCCl_3$) caused twice as high stimulation of ethane exhalation (0.68 p.p.m.).

These results suggest that the extent of chemically induced oxidative stress and effects of lipid peroxidation are linked with both time and dose of $BrCCl_3$ by a non-linear function. The non-linearity may result from limited supply of antioxidants (threshold) and from accumulated with time free radical insult to CYP enzymatic system (suicidal inhibition). Therefore considering biological effects or modeling *in silico* health effects of bromochloromethanes, both variables (time and dose) must be taken into account for realistic predictions and risk characterization.

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SECTION 7

ADVANCED COMPOSITE MATERIALS PROJECT

7.1 SMOKE PRODUCTION AND THERMAL DECOMPOSITION PRODUCTS FROM ADVANCED COMPOSITE MATERIALS

D.L. Courson, C.D. Flemming, K.J. Kuhlmann, J.W. Lane, J.H. Grabau, C.R. Miller¹, J.M. Cline¹, B.J. Larcom², and J.C. Lipscomb²

ABSTRACT

This report describes the smoke production and thermal decomposition products from the combustion of Advanced Composite Materials (ACM) used on high performance aircraft. There are three distinct project phases, however, only Phase I is described in this report. The preliminary work (Phase 0) addressed the physical behavior of burning composites, such as mass loss rates, smoke plume dispersion, and the environmental impact of a burning aircraft.

Phase I involved applying methods developed in Phase 0 to chemical and morphological characterization of the smoke produced in a small-scale wind tunnel. Results from these experiments and analytical findings are presented in this report. The combustion conditions were selected to represent a range of real-world scenarios to evaluate potential health effects from exposure to smoke and thermal decomposition products generated during burning of ACM. An analytical protocol for evaluating the characteristics of smoke produced from controlled combustion of test materials was developed. The combustion apparatus used was a modified form of the cone heater combustion module of the University of Pittsburgh mini-cone calorimeter (UPITT II) method developed at the University of Pittsburgh.

Phase II is currently underway and will describe the *in vivo* effects of acute exposure to smoke from burning composite materials.

INTRODUCTION

The demand for lightweight materials of high strength and improved thermoplastic properties (rigidity, conductivity, thermal expansion) has resulted in the extensive development and application of Advanced Composite Materials (ACM). These materials generally consist of a reinforcing fiber matrix incorporated into an epoxy resin

¹Army Medical Research Unit, Wright-Patterson Air Force Base, OH.

²Armstrong Laboratory, Toxicology Division, Wright-Patterson Air Force Base, OH.

binder (Figure 7.1-1). The current use of graphite-based composites is increasing as manufacturers exploit the unique advantages of this structural material. However, their flammability characteristics and performance are significantly different from their metal counterparts. Use of new synthetic materials, such as ACM, raises concerns about the potential environmental and human health risk resulting from exposure to the chemically complex smoke produced by burning these materials. Although ACM appear to present no danger to human health in their original state, the chemical transformation of this material during combustion is not well characterized. The resins used in binder



Figure 7.1. Typical Cross-Section of Advanced Composite Material

Figure 7.1-1. TYPICAL CROSS-SECTION OF ADVANCED COMPOSITE MATERIAL

material may release potentially harmful gases, vapors, or fibrous materials when burned. Similar situations could occur during operational conflicts for flight line personnel and crew members.

Carbon fiber composites were introduced as a substitute for fiberglass and were found to be stronger than either fiberglass or aluminum panels. The use of carbon composites in aircraft is increasing as manufacturers learn to use the strengths of ACM. Advanced filamentary composite materials provide many advantages over metals and also offer added fatigue and corrosion resistance, controlled thermal expansion, dimension stability, and the ability to form complex-shaped parts without increasing the potential for fatigue.

Two distinct categories of matrix resins are used in advance composite: thermosets and thermoplastics. Historically, continuous fiber reinforced composite applications have been dominated by matrices consisting of thermosetting resins such as epoxies and bismaleimides (BMI). Epoxy resin polymers are known for their superior properties. These include good bonding to several substrates, good chemical resistance, and a wide availability of form to meet the requirements of the manufacturer. In addition, thermosets burn for a shorter period and char rather then soften or melt like a thermoplastic. High performance thermoplastic matrix materials provide alternatives to the

thermosetting matrix composite for aerospace applications. Thermoplastic resins are stable, high molecular weight polymers that retain their chemical stability throughout processing (no chemical reactions occurs during processing). Toughness, durability, strength, and reparability are the driving forces for adapting thermoplastics for use in advanced composites.

Military aviation mishaps have resulted in illness to fire safety personnel as a result of exposure to toxic gases from burning composite materials (Ohlemiller et al., 1993). During an aircraft mishap, the composite structures are subjected to forces that cause them to break into pieces, burn, and subsequently release aggregates or particulates to the local environment. The reinforcement fibers, which are very stiff and give the composite its strength, may be dismembered into smaller elements of varying size causing potential respiratory hazard(s).

The fire hazards of organic composites are not well understood but depend on many factors including actual conditions of use (e.g., geometry and orientation), proximity of other materials, environmental conditions, ignition sources, as well as the intrinsic properties of the composite such as chemical composition, thermal stability, and heat transfer characteristics. Expected variations in decomposition rates will substantially affect the constituency and concentrations of combustion products. Particle distribution and rate of release may also depend on local environmental conditions (Babrauskas et al., 1987). Evidence also indicates that increased smoke and toxic gas generation rates result from conventional attempts to incorporate self-extinguishing or fire retarding formulation in the matrix materials. Under conditions of sustained heat flux and great amounts of entrained air, the ACM will burn more vigorously, generating very high heat energy, smoke, and toxic gas concentrations.

MATERIALS AND METHODS

Materials

A carbon fiber/modified bismaleimide resin ACM (approximate 2:1 ratio by weight) was used in these studies. Specimens were 108 mm square by 2.5 mm thick with a mass of 53.90 ± 0.36 g.

Preliminary Investigation

A modified UPITT II cone calorimeter (Fire Testing Technology Inc.) was utilized in the collection of ACM combusted material for initial analysis (refer to AL/OE-TR-1996-0124). Two samples of combustion products were collected and analyzed in a Perkin-Elmer 910 GC/MS (Gas Chromatograph/Mass Spectrometer) system (see Appendix A, for GC/MS conditions). The first sample was a portion (vapor) of the smoke plume caught in a cold trap, extracted with methylene chloride, and concentrated with a stream of dry nitrogen. The cold trap consisted of a glass impinger in an ice/water bath connected to the end of the calorimeter train. A total of 150 liters (10 L/min for 15 min) of the combustion gas was drawn through the trap. The second sample of deposited soot was collected on a glass wool filter. Soot is defined as a fine particulate material created during combustion and deposited

throughout the apparatus. The standard method for extraction (Soxhlet) could not be employed due to the particle size. Instead, a glass pipette plugged with glass wool was used. The apparatus was cleaned with 50:50 methylene chloride: acetone, the soot added, and the same solvent mix was passed through the soot until no more color was noticed in the solvent as it drained from the pipette. The extract was concentrated to approximately 1 mL by a stream of dry nitrogen and then made up to 20 mL with methylene chloride.

Compound identification was performed quantitatively using the latest available NIST reference library and approximate quantification was performed under EPA Method 8270 protocols. Identification was conducted as a qualitative assessment for preliminary investigation.

Combustion Apparatus & Tunnel Design

A protocol to evaluate the combustion products of ACM was developed using a modified UPITT II method, which permits control of heat flux, (Q), and airflow, (V), the fundamental variables determining combustion conditions, and simultaneously measures mass loss rate (Caldwell et al., 1990a, Miller et al., 1995). A mini-cone calorimeter, similar to that used in the UPITT II combustion apparatus, was used to simulate real world exposure scenarios with experimentally controlled combustion conditions. The mini cone calorimeter (Fire Testing Technology, Inc.) consisting of a truncated cone-shaped heating element which was used to irradiate the ACM sample (approximately 0.01 m² ACM "coupon") at selected heat flux levels. A load cell was incorporated into the sample platform, allowing approximate measurement of mass loss during controlled combustion. The entire apparatus was contained in a ventilated 16 ft² plexiglas containment unit.

A Proportional Integral Derivative (PID) device (Eurotherm) controlled the temperature of the cone calorimeter and was positioned on top of the containment unit. Airflow through the system was maintained by pulling air (unfiltered) through the cone calorimeter and sampling train. Smoke exited the UPITT II via a 3 inch stainless steel tube which was immediately reduced to 2.5 inch. The 2.5-inch tube incorporated a 90° bend and was flanged to an apparatus utilized for collection of gas samples (refer to AL/OE-TR-1996-0124). Main body of flow continued into a 12.5- inch diameter CPVC duct 40 ft in length. Five sampling ports were installed at 10 ft intervals along the length of the tunnel to allow for direct sampling of the smoke plume (refer to AL/OE-TR-1996-0124). Stainless steel tubes ran from the sampling port to the center of the tunnel and were bent 90° into the air stream. Access ports (6-inch holes) were drilled into the tunnel and sealed with removable plugs for ready access to the sampling devices inside the tunnel. Exhaust from the tunnel was pulled through a high efficiency particulate air filter and mist air scrubber and vented into a laboratory hood.

Twenty smoke tests were conducted as part of a cross-classified design of three levels of Q, 38.5, 57.2, and 84.2 kilowatts per square meter (kW/m2) corresponding to 625, 770, and 880 °C, and four levels of V, 340, 370, 400, and 650 liters per minute (L/min). Thermocouples were placed at 10-ft intervals (sampling ports) along the tunnel and

positioned in the center of the air stream to continuously monitor temperature. The time to ignition (Tign), duration of flaming (TFl), and mass loss rate (m) over a 10-min period were determined as described in Caldwell et al., 1990b, 1995; Caldwell and Alarie, 1991.

Statistical Analyses

Two General Linear Model (GLM) one-factor analysis of variance (ANOVA) was used to determine the effect of temperature and the effect of flow rate on mass loss. The Shapiro-Wilk statistic was used to test the normality of the data (Shapiro and Wilk, 1961; Royston, 1982). The equality of variances among the temperatures and among the flow rates was done using Levene's test of equal variances (Levene, 1960). Since most of the twenty smoke tests were unreplicated, a two-factor ANOVA using the two-way (temperature X flow rate) interaction term as an error term was used to determine the effects of temperature and flow rate on gas (CO, CO2, O2) levels (Winer, 1971). Multiple comparisons were done using Bonferroni adjustments (Hochberg and Tamhane, 1987). A three-way contingency table analysis was used to analyze particulates since some of the larger particles influenced the mean. The three-way interaction was significant among temperature, flow rate, and interval; thus, a two-way interaction between flow rate and interval was done for each flow rate, and a two-way interaction between flow rate and interval.

RESULTS

Preliminary Investigation

Numerous organic compounds were tentatively identified by extraction from the soot (refer to AL/OE-TR-1996-0124). The major compounds based on percent weight of the soot at the collection site are listed in Table 7.1-1.

TABLE 7.1-1. APPROXIMATE QUANTIFICATION OF IDENTIFIED COMPOUNDS FOUND IN SOOT

Compound	Soot. Conc.	Soot. Conc.
	µg/g	% weight
2-Hydroxybenzonitrile	214	0.02%
Aniline	2990	0.30%
Phenol	2170	0.22%
3-Methylphenol	46.1	0.00%
3-Methyl-1-isocyanobenzene	2.7	0.00%
1,2-Methylphenol	55.5	0.01%
3-Methylaniline	626	0.06%
2-Methylaniline	578	0.06%

4-Methylphenol	160	0.02%
4-Methylaniline	11.7	0.00%
2-Methylphenol	206	0.02%
4-Methyl-1-isocyanobenzene	15.2	0.00%
N-(1-Phenylethylidene)-methanamine	25.2	0.00%
Naphthalene	256	0.03%
4-Aminostyrene	13.4	0.00%
Quinoline	3480	0.35%
Isoquinoline	186	0.02%
2-Aminoisocyanate	39.3	0.00%
1-Methylnaphthalene	33.2	0.00%
2-Methylnaphthalene	33.2	0.00%
Indole	537	0.05%
2-Propenenitrile,3-phenyl-, (E)-	40.5	0.00%
1-Methylisoquinoline	236	0.02%
8-Methylquinoline	260	0.03%
5-Methylquinoline	, 1200	0.12%
3-Methylquinoline	353	0.04%
7-Methylisoquinoline	141	0.01%
2-Aminobenzonitrile	354	0.04%
3-Methylisoquinoline	201	0.02%
2-Ethenylnaphthalene	529	0.05%
N-Phenylacetamide	156	0.02%
1-Naphthalenemethylphenylacetate	152	0.02%
5-Methylindolizine	121	0.01%
Diphenylether	1050	0.11%
4,8-Dimethylquinoline	12.0	0.00%
5,8-Dimethylquinoline	2.4	0.00%
Benzene, (2,4-cyclopentadien-1-ylidene)me	3.2	0.00%
Diphenylmethane	0.9	0.00%
2-Methoxyethoxybenzene	1660	0.17%
1,2-Dihydro-2,2,4-trimethylquinoline	2210	0.22%
4,8-Dimethylisoquinoline	56.5	0.01%
2-Phenylpyridine	408	0.04%

1-Methyl-4-phenylpyridinium	132	0.01%
4,8-Dimethylquinoline]	1.8	0.00%
1,2-Dihydrocyclobuta[b]quinoline	38.9	0.00%
2-Isocyanonaphthalene	2210	0.22%
4-Methyl-1,1'-biphenyl	19.6	0.00%
Dibenzofuran	1360	0.14%
1-Isocyanonaphthalene	1660	0.17%
1,2,4,6-Tetramethyl-1,4-dihydropyridine-	629	0.06%
2,4,4,6-Tetramethyl-1,4-dihydropyridine-	212	0.02%
Fluorene	584	0.06%
1-Isocyanatonaphthalene	165	0.02%
5-Methylpyrimido[3,4-a]indole	2.8	0.00%
1H-Phenaline	71.1	0.01%
1,1-Diphenylhydrazine	285	0.03%
5H-Indeno[1,2-b]pyridine	87.6	0.01%
2-(Phenylethynyl)pyridine	103	0.01%
1,1'-Biphenyl-2-carbonitrile	130	0.01%
2-Methyl-N-phenylaniline	30.7	0.00%
1,1-Diphenylhydrazine	110	0.01%
Dibenzothiophene	280	0.03%
Anthracene	1700	0.17%
Phenanthrene	521	0.05%
4(1H)-Pteridinone, 2-amino-7-methyl-	286	0.03%
9-Methylacridine	48.9	0.00%
9H-Fluoren-9-imine	352	0.04%
N-Hydroxymethylcarbazole	1290	0.13%
1-(Phenylmethylene)-1H-indene	81.6	0.01%
2-Phenylnaphthalene	238	0.02%
4-Phenylisoquinoline	64.0	0.01%
8-Phenylisoquinoline	128	0.01%
Fluoranthene	1300	0.13%
9-Methoxyanthracene	153	0.02%
9-Phenanthrenecarboxaldehyde, O-acetylox	117	0.01%
Pyrene	701	0.07%

5-Benzoquinoline	98.3	0.01%
Indeno(1,2,3-ij)isoquinoline	564	0.06%
Fluoranthene	832	0.08%
2,4-Imidazolidinedione, 1,3,5-trimethyl-	258	0.03%
Acenaphtho(1,3-B)pyridine	217	0.02%
Phenanthrene, 4-ethyl-5-methyl	124	0.01%
7H-Benzo[c]fluorene	160	0.02%
Benz[a]anthracene, 1,2,3,4,7,13-hexahydr	197	0.02%
Naphthacene	123	0.01%
Chrysene	362	0.04%
Benzo[e]pyrene	550	0.06%
Benzo[j]fluoranthene	205	0.02%
9,10,11,12-Tetrahydrobenzo[e]pyrene	183	0.02%
Benz[e]acenaphthrylene	228	0.02%

During the analytical process it was apparent upon examination of the injection port liner that many of the extracted compounds were not suitable for analysis by GC/MS due to pyrolysis and deposition in the liner. There were a number of Polycyclic Aromatic Hydrocarbon (PAH) peaks in the soot extract which were too low in strength to characterize properly, and were not included in AL/OE-TR-1996-0124. There are probably a considerable number of compounds which either did not extract or did not make it out of the GC injection port.

Mass Loss

The data were normally distributed and variance among flow rates and temperature was equal. The mass loss rate was found to increase from 625 °C to 770 °C (p=0.0512). The greatest mass loss occurred at 770 °C and the least at 880 °C (p=0.0098), however there was no statistical difference between 625 °C and 880 °C. Refer to AL/OE-TR-1996-0124. There was no significant difference among flow rates but an effect was noted due to temperature variation (p=0.0094).

Gas Analysis

There were no significant differences among the temperatures for the maximum value of CO, for the maximum value of CO2, and for the minimum value of O2. Among the temperatures, the data were statistically normal, and the data showed equal variances (refer to AL/OE-TR-1996-0124).

There were no significant differences among flow rates for the maximum value of CO. However, there were significant differences for the maximum value of CO2 (p = 0.0083) and for the minimum value of O2 (p = 0.0017), refer to AL/OE-TR-1996-0124. Among the flow rates, all of the data were normally distributed and had equal variances. The maximum CO2 value decreased and the minimum O2 value increased as flow rate increased.

At 625 °C, the maximal concentration of carbon dioxide is reached at approximately 160 seconds at the lowest flow rate. Increasing the flow rate diminishes the maximal observed concentration of carbon dioxide and prolongs the time required to reach maximal concentration. At 770 °C, the maximal carbon dioxide concentration is reached at approximately 110 seconds. An increase in flow rate from 340 LPM to 370 LPM resulted in a decrease in the time-to-peak. Further increases in flow decreased the maximal observed concentration and lengthened the time to maximal concentration.

At 880 °C, the maximal carbon dioxide concentration is reached at approximately 90 seconds and is highest at the 340 LPM (lowest) flow rate. Increases in flow rate resulted in decreased maximal observed concentrations and increased time required to reach maximal concentrations. Carbon monoxide maximal concentrations were observed at approximately 175 seconds when evaluated at burn temperature of 625 °C. The maximal observed concentration is much lower than the maximal concentration observed at higher temperatures. Increasing the flow rate resulted in decreased maximal concentration and increased time-to-peak.

Maximal carbon monoxide concentrations were observed at approximately 100 seconds when evaluated at 770 °C. Increasing the flow rate resulted in a decreased maximal concentration. At 880 °C, maximal carbon monoxide concentration was observed at approximately 90 seconds. Increasing the flow rate decreased maximal observed carbon monoxide. Oxygen was depleted to a minimum of approximately 14% under these conditions. Increasing the flow rate resulted in higher minimum concentrations of oxygen and a longer time required to produce the effect.

The depletion of oxygen at 770 °C was observed to occur at approximately 125 seconds. Minimum oxygen concentrations of approximately 15% were observed at the lowest flow rate and the minimum oxygen concentration was higher at increased flow rates. Maximal depletion of oxygen observed when combustion was accomplished at 880 °C was observed at approximately 110 seconds. Oxygen was depleted to approximately 15%, roughly the same as in the 770 °C burn and two percent lower than in the 625 °C burn. Increasing the flow rate dramatically reduced the depletion of oxygen.

Particulate Analysis

Both temperature and flow influenced particle size (refer to AL/OE-TR-1996-0124).

At 770 °C for a flow rate of 650 L/min, particle size significantly differed among the temperatures . At a temperature of 770 °C, the percent (26.4%) of particle size was less than one micron and the percent (68.9%) that was greater than ten microns was significantly different than the other temperatures (refer to AL/OE-TR-1996-0124). At a temperature of 880 °C, the percent (53.5%) of particle size that was less than one micron and the percent (39.5%) that was greater than ten microns was significantly different also. All three temperatures showed significant differences in particle size among the flow rates (refer to AL/OE-TR-1996-0124).

Scanning electron micrographs (refer to AL/OE-TR-1996-0124) show amorphous particles of various sizes, specifically the respirable portions, at both the closest (10 ft) and furthest (40 ft) sections of the tunnel.

DISCUSSION

In the present assessment, the combustion-induced disposition of a form of ACM which is intended for use in advanced aircraft was evaluated. The goal of this study was to obtain a qualitative listing of compounds produced from combustion of ACM. As such, we focused on three areas: 1) the material that remained behind following combustion, 2) the volatile compounds which were released following combustion, and 3) the nature of any particulate material released through combustion. Data revealed that the majority (50-75%) of the actual ACM remained behind as a carbon-fiber based material following combustion. This presumably represents the fibers which are embedded in the epoxy binder of the composite. The human health threat of the remaining material has not been characterized.

The balance of the ACM material was combusted to CO, CO2, water, and other material-specific products of combustion. These materials underwent further analysis: 1) vapors produced were trapped in solvent and analyzed by gas chromatography, 2) particulate matter (solid phase soot deposited on filters and plates) was characterized for particle size distribution and extracted with solvent to determine chemical composition. These analyses have identified nearly 90 chemicals produced from the combustion of ACM. The solid (soot) phase was deposited along and at the end of the furnace vent tube. While the intent of this investigation was to determine qualitatively the types of compounds which result from ACM combustion, some quantitation of ACM disposition has been attempted. Subtracting the mass of material remaining in the calorimeter from the starting mass indicated approximately 30% of the starting mass was vented during combustion. The mass loss rate was previously found to be useful for scaling up from laboratory data and permits modeling of the resultant smoke and aerosol plume. The initial analysis of data collected from cascade impactor samples showed that approximately 50% of the particles in the smoke have an aerodynamic diameter less than 10 microns (10µm). The implication of this finding is that the inhalation hazard may be substantial for the combustion products of ACM given the complex organic composition

of the smoke and the small particle size. Particles less than $1.0 \mu m$ have the potential for deposition within the alveoli of the lungs, the site of oxygen absorption. Chemical products of ACM were identified on these particles. The presence of these potentially dangerous chemicals with particulate matter raises the probability of deposition of these chemicals within the alveoli. This may result in increased potential for toxic consequences.

Soot (refer to AL/OE-TR-1996-0124) and vapor (refer to AL/OE-TR-1996-0124) were analyzed for compound identification by mass spectroscopy using spectra library but not confirmed by chemical standards. Of the volatiles trapped in solvent and analyzed by gas chromatography, it appears that eight major constituents can account for 90+% of the volatiles (refer to AL/OE-TR-1996-0124). Of the soot recovered, approximately 30% of the mass was extractable and identifiable by gas chromatography (routine method of analysis). The results of this examination identified over 80 individual compounds. The fourteen major compounds from this analysis accounted for less than 3% of the soot mass. Subjective analysis of the soot revealed a fine, black amorphous powder which packed loosely (1.5 grams occupied 5 mL, where 1.5 grams water would occupy 1.5 mL) and was consistent with elemental carbon.

Known toxicology exists on only a few of the major compounds identified. This preliminary study identified three main classes of combustion products: nitrogenous aromatic compounds, PAHs, and phenols. A summary of the literature search of the significant compounds identified in soot and vapor follows.

ANILINE - Effects include methemoglobinemia (Amdur et al., 1993).

2 AND 3-METHYLANILINE - There is epidemiological evidence that associates occupational exposures to an increase risk of bladder cancer. There is inadequate evidence of carcinogenicity in humans but sufficient evidence in animals. Therefore, it is considered a possible carcinogen to humans. Vapor toxicity is much like aniline with less effect on the Central Nervous System (CNS) and more on the vascular system. Toxicity and biomedical effects include CNS depression, cyanosis, methemoglobinemia, vertigo, headache, and mental confusion (NIOSH, 1990; Hamilton and Hardy, 1974; and Budavari, 1989).

QUINOLINE - Known to cause liver cancer in mice and is mutagenic. Exposure may affect cardiovascular system, CNS, retina, and optic nerve. Quinoline is found in tobacco smoke as well as particulates in urban air. If released to the atmosphere, the vapor is expected to react with hydroxyl radicals with an estimated half-life of 2.5 days (Clayton and Clayton, 1981; Amdur et al., 1993).

1,2-DIHYDRO-2,2,4-TRIMETHYLQUINOLINE - Negative for mutagenicity. Similar effects as quinoline (Zeiger, 1987).

ANTHRACENE - Non-carcinogenic; however, many of its derivatives found as combustion by-products are potent carcinogens. They are commonly found in coal tar, tobacco smoke, and petroleum products. Acute toxicity is presumably due mostly to derivatives and may cause an excess of bronchitis (USEPA IRIS, 1994, Clayton and Clayton, 1981; Amdur et al., 1993).

FLUROANTHENE - Commonly released into the environment from general use in combustion of organic matter, fossil fuels, smoking and charcoal broiling, resulted in generating PAHs. Unabsorbed chemical in air will photolyze with a half-life of approximately 4-5 days. Effects from exposure may include nausea, tachycardia, cardiac arrhythmia's, liver injury, pulmonary edema, and respiratory arrest. NIOSH recommends it be regulated as an occupational carcinogen, however, it is not classified as a human or animal carcinogen (USEPA IRIS, 1994; IARC, 1987; USEPA, 1980).

DIPHENYL ETHER - Not a serious hazard or mutagenic. Diphenyl ether is reportedly released from combustion emissions of plastic manufacturing and turbine engines. It is used in various soaps, detergents, and as a dye carrier in textile operations. Acute oral exposures in rats have shown injury to liver, spleen, kidney, thyroid, and intestinal tract (Clayton and Clayton, 1981; Graedel, 1986).

DIBENZOFURAN - Not considered carcinogenic for either human or animal, but has been associated with chloracne. It is a common emission associated with combustion of coal and fuel. In the gas phase it will degrade rapidly (half-life of approximately 11 hours), but as a particulate, it may be relatively persistent (USEPA IRIS, 1994; Amdur et al., 1993).

N-HYDROXYMETHYLCARBAZOLE - Shown to have mutagenic activity (Ibe and Raj, 1994).

PHENOL - Observed effects from acute exposure may include shock, hypotension, methemoglobinemia, Heinz body hemolytic anemia, pulmonary edema, dark urine, and death due to action on CNS, cardiovascular system, lung, and kidney. Primary site of action is CNS. Phenol is not classified as carcinogenic to humans (Amdur et al., 1993; Gosselin et al., 1984).

It is important to compare the implications of toxicity from this limited-scope evaluation of ACM combustion to the scenario involving the complete aircraft. As with any exposure to burning materials, proximity correlates well with toxicity. The use of personal protective equipment (PPE) may abate the dangers associated with products of combustion, but PPE may not be available to all exposed personnel.

This project has evaluated the combustion-related chemical release from an ACM destined for use in structural components of advanced aircraft. The results of this study provided the expected products of combustion from this

specific ACM matrix. Modifying matrix components will result in altered combustion products, and the potential interaction between combustion products of specific matrix components may also change the pattern of compounds ultimately released by combustion. While the use of ACM in aircraft in development is increasing steadily, ACM presently accounts for less than half the total mass of the most advanced aircraft. In the event of a mishap, the entire airframe may be subjected to combustion. The products released from the combustion of rubber, plastic, fuel, lubricants and hydraulic fluids, and insulation should be considered in any assessment of toxicity risk from a burning aircraft.

APPENDIX A: GC/MS CONDITIONS

Set Points	5			
(Oven Temp 1	35.0	Oven Temp 3	300.0
(Oven Time 1	5.0	Oven Time 3	20.0
(Oven Rate 1	3.0	Oven Rate 3	0.0
V	Valve 1	Off	Valve 3	NONE
(Oven Temp 2	120.0	Oven Temp 4	0.0
(Oven Time 2	20.0	Oven Time 4	0.0
(Oven Rate 2	3.0		
v	Valve 2	NONE	Valve 4	NONE
A	Aux. Zone	220.0	Inj. Temp 1	270.0
I	Pressure 1	13.0	Inj. Temp 2	NONE
I	Pressure 2	0.0	· ·	
Timed Ev	ents			
		Time	Event	Value

	Time	Event	Value
1	-0.10	V1	Off
2	1.00	V1	On
3	•••		

MS Parameters

Starting Mass	35
Ending Mass	650
Multiplier HV	1725 (for instrument)
Runtime (min)	170
Filament delay (sec)	270

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7.2 COMBUSTION OF ADVANCED COMPOSITE MATERIALS - CHEMICAL AND MORPHOLOGICAL ANALYSES

B.J. Larcom¹, J.M. Cline², L.D. Harvey¹, and D.L. Courson

ABSTRACT

Use of new synthetic materials, such as Advanced Composite Materials, raises concerns about the potential human health risks resulting from exposure to the chemically complex smoke produced by burning these materials. We developed an analytical protocol for evaluating the smoke produced from controlled combustion of test materials in a modified cone heater combustion module of the UPITT II apparatus. The objectives of this study were 1) sampling of soot and aerosol for chemical analysis and morphological evaluation using light and electron microscopy, and 2) identification of organic compounds extracted from soot and cold trapped vapor.

INTRODUCTION

Advanced Composite Materials (ACM) offer substantial weight savings in the airframe, ships, submarines, and armored vehicles. Numerous military aircraft, including fighters, bombers, and harriers, contain varying amounts of ACM. The ACM matrix is created by assembling two or more physically distinct synthetic components, reinforced (graphite) and bound with resin (epoxy), and integrated into various configurations to obtain specific characteristics and properties (Roop, 1994). However, the replacement of nonflammable materials (metals such as aluminum) with organic binder resins contributes to fire involvement, which requires consideration of heat fluxes (Ohlemiller et al., 1993).

As a result of wheels-up mishaps involving ACM aircraft, concern has rekindled over the data gaps on the safety and health risks of burned or burning composite materials. The discovery of dead birds around partially-burned aircraft and worker complaints suggest the possibility that toxic off-gassing of the burned composites is occurring during and long after combustion has ceased. In response to concerns voiced at the October 1991 Horizons Conference on Health and Safety Concerns of ACM at Wright-Patterson Air Force Base, researchers initiated an effort to characterize and assess the by-products from burned composites. System Program Offices for aircraft with ACM, ACM Laboratory,

¹Armstrong Laboratory, Toxicology Division, Wright-Patterson Air Force Base, OH.

²U.S. Army Medical Research Unit, Wright-Patterson Air Force Base, OH.

and U.S. Air Force Surgeon General's Office have also expressed concern over the health impact from burned ACM. Although ACM appears to present limited or no human health hazard in its original state, the combustion products, as

well as possible graphite fibers, are not well characterized. For example, over 50 chemicals were identified in the combustion of cellulose, a major component in wood, and over 90 chemicals were identified from combustion of ACM. Following the traditional paradigm of hazard identification, dose-response assessment, exposure assessment, and risk characterization in the case of combustion studies requires an interdisciplinary approach and modification. In this effort we focused on particle size and morphology, soot composition, and volatile organic compounds to expand upon the risk assessment.

MATERIALS AND METHODS

A modified UPITT II cone calorimeter to permit control of heat flux and airflow was used to burn ACM under various test parameters (Courson et al., 1996). Two samples of combustion products were collected and analyzed in a Perkin-Elmer 910 GC/MS (Gas Chromatograph/Mass Spectrometer) system. The first sample was a portion (vapor) of the smoke plume caught in a cold trap, extracted with methylene chloride, and concentrated with a stream of dry nitrogen. A total of 150 liters (10 L/min for 15 min) of the combustion gas was drawn through the trap. The second sample of deposited soot was collected on a glass wool filter. The standard method for extraction (Soxhlet) could not be employed due to the particle size. Instead, a glass pipette plugged with glass wool was used. The extract was concentrated to approximately 1 mL by a stream of dry nitrogen and then made up to 20 mL with methylene chloride. Compound identification was performed quantitatively using the latest available NIST reference library and approximate quantification was performed under EPA Method 8270 protocols.

Glass microscope slides placed adjacent to the scanning electron microscope (SEM) stubs were used as a collection surface for aerosol particles. Particulate samples for evaluation by SEM were dried overnight in a vacuum desiccator, then sputter-coated with a 10-15 nm layer of gold. Photomicrographs were taken of the surfaces using an Amray 1000B SEM at 20-30 kV accelerating voltage.

Algorithms developed for particle analysis were employed using a Quantimet 570c image analysis system (Leica, Inc). Samples collected on glass microscope slides were magnified to 40x, 200x, and 400x on a light microscope and the resulting images captured by a microscope-mounted CCD camera and digitized for image analysis. Photographs obtained by SEM were captured by a CCD camera mounted on a macro-(photo)stand. Particles were detected as "features" in each calibrated digitized image by comparing the gray level of the feature with the background gray level. The identified features were then measured using computer-based image analysis methodologies for area, perimeter, and equivalent circle diameter (Courson et al., 1996).

RESULTS

The major compounds used in this assessment are listed in Table 7.2-1 based on percent weight of the soot at the collection site and Table 7.2-2 of volatiles.

TABLE 7.2-1. IDENTIFICATION AND APPROXIMATE QUANTITATION OF MAJORCOMPOUNDS EXTRACTED FROM SOOT

Identified Compound	Concentration in	Concentration in Soot
	Soot (µg/g)	(% Weight)
Aniline	2990	0.30%
Phenol	2170	0.22%
Quinoline	3480	0.35%
Diphenylether	1050	0.11%

TABLE 7.2-2. APPROXIMATE CONCENTRATIONS OF VAPOR COMPOUNDS

Compound	Air Conc. (µg/m3)
Aniline	571
Phenol	1600
Quinoline	41.5
Diphenyl Ether	190

Both temperature and flow influenced particle size (Table 7.2-3). Particle size significantly differed among the temperatures and at a temperature of 770 °C the percent (68.9%) which was greater than ten microns was significantly different than the other temperatures. At a temperature of 880 °C, the percent (53.5%) of particle size that was less than one micron was significantly different also. All three temperatures showed significant differences in particle size among the flow rates. Scanning electron micrographs show amorphous particles of various sizes, specifically the respirable portions.

TI	EMPERATURE	INTERVAL	COUNT	PERCENT	
FLOW 3	340				
	625	0≤µm≤1	329	37.8	3-Way Interaction
	625	1<µm ≤5	67	7.8	X2 = 152.29
	625	5<µm ≤10	4	0.4	df = 54
	625	<u>10<µm</u>	471	54.1	p = 0.0000
	770	0≤µm≤1	84	19.4	
	770	1<µm≤5	12	2.8	
	770	<µm ≤10	2	0.2	
	770	<u>10<µm</u>	336	77.4	
	880	0≤µm≤1	162	33.8	
	880	1<µm≤5	32	6.7	
	880	5<µm≤10	3	0.6	
	880	10<µm	283	59.0	
FLOW 3	70				

TABLE 7.2-3. THREE WAY INTERACTION FOR TEMPERATURE (°C)/FLOW (L/MIN)/INTERVAL(µM)

625	0≤µm≤1	94	30.1
625	1<µm≤5	27	8.6
625	5<µm≤10	5	1.5
625	<u>10<µm</u>	186	59.6
770	0≤μm≤1	93	30.9
770	l<µm≤5	31	10.3
770	5<µm≤10	1	0.3
770	<u>10<µm</u>	176	58.5
880	0≤µm≤1	166	38.6
880	1<µm≤5	35	8.2
880	5<µm≤10	7	1.6
880	10<µm	222	51.6

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FLOW 400

625	0≤µm≤1	161	50.2
625	1<µm≤5	48	14.9
625	5<µm≤10	- 3	0.9
625	<u>10<µm</u>	109	34.0
770	0≤µm≤1	93	33.1
770	1<µm <u>≤</u> 5	29	10.3
770	5<µm≤10	8	2.9
770	<u>10<µm</u>	151	53.7
880	0≤µm≤1	93	31.5
880	1<µm≤5	29	9.9
880	5<µm≤10	2	0.6
880	10<µm	171	58.0

FLOW 650

625	0≤µm≤1	306	44.2
625	l<µm≤5	38	5.5
625	5<µm≤10	3	0.4
625	<u>10<µm</u>	345	49.9
770	0≤µm≤1	107	26.4
770	1<µm≤5	18	4.4
770	5<µm≤10	1	0.2
770	<u>10<µm</u>	279	68.9
880	0≤µm≤1	267	53.5
880	1<µm≤5	31	6.2
880	5<µm≤10	4	0.8
880	10<µm	197	39.5

DISCUSSION

In the present assessment, the combustion-induced disposition of a form of ACM which is intended for use in advanced aircraft was evaluated. The goal of this study was to identify potential health hazards produced from combustion of ACM. As such, we focused on two areas: 1) the volatile compounds which were released following combustion, and 2) the nature of any particulate material released through combustion. Our data revealed that the majority (50-75%) of the actual ACM remained behind as a carbon-fiber based material following combustion. This presumably represents the fibers which are embedded in the epoxy binder of the composite. The human health threat of the remaining material has not been characterized.

The balance of the material was combusted to CO, CO2, water, and other material-specific products of combustion. These materials underwent further analysis: 1) vapors produced were trapped in solvent and analyzed by gas chromatography, 2) particulate matter (solid phase soot deposited on filters and plates) was characterized for particle size distribution and extracted with solvent to determine chemical composition. These analyses have identified nearly 90 chemicals produced from the combustion of ACM. We have shown that approximately 30% of the starting mass is vented during combustion. The initial analysis of data collected shows that approximately 50% of the particles in the smoke have an aerodynamic diameter less than 10 microns (10um). The implication of this finding is that the inhalation hazard may be substantial for the combustion products of ACM given the complex organic composition of the smoke and the small particle size. Particles less than 1.0 μ m have the potential for deposition within the respiratory tract. We have clearly identified chemical products of ACM on these particles. The presence of these potentially dangerous chemicals with particulate matter raises the probability of deposition of these chemicals within the alveoli. This may result in increased potential for toxic consequences.

Soot (Table 7.2-1) and vapor (Table 7.2-2) were analyzed for compound identification by mass spectroscopy. Of the volatiles trapped in solvent and analyzed by gas chromatography, it appears that eight major constituents can account for 90+% of the volatiles. Of the soot recovered, approximately 30% of the mass was extractable and identifiable by gas chromatography. The results of this examination identified over 80 individual compounds. Subjective analysis of the soot revealed a fine, black amorphous powder which packed loosely (1.5 grams occupied 5 mL, where 1.5 grams water would occupy 1.5 mL) and was consistent with elemental carbon. This preliminary study identified three main classes of combustion products: nitrogenous aromatic compounds, PAHs, and phenols.

In this study we were able to address the potential hazards associated with combustion of ACM. Rather than the anticipated fibers, we identified amorphous carbon soot as the inhalation hazard. The magnitude of organic compounds in vapor was expected, however preliminary identification was completed. The route of exposure was determined to be by inhalation and the target receptors to be crew and fire fighters. Based on the compound with the lowest LD50 (aniline) using an occupational exposure the Threshold Limit Value was not exceeded. Toxicity of combustion products using a bioassay has been developed and will be completed in the following effort.

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7.3 INITIAL CHARACTERIZATION OF ADVANCED COMPOSITE MATERIALS (ACM) COMBUSTION BY SCANNING ELECTRON MICROSCOPY AND COMPUTER-BASED IMAGE ANALYSIS

C.D. Flemming, J.H. Grabau, and J.W. Lane

ABSTRACT

The combustion of Advanced Composite Materials (ACM), comprised of a reinforcing fiber matrix core and an epoxy resin binder, produces a complex smoke of potentially harmful gases and particulate material. Using computer-based morphometric analysis, the size distribution and shape of particulates, which were derived from the combustion of ACM at three combustion temperatures (625 °C, 770 °C and 880 °C) and four flow rates (340, 370, 400, and 650 L/min) through a modified UPITT II combustion chamber, were statistically analyzed. All temperatures showed a significant difference in particle size distribution among flow rates. Generally, for a given temperature, increasing the flow rate increased the fraction of particles with a mean diameter less than one micron. Also, flow rate more often affected particle size distribution than did temperature. Data indicate that variations in flow rate exhibit a greater influence on particulate shape and size of ACM aerosol than combustion temperature. These data will be valuable in predicting inhalation hazards associated with human exposure.

INTRODUCTION

In this initial portion of the combustion study results on the morphological characterization of the smoke residue produced in a small-scale wind tunnel are presented. These morphological characteristics should help determine the fate of particulates in the air passages, as well as how far the particulates travel from their point of origin before being deposited as residue. Another consideration is the potential release of adsorbed chemicals on the surfaces of the particulates; previous studies have identified numerous toxic compounds released from Advanced Composite Materials (ACM) combustion including nitrogenous aromatic compounds, polycyclic aromatic hydrocarbons and phenols (Caldwell et al., 1995). ACM in its natural state appears to present no danger to human health (Ohlemiller et al., 1993), but, if the material is combusted, fire safety personnel became ill after being exposed to ACM combusted smoke. During an aircraft mishap, the composite structures are subjected to forces that cause them to break into pieces, burn, and subsequently release particulates to the local environment. The particle distribution and rate of release may depend on local environmental conditions (Babrauskas et al., 1987).

The combustion apparatus used in this study is a modification of the cone heater combustion module of the UPITT II method developed at the University of Pittsburgh (Caldwell et al., 1995) attached to a 40-ft. long horizontal isoaxial chimney. Several different burn conditions involving fluctuations in combustion temperature and air flow rate were selected to characterize the smoke. The methodology in this study included: 1) the collection of samples at four different ports along the combustion apparatus, 2) sample analysis using a scanning electron microscope (SEM) and light microscope (LM), 3) multivariable measurement of particles using computer-based image analysis, and 4) statistical analysis of the data.

To morphologically characterize the smoke residue, several image analysis measurement variables were used initially to assess the size and shape of ACM combustion particulates. These variables were narrowed by statistical analysis to yield four measurement variables best describing the smoke residue morphologically. These variables correlate strongly with geometric variables determined in similar studies (Farrants et al., 1989; Stevens and Moyer, 1989). A thorough characterization of the smoke over several burn scenarios will help in the evaluation of potential environmental and health risks of ACM smoke and in the description of the *in vivo* effects of acute exposure to ACM smoke.

MATERIALS AND METHODS

Particulate Sampling

Four sampling ports were placed at 10-ft. intervals along the isoaxial chimney. Clean aluminum SEM stubs coated with adhesive and pre-cleaned glass slides were mounted in special holders and positioned on the bottom of the chimney. Holders were positioned parallel to the airstream and slightly upwind of the access ports just prior to the combustion, which prevented residual material from the chimney top from depositing on the sampling surfaces (Figure 7.3-1).



Figure 7.3-1. - Combustion System

LM analysis

Glass slides for LM evaluation were examined on an Olympus VH2 light microscope at magnifications of 100x, 200x, and 400x. A single image at each magnification (100x, 200x, and 400x) was obtained from five random locations on each glass slide. Counts were normalized for area analyzed. The resulting images were captured by a microscope-mounted CCD camera and digitized for image analysis.

SEM Analysis

Aerosol samples for SEM analysis were placed into storage boxes and stored overnight in a vacuum desiccator. The samples were sputter-coated with a 10-15 nm layer of gold/palladium and imaged with an AMRAY 1000B SEM at 20-30 kV accelerating voltage. Photomicrographs of random areas on the stub surface were taken at magnifications of 20x, 200x, 2000x, 10000x, and 25000x. Representative photomicrographs were submitted for computer-based image analysis.

Image Analysis

Both scanning electron micrograph and LM slide field were digitized using the Leica Quantimet 570c Image Analysis System (Deerfield, IL) and stored as an 8-bit computer image file. A Sony CCD camera operating in gray scale mode captured the images from a gross mounting stage. An analog input for digital conversion and image buffering were generated within the memory planes of the Quantimet.

Each micrograph contained a nanometer calibrated scale bar that was placed on the micrograph at the time of exposure during electron microscopy. This bar was utilized to calibrate the Quantimet. It should be noted that the instrument autocalibration procedure of the electron microscope has an estimated error between 5% and 10%. Particulates identified in the digitized image files were measured using variables that defined size and shape.

Statistical Analysis

A three factorial multivariate analysis of variance was used to determine statistical differences among temperatures, flows, and distances from the source of the burn in size and shape morphometric variables (Johnson and Wichern, 1988). For a significant statistic, the p value was less than 0.05. Using the statistical programs BMDP 4M (factor analysis) and BMDP 2R (stepwise regression), the number of size and shape morphometric variables was reduced from 16 to 4. The criterion for a variable to enter the stepwise regression model was the largest partial correlation between each variable and the distance from the source of the burn. The criterion for a variable to leave the stepwise regression model was the smallest partial correlation between each variable and the distance from the source of the burn. This procedure was continued until there were no remaining variables to add or remove.

RESULTS

Through factor analysis and stepwise regression, the measurement variables that provided the best morphological description of the smoke residue were area, perimeter, equivalent circular diameter, and roundness. These variables strongly correlate with those found in a similar combustion study (Farrants et al., 1989).

The analysis of variance for the area variable indicated that there were significant differences in the interaction of temperature, flow, and distance to the burn. Hence, the area of a particle simultaneously depends on the temperature, flow, and distance from burn (port). At the temperature of 880 °C, the areas were among the largest; the largest mean area occurred at 340 L/min and port 1. The second largest mean area occurred at 400 L/min and port 1. See Figures 7.3-2, 7.3-3, and 7.3-4. The perimeter values also showed significant differences in the interaction among temperature, flow, and distance to the burn. The largest perimeter occurred at 880 °C, 340 L/min and port 1. See Figures 7.3-2, 7.3-3, and 7.3-4. Both the mean area and the perimeter were the smallest at 770 °C, 400 L/min and port 1. See Figures 7.3-2, 7.3-2, 7.3-3, and 7.3-4. The equivalent circle diameter differed statistically in the interaction of temperature, flow, and distance to the burn simultaneously effects equivalent circle diameter. The largest mean of the equivalent circle diameter was at 880 °C, 400 L/min and port 1.

At 770 °C, 400 L/min and port 1, the mean of the equivalent circle diameter was the smallest. See Figures 7.3-5, 7.3-6, and 7.3-7. There were differences in the interaction among the temperature, flow, and distance to the burn in the mean (port) of the roundness; therefore, roundness is affected by temperature, flow, and distance to the burn simultaneously. Since the mean of the roundness is less than 1000, the shape of the particles were generally regular with no sharp corners; therefore, the particles appeared to be a sphere. The largest mean for the roundness occurred at 770 °C, 400 L/min and port 4. Most of the roundness means were less than or equal to two. See Figures 7.3-5, 7.3-6, and 7.3-7.



D1 is flow 650 L/min and port 1. D4 is flow 650 L/min and port 4.

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Figure 7.3-2. Mean Area and Perimeter at 625 °C



At is flow 340 L/min and port 1. A2 is flow 340 L/min and port 4. B1 is flow 370 L/min and port 1. B4 is flow 370 L/min and port 4. C1 is flow 400 L/min and port 1. C4 is flow 400 L/min and port 4. D1 is flow 650 L/min and port 1. D4 is flow 650 L/min and port 4.

Figure 7.3-3. Mean Area and Perimeter at 770 °C



Figure 7.3-4. Mean Area and Perimeter at 880 °C






Figure 7.3-6. Equivalent Circle Diameter and Roundness at 770 °C





DISCUSSION

The combination of the area and perimeter estimates the three-dimensional surface area of the particles. The three-dimensional surface area measures the potential of the particles to carry the organic compounds into the respiratory tract of an animal or human. Hence, the area and perimeter variables proved to be critical measurements in morphologically characterizing the particles; the higher that these variables are implies the higher potential of carrying organic compounds. Therefore, since 880 °C, 340 L/min and port 1 had the largest area and perimeter, they could contain the largest amount of chemicals adsorbed on their surface or within internal spaces. Since the particles had a small roundness, they were spherical. A sphere has a diameter which was estimated by the equivalent circle diameter. For this burn, the particles were spherical, had a large surface area, had about 50% with an equivalent circle diameter which was greater than 10 μ m, and had about 50% with an equivalent circle diameter which was less than or equal to 5 μ m. For this burn, the associated human health risk would be lower than other burns. Although the particles might contain greater amounts of associated organic compounds due to their increased surface area, these larger particles have a lower probability of getting into the alveolar portions of the lung.

Since 770 °C, 400 L/min and port 1 showed the smallest area and perimeter, that burn scenario is characterized by particulates having the smallest three-dimensional surface area and probably containing a lesser amount of associated chemicals per particle. This burn had a small average roundness; hence the particles were spherical. For this burn, 90.5 % of the particles had an equivalent circle diameter which was less than 5 μ m. Hence, these particles have a larger probability of getting into the deep lung with their associated adsorbed chemicals; the potential human health risk due to inhalation exposure is probably highest for ACM combustion at these levels.

The roundness variable compares the particle to a circle. If the roundness is small, the particle is circular. If the roundness is large (> 1000), the particle is fibrous. 770 °C, 400 L/min and port 4 showed the largest average roundness, and 96% of the particles from this burn were between 1 and 5 in roundness. This indicates that most particulates were circular in shape and that there were very few particles with sharp edges. The experimental results showed that for the burn scenarios which were investigated, fibers were either not released into the smoke plume (remained intact as non-combusted coupon left behind after burn) or that fibers were altered during the combustion process to form particulates that were fairly round and regular in shape.

In this study, particulates were characterized from the combustion of ACM using 16 morphometric variables which were statistically reduced to 4 variables: area, perimeter, equivalent circle diameter, and roundness. A combination of area and perimeter estimated the three-dimensional surface area of particulates. The roundness of a particle defined whether it was a sphere or not. If the particle was a sphere, the equivalent circle diameter gave an estimate of the diameter. The results demonstrated that the smoke residue was comprised of fairly round

particulates with a wide range of particulate sizes. In addition to surface area for chemical adsorption, these particulates probably contained internal spaces that could contain potentially hazardous toxicants. These results should help to develop a model of the smoke plume and aid in the interpretation of the ongoing bioassay portion of this study.

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SECTION 8

TRIMETHYLOLPROPANE PHOSPHATE TOXICITY PROJECT

8.1 REPEATED EXPOSURE TO TRIMETHYLOLPROPANE PHOSPHATE (TMPP) FACILITATES ELECTRICAL STIMULATION OF MYGDALOID COMPLEX INDUCED SEIZURES IN MALE RATS

J. Lin, J. Cassell^l, and J. Rossi III^l

ABSTRACT

Actions of a potent organophosphate convulsant trimethylolpropane phosphate (TMPP) were tested on the amygdaloid complex of freely moving rats using in vivo stimulating-recording electrophysiological and electrical kindling techniques. The effects of known convulsants pentylenetetrazol (PTZ) and N-Methyl-B-carboline-3carboxamide (FG-7142) were also tested and compared with that of TMPP. Stimulating/recording electrodes were implanted in the left amygdala, right amygdala, and left bed nucleus of stria terminalis of male Sprague-Dawley rats. TMPP (0.275 mg/kg), PTZ (20 mg/kg), FG-7142 (7.5 mg/kg), or vehicle (0.5 ml/kg) were administered i.p. to rats 3 times/week for 10 weeks. Single pulse stimulation was applied to left amygdala and local EEGs were recorded from left bed nucleus and right amygdala 24 h following the drug administration. Electrical stimulation (0.1 Hz, 0.1 ms duration, 280-1500 µA, 20 pulses) of left amygdala evoked epileptiform after discharges in left bed nucleus and right amygdala after 7 weeks of TMPP (0.275 mg/kg) or PTZ (20 mg/kg) treatment and after 9 and 19 weeks of FG-7142 (7.5 mg/kg) treatment from left bed nucleus and right amygdala, Triweekly administration of TMPP, PTZ, or FG 7142 produced an increased incidence of respectively. myoclonic jerks by the 5^{th} dose of treatment, and 37.5% and 33% of animals exhibited clonic seizures by the 10^{th} dose of TMPP and PTZ treatment, respectively. Control animals showed neither behavioral nor electragraphic seizure activities to vehicle solution injection and single pulse electrical stimulation of left amygdala. Electrical kindling stimulation applied in left amygdala (60 Hz, 2 sec train duration, 20-1500 µA, 0.1 ms pulse duration) evoked epileptic seizure after discharges from both the stimulation site (left amygdala) and secondary recruiting sites (left bed nucleus and right amygdala). Repeated treatment of animals with TMPP, PTZ, or FG 7142 decreased both the threshold and the latency to onset of afterdischarges in both the primary site (left amygdala) and secondary sites (left bed nucleus and right amygdala). The present study indicates that long-term exposure of rats to low doses of TMPP, PTZ, and FG-7142 induces sensitization of the amygdaloid complex and TMPP may play a similar role with gamma-aminobutyric acid (GABA)-benzodiazepine receptor antagonist PTZ and FG7142 on inducing seizure activities by sensitizing the amygdaloid pathways.

¹Naval Medical Research Institute/Toxicology Detachment, Wright-Patterson Air Force Base, OH.

INTRODUCTION

Amygdala complex is one of the brain structures possessing the highest susceptibility to kindling and seizure production (Goddard et al., 1969). The network of interamygdala and intraamygdala connection is well related to the outstanding sensitivity of the amygdala to epileptiform-inducing procedures or agents. Amygdala kindling has been used as a model of epilepsy in which both complex partial (focal) seizures and generalized motor seizures may be induced and the effects of proconvulsant or anticonvulsant compounds may be studied (McNamara, 1984). Neurotoxic compound trimethylolpropane phosphate (TMPP), generated from the pyrolysis of phosphate esterbased lubricants, is a concern to the military tri-service. Completed and ongoing work at the Naval Medical Research Institute, Toxicology Detachment has shown that TMPP induces EEG paroxysms, sub-clinical seizures, clinical seizures and lethality (Rossi et al., 1993), as well as long-term central nervous system (CNS) sensitization/kindling including progressive increases in EEG paroxysms, audiogenic seizure susceptibility and, hyperreactivity to amphetamine challenge in rats (Bekkedal et al., 1996; Ritchie et al., 1995). It is hypothesized that TMPP-induced effects reflect chemical modulation of specific neural pathways. Furthermore, it has also shown that TMPP binds to benzodiazepine-GABA_A receptor complex (Ritchie et al., 1995) indicating that its pharmacological effects are mediated via a specific interaction with the GABA receptor. Since basolateral amygdala nucleus projects heavily to the bed nucleus of the stria terminalis, the purpose of the present experiments was to determine if long-term TMPP treatment sensitized this amygdala afferent pathway and left amygdala to right amygdala pathway by using electrophysiological techniques in awake, freely moving rats. The present study was also designed to investigate the mechanism of TMPP-induced sensitization by comparing the effects induced 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 in the same pathways.

MATERIALS AND METHODS

Subjects and Surgical Procedures

Twenty four 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, Virginia), were implanted bilaterally in basolateral nucleus of amygdala at the following coordinates (mm): -2.9 AP; 4.8 ML; 8.2 DV, and in left bed nucleus of stria terminalis at the following coordinates (mm): -0.3 AP; 1.5 ML; 7.0 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 subjected to drug treatment and electrophysiological assessments.

Drug Studies and Behavior Observation

Animals were divided into four groups (n=5 for FG 7142 and vehicle groups, n=6 for PTZ group, and n=8 for TMPP; group size arranged by way of preparing animal lost) 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), respectively, three times per week for ten weeks (30 total administrations). Rats received the same dose of the same treatment at the same time of day throughout the 10-week period. EEG were recorded from left bed nucleus of stria terminalis and right amygdala, 24 h following each dose of injection. Behavioral changes were recorded into two categories as myoclonic jerks and clonic seizures immediately after the injection and observed for a 1-h period.

Electrical Stimulation

Rats in all groups were electrically stimulated. Single pulse electrical stimulation was delivered through a bipolar stimulating electrode to left amygdala (monophasic square wave, 0.1 ms pulse duration, 0.1 Hz at 280-1500 μ A) by Grass stimulator-isolator. Twenty pulses were presented to rats, 25 min and 24 h following the test drugs and vehicle administration. Behavioral changes were also recorded.

Electrical Kindling Stimulation

Four weeks following the last dose of treatment, all rats were subjected to electrical kindling stimulation. High frequency train electrical stimulation (60 Hz, 2 sec train duration, 20-1500 μ A, 0.1 ms pulse duration) was delivered through a bipolar electrode to left amygdala by Grass stimulator-isolator. Responses were recorded from left bed nucleus, right amygdala, and left amygdala. Afterdischarge (AD) (electrographaphic seizure) threshold was determined by stimulating animals at an intensity of 20 μ A and increasing in 50 μ A steps at 1-min intervals until an AD of 5 sec or longer was observed. Duration of evoked AD and behavioral seizure was recorded following each stimulation.

Chemicals and Drugs

Vehicle contained Dimethyl Sulfoxide: Propylene Glycol: Saline (1/4:1/4:1/2). Pentylenetetrazol (PTZ) (RBI), TMPP (gift from Dr. Paul Servé, Department of Chemistry, Wright State University) and N-Methyl-β-carboline-3-carboxamide (FG7142) (RBI) were dissolved in the vehicle solution.

Data Acquisition and Data Analysis

Electrical signals from left amygdala, left bed nucleus, and right amygdala were directly recorded on a digital tape recorder and continuously displayed on an oscilloscope and a thermal chart recorder. Paired t tests were used to analyze the electrographic data.

RESULTS

Sensitivity to TMPP, PTZ, FG 7142, and Electrical Stimulation After Repeated Exposure to Chemicals.

Electrical stimulation (0.1 Hz, 0.1 ms duration, 280-1500 µA, 20 pulses) of left amygdala evoked field potentials in left bed nucleus and right amygdala in 87.5% (21 of 24) and 83.3% (20 of 24) of naive rats tested, respectively (Figure 8.1-1a). No observable behavioral change could be obtained from these rats upon electrical stimulation. However, after 7 weeks of TMPP (0.275 mg/kg) or PTZ (20 mg/kg) treatment, when recorded from left bed nucleus and right amygdala 24 h following the drug administration, electrical stimulation of left amygdala induced epileptiform after discharges (Figure 8.1-1b, Figure 8.1-2a,b). The responses from FG 7142 (7.5 mg/kg) treated rats were much delayed (from left bed nucleus, 9 weeks of treatment and from right amygdala, 19 weeks of treatment) (Figure 8.1-2a,b). Myoclonic jerks evoked by stimulation of left amygdala 24 h following the drug injection could be observed after 9 weeks of TMPP or PTZ treatment or 13 weeks of FG7142 treatment (Figure 8.1-2c). After 30 doses of TMPP, PTZ, or FG 7142 treatment, the total percentage of rats exhibited epileptiform after discharge in left bed nucleus and right amygdala or myoclonic jerks when left amygdala was stimulated is summarized in Figure 8.1-3. Electrical stimulation of left amygdala, however, did not induce any epileptiform after discharge in left bed nucleus and right amygdala nor behavioral jerks or seizures in all vehicle treated rats in 10 weeks of experimental period.

Triweekly administration of TMPP, PTZ, or FG7142 produced an increased incidence of myoclonic jerks by the 5^{th} dose of treatment. By the 10^{th} dose of treatment, all drug treated animals exhibited myoclonic jerks (Figure 8.1-4). In contrast, clonic seizures were developed much slower. By the 10^{th} dose of treatment, 37.5% of TMPP treated, 33% of PTZ treated and no FG7142 treated animals exhibited clonic seizures. However, by the 30^{th} dose of treatment, all TMPP and FG 7142 treated, and 50% of PTZ treated animals exhibited clonic seizures (Figure 8.1-4). Vehicle treated animals exhibited neither myoclonic jerks nor clonic seizures in the whole treatment period.

Sensitivity to Electrical Kindling

Electrical kindling stimulation (60 Hz, 2 sec train duration, 20-1500 μ A, 0.1 ms pulse duration) was applied to left amygdala of drug and vehicle treated animals after a 4-week rest period without further dosing. Repeated dosing with TMPP facilitated the electrical kindling which indicated by decreasing afterdischarge threshold and the latency to onset of afterdischarge significantly in both the primary stimulating site (left amygdala) (Table 8.1-1) and the secondary recruiting sites (right amygdala and left bed nucleus of stria terminalis) (Table 8.1-2 and Table 8.1-3) when compared to vehicle treated animals. No effects were seen on the duration, amplitude, and frequency of afterdischarge (Tables 8.1-1, 8.1-2, and 8.1-3). Onset of generalized clonic seizures was closely related to the onset of afterdischarge in the secondary recruiting sites. When afterdischarge was evoked only at the stimulation site (left amygdala), the animals were usually immobilized. Repeated FG 7142 and PTZ treatment resulted in a similar effect on threshold and latency of afterdischarge threshold in the primary site (left amygdala, Table 8.1-1) was not decreased when compared to vehicle treated animals. Figure 8.1-5 represents an example of electrical kindling stimulus evoked electrographic responses recorded from the stimulating site (left amygdala) and the secondary sites (right amygdala and left bed nucleus) of TMPP treated animals.



Figure 8.1-1. Continuous recording of electrical activities from left bed nucleus (top traces) and right amygdala (bottom traces) of one rat upon electrical stimulation of left amygdala (single pulse, 0.1 ms duration at 560 μA for a and 280 μA for b, respectively) before (a) and after six weeks of TMPP (0.275 mg/kg, i.p.) treatment (b). Downward and upward deflections in (a) and (b), stimulating artifact.

TABLE 8.1-1.	MEAN AFTERDISCHARGE (AD) THRESHOLD, AD DURATION, AD AMPLITUDE, AD
	FREQUENCY, AND LATENCY ONSET AD FROM LEFT AMYGDALA IN RESPONSE TO
	LEFT AMYGDALA STIMULATIONS IN TMPP, PTZ, FG7142, AND VEHICLE TREATED
	ANIMALS

	TMPP Mean±SEM (n=8)	PTZ Mean±SEM (n=6)	FG7142 Mean±SEM (n=5)	Vehicle Mean±SEM (n=5)
AD Threshold (µAmps)	245.0±35.0*	552.0±73.1	196.0±34.2*	397.0±37.9
AD Duration (s)	24.5±7.5	74.0±31.0	39.8±25.8	21.8±7.4
AD Amplitude (µV)	657.5±120.6	380.0±112.4	1166.0±392.7	850.0±272.3
AD Frequency (Hz)	2.3±0.6	2.2±0.6	3.4±1.0	2.9±0.8
Latency Onset AD (s)	0.6±0.3**	0.4±0.2**	0.5±0.2**	2.5±0.5

* or ** indicates significantly different from vehicle treated group at the level of p < 0.05 or p < 0.01, respectively, by paired t test analysis.

TABLE 8.1-2.MEAN AFTERDISCHARGE (AD) THRESHOLD, AD DURATION, AD AMPLITUDE, AD
FREQUENCY, AND LATENCY ONSET AD FROM *RIGHT AMYGDALA* IN RESPONSE TO
LEFT AMYGDALA STIMULATIONS IN TMPP, PTZ, FG7142, AND VEHICLE TREATED
ANIMALS

	TMPP Mean±SEM (n=8)	PTZ Mean±SEM (n=6)	FG7142 Mean±SEM (n=5)	Vehicle Mean±SEM (n=5)
AD Threshold (µAmps)	610.0±85.4**	600.0±88.5**	408.0±73.1**	1122.0±122.6
AD Duration (s)	57.5±18.1	100.6±33.7*	54.4±13.5	22.0±1.9
AD Amplitude (µV)	825.0±92.4**	507.6±95.0	423.0±35.3	262.5±45.1
AD Frequency (Hz)	4.25±0.7	3.34±0.9	5.7±0.9	4.0±0.8
Latency Onset AD (s)	2.0±0.5**	7.1±1.9**	26.9±7.9	24.3±2.6

* or ** indicates significantly different from vehicle treated group at the level of p < 0.05 or p < 0.01, respectively, by paired t test analysis.

TABLE 8.1-3.MEAN AFTERDISCHARGE (AD) THRESHOLD, AD DURATION, AD AMPLITUDE, AD
FREQUENCY, AND LATENCY ONSET AD FROM LEFT BED NUCLEUS IN RESPONSE TO
LEFT AMYGDALA STIMULATIONS IN TMPP, PTZ, FG7142, AND VEHICLE TREATED
ANIMALS

	TMPP Mean±SEM	PTZ Mean±SEM (n=6)	FG7142 Mean±SEM	Vehicle Mean±SEM
	(n=8)		(n=5)	(n=5)
AD Threshold (µAmps)	440.0±42.9**	496.0±69.9**	280.0±9.0**	915.0±96.0
AD Duration (s)	15.5±3.2	45.8±21.0	44.0±24.8	15.8±4.6
AD Amplitude (μ V)	387.5±112.5	306.0±87.9	233.0±39.2	475.0±242.8
AD Frequency (Hz)	3.3±0.9	2.4±0.5	4.2±1.2	3.2±1.1
Latency Onset AD (s)	2.5±0.5**	7.9±1.3	2.0±0.2**	7.3±0.3

* or ** indicates significantly different from vehicle treated group at the level of p < 0.05 or p < 0.01, respectively, by paired t test analysis.

DISCUSSION

The data show that repeated systemic administration of low doses of TMPP, PTZ, or FG7142 produced an increase in behavioral and electrographic sensitivity over time. A chemical kindling mechanism for the increased sensitivity of animals to repeated administration of TMPP, PTZ, or FG 7142 is supported by the increased incidence of seizure behavior over the treatment period. Sensitization of the amygdala complex and the connecting structures is indicated by the conversion of single pulse stimulation evoked field potentials to epileptiform discharge after repeated exposure of animals to TMPP, PTZ, or FG 7142. Persistent sensitization of the amygdala complex and facilitation of amygdala kindling are also indicated by increased sensitivity to electrical kindling stimulation of left amygdala in animals treated with TMPP, PTZ, or FG 7142 and rested for up to 4 weeks without additional dosing. Facilitation of electrical kindling of the amygdala in a similar proconvulsant paradigm has been demonstrated with a variety of pharmacological agents including PTZ, FG 7142, bicuculline, cocaine, and lidocaine (Bowyer and Albertson, 1982; Corda et al., 1986; Joy, 1985; Kalichman et al., 1981; Kilbey et al., 1979; Kokkinids and McCarter, 1990 and Weiss et al., 1990) and pesticides like endosulfan, amitraz, chlordimeform, dieldrin, and lindane (Gilbert, 1988; Gilbert, 1992; Gilbert, 1995; Gilbert and Mack, 1989; Joy et al., 1980; Joy et al., 1983). The mechanism whereby most of the chemicals mentioned above are thought to be related to their interaction with GABAergic system. Although the mechanism of TMPP action is presently unknown, its convulsant properties were tied to an action on GABAergic inhibitory function in the CNS. TMPP has been shown to interact with GABA₄-benzodiazepine receptor complex in rats (Ritchie et al., 1995). PTZ and FG7142 are known GABA antagonists. It is not surprising to find TMPP, PTZ, and FG 7142 exhibiting a similar result.

As is the case with TMPP, most of the GABA antagonists like PTZ (Cain, 1981; Cain, 1982; Mason and Cooper, 1972; Nutt et al., 1982), picrotoxin (Cain, 1987; Corda et al., 1990), bicuculline (Uemura and Kimura, 1990), and GABA-benzodiazepine receptor ligand FG 7142 (Little et al., 1984) are also proconvulsant to electrical kindling when administered prior to each stimulation. Theses drugs are readily cleared from the body (elimination half-life of 30 min to 3 h) and thus are not likely promoting seizures by accumulation in the brain or other tissue with repeated administration (Hardman et al., 1996). It is known that the elimination half-life of TMPP in the brain has been estimated to be 6 h. Repeated daily exposure of animals with TMPP 0.5 mg/kg did not result an accumulation of TMPP in the brain or other tissues. Also, no TMPP metabolites were detected during and after the course of clearance (Jung et al., 1995). Thus, it suggests that sensitization of amygdala complex by TMPP was neither due to the bioaccumulation of the TMPP nor due to TMPP metabolites. The evidence of a persistent sensitization to electrical stimulation of amygdala and electrical kindling of amygdala was present long after the chemical had cleared out of the body, indicating that increases in amygdala complex excitability produced by TMPP are not simply temporarily augmenting the response to stimulation, but may be due to more complicated evolution of the sensitization process.

In conclusion, our results indicate that repeated intermittent exposure of rats to low doses of GABAbenzodiazepine receptor antagonist TMPP, PTZ, or FG-7142 induces sensitization of amygdaloid complex and connecting structures which indicated by gradual development of sensitization to electrical stimulation and long lasting proconvulsant ability to electrical kindling of this pathway. Although the mechanism of TMPP proconvulsant property and neurotoxicity is unknown, the results also indicate that TMPP may play a similar role with GABA-benzodiazepine receptor antagonist PTZ and FG7142 on inducing seizure activity by sensitizing the amygdaloid complex and pathways.

Acknowledgments

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Figure 8.1-2. Epileptic discharges recorded from left bed nucleus (a) and right amygdala (b) and myoclonic jerks evoked by single pulse electrical stimulation of left amygdala 24 h following TMPP (0.275 mg/kg), PTZ (20 mg/kg), or FG 7142 (7.5 mg/kg) treatment. Height of columns represents percentage of animals tested exhibiting epileptic discharges (a, b) or myoclonic jerks (c). Abscissa, number of treatment in doses.





Figure 8.1-3. Epileptic discharges from left bed nucleus, right amygdala, and myoclonic jerk behavior recorded 24 h after TMPP (0.275 mg/kg), PTZ (20 mg/kg), or FG 7142 (7.5 mg/kg) treatment upon single pulse electrical stimulation of left amygdala. Height of column represents percentage of animals in total which exhibited epileptic after discharge or myocolonic jerks in ten weeks (30 doses) of treatment.



Figure 8.1-4. Cumulative incidence of colonic seizures (a) and myoclonic jerks in animals treated triweekly with TMPP (0.275 mg/kg), PTZ (20 mg/kg), FG 7142 (7.5 mg/kg), or vehicle (0.5 ml/kg). Ordinate, percentage of animals tested. Abscissa, number of treatment in doses. Behavioral observations were performed for 1 h after each dosing.



Figure 8.1-5. Continuous recording of EEG from left bed nucleus (LBN, top traces), right amygdala (RAD, middle traces) and left amygdala (LAD, bottom traces) of rats 4 weeks after the last dose of triweekly TMPP (0.275 mg/kg) treatment for 10 weeks following a 60 Hz, 2 sec. train stimulation of LAD (280 μ A, 0.1 ms duration). Note: the stimulating artifacts were truncated.

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SECTION 9

1

METHODS DEVELOPMENT

9.1 CONFOCAL MICROSCOPIC ANALYSIS OF THE REGION OF APOPTOSIS IN MURINE LIMB BUDS FOLLOWING EXPOSURE TO ALL-TRANS RETINOIC ACID

L.J. Graeter, K.W. Dean¹, D.A. Warren¹, and J.R. Latendresse

ABSTRACT

Confocal microscopy techniques are powerful tools when used to assess biological events in living tissue. The goal of this study was to use this novel approach in evaluating the region of cell death in murine limb buds following exposure to retinoic acid (RA). Pregnant dams were orally dosed with 0 or 60 mg/kg all-trans RA on gestation day 11. Live embryos were harvested 24 hours post dosing, and placed immediately into culture in medium containing 25μ g/mL propidium iodide (PI) for 30 minutes. A Meridian InSight-IQ Laser scanning confocal microscope (LSCM) was used to analyze the region of the forelimbs stained with PI, which has an excitation/emission spectrum of 536/617, respectively. We used the following LSCM parameters: an argon laser setting of 40 mW, a green 514 dichroic cube, and a 605LP emission filter. Analysis was accomplished by discrete image analysis with the Meridian InSight-IQ V1.2 software. The intensities/area were 7292/µm², 3127/µm², and 1291/µm² at 4 h post dose for 100, 60, and 0 mg/kg RA, respectively; 12798/µm², 2111/µm², and 170/µm² at 24 h post dose of 100, 60, 0 mg/kg RA, respectively. Staining was predominant in the base of the limb buds, proximal to the large artery and distal to the nerve trunk that extends into the limb bud. There was negligible staining in this region of the forelimbs in the control group. The region of cell death in the limb buds correlates with the region of cell death we have previously seen in hematoxylin/eosin stained paraffin sections. These data suggest that LSCM is a useful technique in evaluating cell death in limb bud tissue.

INTRODUCTION

The number of female military personnel is increasing, as well as the scope of their duty assignments. Some of the compounds that women may be occupationally exposed to are possible teratogens. Chemical contamination of military installations may pose an additional threat to the civilian population. It is important for the DoD to address the reproductive/developmental health of these populations. The development of a biologically based model that will aid in determining the risk associated with exposure of a compound on the developing fetus will provide a useful tool for risk assessment and in setting guidelines for clean-up of contaminated sites.

¹Armstrong Laboratory, Toxicology Division, Wright-Patterson Air Force Base, OH.

All-Trans Retinoic acid (RA) is a well characterized teratogen; therefore, it was used as a model compound in this study. Accurate measurements of biological endpoints following exposure to RA will aid in the development of the model.

RA is a well-characterized teratogen when a fetus is exposed to high concentrations during embryogenesis. Limb dysmorphogenesis is only one of many malformations that RA is known to mediate. Vertebrate limbs are of mesenchymal origin; a limb arises from a small bud of mesenchymal tissue enclosed in an epithelial sheath on the embryonic flank. The epithelium at the limb bud margin forms the apical ectodermal ridge; after the ridge is formed, the bud elongates and the mesenchyme terminally differentiates into bone, cartilage, muscle, and epithelium that form the limb (Eichele, 1989). Programmed cell death (PCD), or apoptosis, is a normal component of limb formation. However, exposure to high concentrations of RA alters the pattern of apoptosis resulting in varying degrees of limb dysmorphogenesis. One theory is that RA causes an expansion of the region of PCD within the limb bud, thereby inducing limb malformations (Mendelsohn et al., 1992). Historically, levels of apoptosis in tissues were analyzed by immunohistochemical staining or histopathological evaluation of embedded, sectioned, and stained slides. Levels of tissue tranglutaminase have also been used to assess the degree of apoptosis. These methods do not provide an accurate measure of the expanded region of apoptosis in the limb bud following exposure to RA. Confocal microscopy (CM) offers a novel approach in solving this problem. The region of apoptosis (PCD) in the limb bud will be fluorescently stained. Optical sections will be obtained through the entire depth of the bud. The optical sections will be reconstructed into a 3-D image and the region of apoptosis (PCD) quantitated.

MATERIALS AND METHODS

Timed-pregnant female Crl:CD-1[®](ICR)BR mice were ordered from Charles River Laboratories to arrive at WPAFB on gestation day 8. The females were dosed via oral gavage with 0, 60, or 100 mg/kg RA in a vegetable oil vehicle on gestation day 10.75.

The females were sacrificed 4 or 24 h post dosing via cervical dislocation. The abdominal cavity was opened and the uterine horns removed to a sterile petri dish. A slit was made with microscissors in the uterus at each implantation site. The fetus was removed and rinsed in sterile PBS. The fetus was placed in 0.25 g propidium iodide/mL PBS for 45 min at 37°C. The fetus was rinsed in two washes of PBS and placed in a 25% glycerol/PBS solution.

Confocal Microscopy: The fetus was placed in a hanging drop slide and coverslipped for CM scanning. Optical sections were obtained at 5 micron increments through the forelimb bud. The CM settings were: an argon laser set at 40mW, a 605LP emission filter, and a 488 blue dichroic mirror.

Electron Microscopy: Six limb buds/dose group were submitted for electron microscopy for ultrastructural analysis of the apoptotic region.

Image Analysis: Image analysis will be used to reconstruct the confocal microscopy optical sections (z-scans) into a 3-D image. The volume of the apoptotic region in the limb bud will be quantitated.

RESULTS

The effects of all-trans retinoic acid on the region of cell death in the developing limb buds was dose- and timedependent. Fluorescently stained regions of the limb buds included portions of the apical ridge, and regions of the base of the limb bud; the area of the stained region at the base of the limb bud increased with dose over time (Tables 9.1-1 and 9.1-2). Preliminary data suggest that minimal staining is present in this region in control limb buds (Figure 9.1-1). This region was stained at 4 and 24 h in the 60 and 100 mg/kg groups (Figures 9.1-2 and 9.1-3). There was a significant increase in the area of the stained region in the 100 mg/kg group when comparing the 4- and 24-h time points. By contrast, there was not an increase in the 60 mg/kg group. The area of cell death in the two treated groups was significantly larger than the control level at both time points.

The PI-stained regions of the limb bud correlate with the region of apoptosis noted in hematoxylin and eosin stained paraffin sections (data not shown). The PI-stained area of the limb bud is dependent upon depth as illustrated in Figure 9.1-4. This figure shows a z-scan series through the limb bud, and the changing area of cell death.

Additional experiments are underway. Methodology for image analysis of the z-scans is under development (Stevens, 1994). The z-scan series will be reconstructed into a 3-D image using the Silicon Graphics Workstation. The volume of the fluorescently stained region will be quantitated.



Figure 9.1-1. Limb bud from a control animal 24 h post vehicle dosing.



Figure 9.1-2. Stained region at the base of a limb bud 24 h after dosing with 60 mg/kg RA.



Figure 9.1-3. Stained region at the base of the limb bud 24 h after dosing with 100 mg/kg RA.



Figure 9.1-4A. Z-scan of a limb bud harvested at 4 h at 0 μ m Figure 9.1-4B. Z-scan of the same limb bud at 200 μ m Figure 9.1-4C. Z-scan at 400 μ m

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	Control	60 mg/kg	100 mg/kg
Intensity/ micron ²	5120	3556	7279
	2477	3339	2693
	0	4800	3481
	150	2395	10221
	0	645	12785
		6009	
		1146	
Mean	 1291	3127	7291
SEM	864	723	1929

TABLE 9.1-1. FLUORESCENT INTENSITY/MICRON² IN MURINE FORELIMB BUDS 4 HOURS POST DOSE

TABLE 9.1-2.FLUORESCENT INTENSITY/MICRON² IN MURINE FORELIMB BUDS24 HOURS POST DOSE

۶	Control	60 mg/kg	100 mg/kg
Intensity/ micron ²	678	1966	1539
	0	1206	9141
	0	4604	12467
		1684	16851
		1373	23994
		1838	
Mean	170	2111	12798
SEM	170	512	3755

DISCUSSION

Confocal microscopy offers a novel approach in the assessment of biological events in tissue samples. In these experiments, CM was used to evaluate the region of cell death in developing murine forelimb buds following maternal exposure to RA. Traditionally, apoptosis has been evaluated by immunohistochemical techniques using embedded and sectioned samples or by monitoring the level of tissue transglutaminase, an enzyme induced during the apoptotic process. The level of apoptosis in the limb bud is dependent upon the location within the bud.

Therefore, traditional methods such as 2-D analysis of a 5-micron thick section or biochemical analysis may not provide the most accurate assessment of the effects of a toxicant on the region of apoptosis.

In this study the fluorescent fluorophore propidium iodide (PI) was used to tag the apoptotic region. There were significant differences in the regions of cell death in both treated groups compared to the control groups at both time points, suggesting that RA does cause an expansion in the area of programmed cell death. This effect on the area of PCD appears to be dose-dependent. In the 'Biologically Based Dose-Response Modeling of Retinoic Acid-Induced Limb Dysmorphogenesis in the Developing Mouse' project currently underway at the Toxicology Division, apoptosis was evaluated using hematoxylin and eosin-stained paraffin sections. The region of apoptosis noted on the histological slides correlates with the PI-stained region of the CM images. However, PI stains cells whose membranes are damaged, so it does not distinguish between necrotic and apoptotic cells. Electron microscopy studies are underway to confirm the region of cell death as apoptosis.

Additional animal exposures are in progress. The limb buds were optically sectioned via confocal microscopy. The resulting optical sections will be reconstructed into 3-D images; image analysis techniques will be used to quantitate the volume of the apoptotic region.

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SECTION 10

CONFERENCE SUPPORT

10.1 1996 TRI-SERVICE TOXICOLOGY CONFERENCE ON ADVANCES IN TOXICOLOGY AND APPLICATIONS TO RISK ASSESSMENT

L.A. Doncaster

The Conference on Advances in Toxicology and Applications to Risk Assessment was held at the Hope Hotel and Conference Center, Wright-Patterson Air Force Base, OH, from 23 through 25 April 1996. The conference was sponsored by Tri-Service Toxicology, Wright-Patterson Air Force Base; the Office of Research and Development, 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.

The goals of the conference were (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.

Session I of the conference dealt with the Environmental Risk Assessment Program (ERAP) and included presentations on an overview of the ERAP (W.B. Peirano); assessment of environmental hazards of 1,3,5-trinitrobenzene (G. Reddy/H. Choudhury/T.V. Reddy); trichloroethylene - what are the appropriate standards (E.A. Maull); an evaluation of the effects of ionizing radiation on terrestrial biota - implications for their protection (L.W. Barnthouse/H. Beckert)); human health risk assessment for petroleum hydrocarbon contaminated sites (W.H. Weisman, Jr.); and an overview of the Rocket Emissions Group (REWG) (J.P. Hinz).

The subject of Session II was ecological risk - assessment, incorporation, and application. Included were discussions on the tri-service coordination of ecological risk assessment activities (R.S. Wentsel); the Army Corps ecological risk assessment guidance document (T.L. Walker); application of ecological risk assessments and case studies in the Army (J.E. Whaley/M.S. Johnson/L.V. Tannenbaum); ecological risk assessment applications in the U.S. Air Force (R.C. Porter); a perspective on ecological risk assessment at EPA (S.B. Norton); and using ecological risk assessment as a tool in environmental decisionmaking - a case study of the National Gypsy Moth Management Program (L.C. Abbott).

Session III concerned biologically based modeling applications in risk assessment of toxic substances and included discussions on the application of pharmacokinetic and dose-response modeling in noncancer risk

assessment (H.J. Clewell III); bromodichloromethane - cancer risks, mechanisms, and modeling approaches (R.A. Pegram); physiological "constants" for PBPK models for pregnancy (J.F. Young); and filling data gaps with physiologically based pharmacokinetic models (R.H. Reitz).

Presentations in Session IV dealt with risk-based guidelines (applications of reference values) and included discussions on the National Center for Environmental Assessment research with uncertainty factors (T. Harvey); ADI, BMD, CEL - the alphabet soup of methods for dose-response assessment (M.L. Dourson); an overview of the revisions to the Exposure Factors Handbook (J. Moya); the role of health guidance values in public health practice (C.T. DeRosa); and recent advances in quantitative noncancer risk assessment methods - toxicological and mechanistic considerations (E.M. Faustman).

Session V discussed risk communication in the federal government and included presentations on environmental risk communication (S.E. Williams); a federal perspective on the role of risk communication policies and practices (T.L. Tinker); methyl parathion contamination of private residences - risk assessment, risk management, and risk communication approaches (J.M. Clark); chemical UXO on the installation boundary - dealing with public concerns (J.T. Paul, Jr.); when simple language fails - addressing lay theories through risk communication (K.E. Rowan); and risk communication in the federal government - a citizen's perspective (M.T. Flickinger).

Evening poster and database sessions provided additional scientific information exchange in an informal manner. There were 27 oral presentations, 55 poster presentations, four database presentations, and 292 participants in the three-day conference. The conference was highly rated by the participants. Proceedings of the conference will be published as a technical report.

10.2 1997 TRI-SERVICE TOXICOLOGY CONFERENCE ON ISSUES AND APPLICATIONS IN TOXICOLOGY AND RISK ASSESSMENT

L.A. Doncaster

Planning was initiated in May 1996 for the 1997 toxicology conference, "Conference on Issues and Applications in Toxicology and Risk Assessment". It will be held 7 through 10 April 1997 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 ManTech Environmental Toxic Hazards Research.

The goals of the conference are (1) exploration of new methodologies for combustion, GI tract, and developmental toxicology; (2) application of guidelines in the risk assessment process; and (3) examination of the issues and approaches for ecotoxicology and pollution prevention.

The ManTech Environmental 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.

An initial conference announcement has been placed in a major scientific journal and the conference announcement has been mailed to over 3100 individuals.

SECTION 11

RESEARCH SUPPORT

11.1 RESEARCH ENGINEERING/FABRICATION SPECIAL PROJECTS

W.B. Sonntag

Construction of Organ Bath and Rings

The Navy Neurobehavioral Unit requested the design and fabrication of two organ baths. The baths are used to investigate organ tissue in process under a microscope. The rings are to secure the organ tissue in place. The plexiglas baths are $(1 \ 3/4 \ in. x \ 1 \ 2 \ in.)$ with three holes on top connected by a series of channels and canals. The first hole (2.8 mm diameter x 3.5 mm depth), borders with the second hole (12.5 mm diameter x 5 mm depth), and are connected at the bottom with a 2 mm channel for liquid flow. The third hole (4 mm diameter x 2.5 mm depth) is connected to the second hole with a canal to keep constant flow. The liquid is extracted with a 2-mm tube channel on the opposite side of the bath and runs to the bottom of the third hole.

Construction of a Hydra Farm

The Research Engineering/Fabrication group (RE/F) was tasked to construct a hydra farm. The task consisted of the construction of farm screens and a newly designed farm to correct problems encountered with the first farms.

The hydra farm consisted of a plexiglas aquarium (2 in. x 13 in. x 48 in.) standing on 8 in. legs (changed from 6 in.), and was constructed to house hydra in a clean, natural environment. On one end, two ports allowed water to enter the farm where a deflector screen slowed the flow of the water into the farm. A dam placed in back of the deflector screen allowed the stream of water to run smoothly into the aquarium and not disturb the hydra. Constant fresh air was 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 gate's foundation was fabricated out of 1/2 in.-thick plexiglas (to correct the problem of cracking) and was secured to the aquarium with glue and screws. The end also had a 1/2 in.-thick piece of plexiglas (the same width as the first leg) to solve the problem of cracking.

Hydra require a cool environment to thrive. A constant-flow water chamber was fabricated underneath the aquarium to use as an emergency cooling system. This aquarium was tested, and the problems were corrected.

Fabrication of a Stainless Steel Nose-Only Inhalation Chamber

The Advanced Composite Combustion Group requested the RE/F to design and fabricate a stainless-steel nose-only inhalation chamber. This chamber is similar to a Cannon chamber and was connected in-line with the combustion atmosphere inhalation exposure system.

The main chamber body (3.5 in. x 3.0 in.) was constructed with 12 ports in 3 sets that are 90° apart and offset 45° . There were three 1/8 in.-pipe ports; one on top for a thermocouple and two on the bottom for collecting off-gassing and a thermocouple. The airtight inhalation system has an intake flow going into a 1-inch stainless-steel inner tube that follows an in-line port matching the outside chamber and connected by ¹/₄-in. tubes.

Plethysmographs were linked together and exhausted through the bottom of the chamber. The chamber is attached to a 6-in. high turntable stand which allows the chamber to rotate 360°. Two Legris fittings are used to join this system to the combustion inhalation unit.

Improved Rat Plethysmograph

Several Tri-Service Toxicology investigations required the design and development of an improved plethysmograph that would: 1) provide a less-stressful confinement of animals, 2) allow easier training of animals, and 3) the ability to attach the chest capsule design to the Cannon chamber. A new space for the forelimbs directly below the head provided animals less resistance and prevented animals use of their forelegs to push backwards. The new design also provided more room around the animals' eyes and ears, plus had several different size inserts to accommodate different animal sizes.

The design of this plethysmograph had four parts: head chamber, chest capsule, body chamber, and tail cap. The nose cap has been fabricated to fit snugly in a Cannon chamber with a 90° flattened surface to keep the plethysmograph from slipping out. The inside head space had a long taper to create space for the eyes and ears. The outside area was cut to allow adequate space for toes, to lock the forearms in place, and to seal it to the chest capsule.

The chest capsule gave the rat enough area so it is not constrictive, yet the animal remained confined. The spacers fit into the back of the capsule to size the animal properly. In the middle of the capsule, a taper was added causing the animal to step down and maintaining the forelegs directly below the animal's head, allowing locking with the head chamber.

The body chamber fits air-tight over the chest capsule to help size the animals comfortably. This part also has all the ports that connect to the instrumentation needed for each project. The tail cap connects to the body chamber with a hollow push rod with nylon inserts to adjust the animal securely. This rod also acts as the temperature control for the rodents.

Rodents freely enter the chamber and place their heads and forelimbs through the taper. The handler can control the animal by grasping it firmly at the base of the tail while loading. When the head and forelimbs are in place, the handler slowly connects the head cap making sure the animal's feet and legs are in place; then puts the tail cap on, making sure to seal the tail area.
11.2 MATHEMATICAL AND STATISTICAL SUPPORT

C.D. Flemming

The THRU's Biometry staff was actively involved in the support of several research projects. All protocols which were produced by the contractors or Air Force personnel were read and signed by the statistician. Thus, the statistician adviced investigators in the statistical design of most studies conducted by AL/OET. Sample size for new protocols were found by power analysis.

Programming in FORTRAN, BMDP, and RPL was done when required. Ten percent of the time was used for programming. Most of the programming was performed to transform data.

Informal training in mathematics, statistics, programming, and computers was provided to individuals in the laboratory. A formal course was offered three times on "Experimental Design for Research and Development".

The statistician served on two Air Force Institute of Technology student thesis committees. The statistician was the statistical advisor for one of the students.

The statistician has been asked by various laboratory personnel to evaluate the statistical validity of research papers which they were asked to review. The statistician was also requested to evaluate various research papers.

11.3 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 animals. Necropsy procedures included determination of terminal body weights, detailed dissection, weighing of required organs, and collection and fixation of gross lesions and other required tissues for light microscopic examination.

Histologic 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 continue to implement their 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.

Processing of tissues for transmission electron microscopy included trimming, fixation, en-block staining, dehydration, plastic embedding, microtomy, and ultramicrotomy of tissues to specified thickness, and routine and advanced post-staining of tissue sections. Specimen processing for scanning electron microscopy and X-ray microanalysis included fixation, dehydration, critical-point drying, and gold sputtering or carbon coating. Specimens were examined on a JEOL 1200 TEM or AMRAY 1000B SEM, and resulting micrographs were reviewed by veterinary pathologists.

The electron microscopy laboratory continues to function as a diagnostic and research support tool in support of several current research projects. EM personnel provided various ultrastructural and X-ray microanalysis support for the following projects:

- 1) Peroxisome proliferation in $B_6C_3F_1$ mouse hepatocytes following exposure to trichloroethylene.
- 2) Confirmation of apoptosis in developing mouse limb buds following exposure to retinoic acid.

- SEM analysis of smoke residue from the combustion of advanced composite materials (ACM) including particle shape and size analysis and X-ray microanalysis.
- 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) Predictive Toxicology: TEM analysis of ultrastructural damage to perfused rat livers.

Necropsy, histology, and electron microscopy personnel continue to play a significant role in keeping our laboratories in 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, and helped facilitate the ongoing transfer of all HM to a centralized issue point (AFMC Pharmacy concept). Pathology staff members processed the following research animals and provided the following research support during the past reporting period (Tables 11.3-1 through 11.3-4).

TABLE 11.3-1.	TOTAL ANIMALS LISTED BY SPECIES AND RESULTING NUMBER	: OF
	SLIDES	

Species	Animals	Slides
Rat	746	4075
Mouse	314	4953
G. Pig	96	195
Ferret	4	231
Monkey	2	61
Cat		82
Gerbil	1	30
Human	3	17
Rabbit	19	
Dog		4
Fish		1

	Number of Animals	Number of Slides
Date	(in Species)	(in Type)
October 1995	92 Rat	158 Rat
	59 G. Pig	20 G. Pig
	1 Rabbit	33 Mouse
		37 Monkey
		17 Human
		7 Cat
November 1995	64 Rat	290 Rat
	10 Mouse	9 Mouse
		44 G Pig
		17 Ferret
		1710100
December 1005	103 Pat	180 Pat
Detember 1995	175 Kat	
	22 G Big	29 Mouse
	22 G. Fig	of G. Fig
	18 Rabbit	60 ML MA 401
	I Ferrei	
		2 Dog
January 1006	52 Pat	547 Pat
January 1990	J2 Kat	27 Mouro
	1 Monkey	57 Mouse
	1 Wonkey	
	====	
		i Fish
		2 Special
February 1006	13 Pat	674 Rat
1 coluary 1990	12 Mouse	24 Mouse
March 1996	47 Rat	919 Rat
	59 Mouse	90 Mouse
4	13 G. Pig	9 G. Pig
		24 Monkey
		5 Special
April 1996	41 Rat	52 Rat
	46 Mouse	30 Mouse
		2 Dog
		11 Special
		14 Special

TABLE 11.3-2. NUMBER OF ANIMALS PROCESSED BY MONTH AND RESULTING NUMBER OF SLIDES

	Number of Animals	Number of Slides
Date	(in Species)	(in Type)
May 1996	14 Rat	193 Rat
-	12 Mouse	86 Mouse
	1 Ferret	200 Ferret
June 1996	20 Rat	158 Rat
	41 Mouse	10 Mouse
	1 Ferret	7 Ferret
		7 Gerbil
July 1996	19 Rat	
	41 Mice	3520
	1 Gerbil	
	1 Ferret	
August 1996	134 Rat	764 Rat
	49 Mouse	1019 Mouse
	1 G. Pig	
	1 Ferret	7 Ferret
Sentember 1006	27 Pat	/ 1/0 Rat
September 1990	14 Mouse	66 Mouse
	1 G Pig	
	1 Monkey	
	3 Human	
		23 Gerbil
		75 Cat

TABLE 11.3-2. NUMBER OF ANIMALS PROCESSED BY MONTH AND RESULTING NUMBER OF SLIDES (Cont'd)

Project Title	Sample Type	Number of Samples	Number of Micrographs	Number of Spectra
CeramicsWSU/UD	Bioceramics	8	32	4
ACM Combustion	ACM Smoke Residue	5	27	N 22 2 2
SEM Tours/ Training	Various	8	12	12
SEM Maint./Calib.	Standards	6	70	10
TOTALS		27	141	26

TABLE 11.3-3. SEM SUPPORT LISTED BY PROJECT

TABLE 11.3-4. TEM SUPPORT LISTED BY PROJECT

	Section Trees	Number of	Number of	Number of
Project 1 itle	Specimen Type	Specimens	Shaes	Micrographs
04F	Mouse Liver	1230	183	332
11F	Mouse Embryo Limb Buds	120	20	++
09F	Rat Liver	290	35	+-+
05N	Mouse Liver	60	23	35
TEM Tech. Training	Various	6		
TEM Maint./Calib.	Standards	2		20
TOTALS		1708	261	387

note: ++ denotes on-going study

11.4 QUALITY ASSURANCE

M.G. 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 where training covering auditing clinical and pathology aspects of GLP studies was received. The QA Associate (QAA) participated on the program management team during the Conference on Risk Assessment for Sensitive Human Populations.

SOP ACTIVITIES

The QAA headed an Ad-Hoc Committee of the Group Administrators which developed policy and procedures for SOPs for the Toxicology Division. Twelve new or revised SOPs were reviewed, prepared for approval, distributed, and placed on the Division LAN system. This allowed SOP access to all personnel.

STUDY REQUEST AUDITS

The QAC conducted protocol reviews, and procedure, data, and final report audits for the following studies:

Unicharge Toxicity Decontamination Solution Toxicity Reference Dose Level JP-4 Ammonium Dinitramide Toxicity CF₃I Subchronic Toxicity Halon Replacement Quadricyclance Toxicity Advanced Composite Materials Projects Dermal Hazard of Modular Artillery Charge System PBr₃ Toxicity

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TECHNICAL EDITING

The QAA was principal editor for the 1995 THRU Annual Report and coordinated processing of the annual report by ManTech Technical Publications. Seven manuscripts for peer-reviewed publication were edited by the QAA for the following studies:

Pharmacodynamic Description of Biological Effects Nitrates Biological Effects of TCE PKPD Description of Biological Effects Biological Effects of TCE Predictive Toxicity

Eight technical reports were edited by the QAA for the following studies:

ADN SIDS Reproductive Nitrates Unicharge Acute Toxicity Pharmacodynamic Description of Biological Effects JP8 + 100 Toxicity PKPD Description of Biological Effects Quadricyclane Toxicity Defense Women's Health Research Program

11.5 HEALTH & SAFETY

M.G. Schneider

GENERAL

The Health and Safety Representative (HSR) provided occupational health and safety support for ManTech staff and was the Unit Safety Representative for AL/OET, Toxicology Division.

TRAINING

ManTech staff who worked in laboratory areas received annual 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. ManTech staff who were designated as hazardous waste initial accumulation point (IAP) managers or accumulation site (ACCS) managers received annual training provided by WPAFB Environmental Management for Environmental Protection Agency Resource Conservation Recovery Act (RCRA) hazardous waste and biannual training on Department of Transportation (DOT) hazardous materials transportation regulations. The HSR provided safety orientation training for all new staff (DoD and Contractor) assigned to the Toxicology Division. The HSR received special training in explosive safety, radiation safety, Environmental Management hazardous waste regulations (RCRA 8-hour), DOT hazardous material regulations, Environmental Management 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 completed. The HSR completed the turn-in process for excess hazardous material (QTIP) being stored in the Toxicology Division. The HSR completed the annual review and revisions of the Chemical Hygiene Plan and Bloodborne Pathogen Exposure Control Plan to comply with OSHA and Air Force regulations.

EXPLOSIVES SAFETY

The HSR was assigned responsibility as alternate for the AL/OET explosives safety program. Each item stored in the facility required licensing by WPAFB. The HSR assisted in the training of the investigators and technicians using explosive materials. All class 1.1 and 1.2 explosives were moved to secure storage outside the Toxicology Division. This had minimal impact on investigator access to the materials for toxicity testing purposes.

HAZARDOUS WASTE

The HSR closed operation of the accumulation site located in Building 77. He coordinated the removal of the 130 containers of hazardous waste to the WPAFB storage facility.

PERSONNEL SAFETY

ManTech 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 and exit), follow-up examinations on work-related injury or illness for work release, consultation for pregnant staff, or suspected work-related illness, and initiation of Workers Compensation paperwork was continued. Annual physicals for staff working in laboratories and with animals were provided by the Air Force. Hepatitis B immunization was offered to staff who might encounter bloodborne pathogens in their jobs. One ManTech employee required treatment related to an on-the-job injury.

The HSR coordinated the AL/OET respiratory protection program. DoD personnel were trained and fit tested for respirator wear by Base Occupational Health, while Contractor staff were trained and fit tested by the HSR. Permanent welding permits were established for ManTech shop areas through the WPAFB Fire Department.

INSPECTIONS

Work areas occupied by ManTech staff were subject to annual inspections by Air Force safety personnel from HSC, Brooks AFB, WPAFB (Environmental Management, 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 in its areas of responsibility was always timely. The HSR also conducted spot and monthly safety inspections, including testing eyewashes and showers. The HSR inspected laboratory spill kits semi-annually. Issues of immediate concern were addressed with staff and supervisors on-thespot. Work requests were submitted through the OET Building Superintendent to correct building-related safety issues.

SECTION 12 APPENDICES

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APPENDIX A

MANTECH ENVIRONMENTAL TECHNOLOGY, INC. LIST OF PERSONNEL (SEPTEMBER, 1996)

Angell, MaryAnn Brashear, Wayne, Ph.D. Buttler, Gerry Byczkowski, Janusz, Ph.D. Courson, Dave Dodd, Darol, Ph.D. Doncaster, Lois Ellis, Dave Feldmann, Marcia Flemming, Carlyle Godfrey, Dick Godfrey, Susie Graeter, Linda, Ph.D. Greenberg, Marc Kuhlmann, Karl Lane, John Latendresse, John, Ph.D., D.V.M. Leahy, Harry Lin, Jan, Ph.D. Mahle, Deirdre Malcomb, Willie Neely, Gloria Nicholson, Jerry Parish, Peggy Pollard, Dan Schneider, Matt Sonntag, Bill Vinegar, Al, Ph.D. Wolfe, Robin Zhang, Hong, Ph.D.

GEO-CENTERS, INC., LIST OF PERSONNEL (SEPTEMBER, 1996)

Abbas, Richat, Ph.D. Caracci, Melanie Confer, Patricia Garrett, Carol Geiss, Kevin Grabau, John, D.V.M. McDougal, James, Ph.D. Narayanan, Latha

APPENDIX B

PRODUCTS LIST FOR 1996

October 1, 1995 - September 30, 1996

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PRESENTATIONS AT SCIENTIFIC MEETINGS

INVITED PRESENTATIONS

Byczkowski, Chemically Induced Oxidative Stress, Polish Academy of Science, Warsaw, Poland, 26 Jul 1996.

Byczkowski, Chemically Induced Oxidative Stress, Polish Society of Toxicology, Gdansk, Poland, 30 Jul 1996.

Byczkowski, 10/25/96, Pharmacodynamic Modeling of Chemically-Induced Oxidative Stress, Ohio Valley Society of Toxicology, Indianapolis, IN.

Latendresse, Toxic Responses of the Adrenal Cortex, 1997, to be published by *Comprehensive Toxicology*, Elsevier Science.

Vinegar, Jepson, The PBPK Model: Analysis of Halon Substitutes, 22-25 October 1995, International CFC and Halon Alternatives Conference, Washington, D.C.

Vinegar, Jepson, Blood Concentration Time Courses to Cardiac Sensitization Thresholds Predicted by Physiologically Based Pharmacokinetic Modeling, 12-15 November 1995, 16th Annual Meeting of the American College of Toxicology, Vienna, VA.

Vinegar, Jepson, Pharmacokinetic Modeling and Its Role in Cardiac Sensitization, 18-19 December 1995, U.S. EPA Conference on the Physiological Effects of Alternative Fire Protection Agents, Bethesda, MD.

Vinegar, Jepson, Pharmacokinetics of Hydrofluorocarbons and Hydrochlorofluorocarbons as Related to Cardiac Sensitization, 7-8 March 1996, National Research Council, Committee on Toxicology Workshop: Toxicity of Alternatives to Chlorofluorocarbons (CFCs), Irvine, CA.

ORAL PRESENTATIONS

Garrett, Mahle, Lipscomb, Comparison of the *In Vitro* Metabolism of Trichloroethylene in Three Species: Rat, Mouse, and Human, 1996 SOT.

Caracci, Vinegar, Jepson, Determination of Blood-Air Partition Coefficients for Poorly Soluble Chemicals, SOT 1996.

Caldwell, Grabau, Kuhlmann, Miller, Combustion Products of Advanced Composite Materials, INTERFLAM 96 Fire Science and Eng Conference, St John's College, Cambridge, ENG, 26-28 Mar.

Steel-Goodwin, Music, Dean, Pace, Carmichael, Flemming, Spin Label Assays Using Protein Conjugates to Measure Receptor Binding by Electron Paramagnetic Resonance, SAFMLS Meeting, Washington, DC, 3 Mar.

Vinegar, Jepson, Pharmacokinetics of Hydrofluorocarbons and Hydrochlorofluorocarbons as Related to Cardiac Sensitization, AL/OE-TR-1995-0132, National Research Council Committee on Toxicology Workshop, Mar 7-8, Irvine, CA.

Vinegar, Jepson, Pharmacokinetic Modeling and Its Role in Cardiac Sensitization, 16th Annual Meeting of American College of Toxicology, Vienna, VA, 12-15 Nov.

Vinegar, Jepson, Pharmacokinetic Modeling and Its Role in Cardiac Sensitization, U.S.E.P.A. Conference on the Physiological Effect of Alternative Fire Protect Agents, Bethesda, MD, 18-19 Dec.

POSTER PRESENTATIONS

Abernathy, Flemming, Measurement of Cardiovascular Response of Male Sprague-Dawley Rats to Modular Artillery Charge System Using Radiotelemetric Implants, SOT 1996.

Barton, Frazier, McCafferty, Mahle, Pharmacokinetics of [14C] Trichloroacetate in Male Fischer 344 Rats, SOT 1996, Anaheim, CA, Mar 10-14.

Brashear, Bishop, Abbas, Electrospray Analysis of Biological Samples for Trace Amounts of Trichloroacetic Acid, Dichloroacetic Acid and Monochloroacetic Acid, American Chemical Society Mid-West Regional Meeting, Dayton, OH, 9-12 Jun 1996.

Byczkowski, Seckel, Development of Physiologically Based Pharmacodynamic Model for Ethane Exhalation, American Chemical Society Mid-West Regional Meeting, Jun 96, Dayton, OH.

Byczkowski, Pravecek, Channel, Computer Simulation of Lipid Peroxidation in Precision Cut Mouse Liver Slices and Experimental Calibration of the Mathematical Model, American Chemical Society Mid-West Region Meeting, Jun 96, Dayton, OH.

Byczkowski, Flemming, Computer-Aided Description of Chemically Initiated Oxidative Stress In Vitro, Conference on Advances in Toxicology and Application to Risk Assessment, WPAFB, OH, 23-25 Apr.

Byczkowski, Flemming, Computer-Aided Description of Chemically Initiated Oxidative Stress In Vitro Ohio Valley SOT, Cincinnati, OH, 8 Dec.

Byczkowski, Flemming, Computer-Aided Dose-Response Characteristics of Chemically Initiated Oxidative Stress In Vitro, SOT 1996.

Byczkowski, Flemming, Mathematical Modeling of Oxidative Stress *In Vitro*, American Chemical Society Mid-West Regional Meeting, June 96, Dayton, OH.

Caracci, Vinegar, Jepson, Determination of Blood-Air Partition Coefficients for Poorly Soluble Chemicals, Conference on Advances in Toxicology and Application to Risk Assessment, WPAFB 23-25 Apr. Carmichael, Music, Dean, Flemming, Steel-Goodwin, Changes in Receptor Binding as a Biomarker, 1996 Toxicology Conference, WPAFB, 23 April.

Confer, Buttler, Bandiera, Lipscomb, Trichloroethylene Metabolism in Hepatic Microsomal and S9 Protein of the Japanese Medaka, Conference on Advances in Toxicology and Applications to Risk Assessment, WPAFB, 23-25 Apr.

Confer, Buttler, Bandiera, Lipscomb, Trichloroethylene Metabolism in Hepatic Microsomal and S9 Protein of the Japanese Medaka, 28th ACS Central Regional Meeting, Dayton, OH, 9-12 Jun.

Courson, Malcomb, Walsh, Improved Combustion Toxicology Testing through Automated Data Acquisition on Control, 1996 Annual Meeting of Society of Tribologists & Lub. Eng., Cincinnati, OH, Mar 96.

Ellis, Dodd, Wolfe, Weisman, Seven Day Dose Range Finding Studies for Toxicity Assessment of Long Chain Petroleum Hydrocarbons, Conference on Advances in Tox and App to Risk Assessment, WPAFB, 23-25 Apr.

Frazier, Toxopeus, A Biologically Based Kinetic Model for the Isolated Perfused Rat Liver, SOT 1996.

Garrett, Mahle, Lipscomb, Comparison of the *In Vitro* Metabolism of Trichloroethylene in Three Species: Rat, Mouse, and Human, Conference on Advances in Tox and App to Risk Assessment, WPAFB, 23-25 Apr.

Graeter, Wolfe, Kinkead, Flemming, Cooper, Preimplantation Effects of Ammonium Dinitramide (ADN) Administered in the Drinking Water of Sprague-Dawley Rats, Conference on Advances in Tox and App to Risk Assessment, WPAFB, 23-25 Apr.

Graeter, Wolfe, Kinkead, Flemming, Cooper, Preimplantation Effects of Ammonium Dinitramide (ADN) Administered in The Drinking Water of Sprague-Dawley Rats, 1996 Annual Society of Toxicology Meeting, Anaheim, CA, 10-14 March.

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Greenberg, Abbas, Fisher, Determination of Metabolis Constants for Trichloroethylene in the B6C3F1 Mouse from Gas Uptake Studies, Conference on Advances in Tox and App to Risk Assessment, WPAFB, 23-25 Apr.

Greenberg, Use of Bioenergetics-based Toxicokinetic Modeling to Predict Tissue Burdens of Benzo[a]pyrene in a Vertebrate Species, OH Valley Chapter of Society of Toxicology, Annual Meeting, Dec 8.

Kinkead, Wolfe, Freedman, Flemming, Caldwell, Reproductive Screen of Modular Artillery Charge System Administered in the Diet of Sprague-Dawley Rats, Conference on Advances in Tox and App to Risk Assessment, WPAFB, 23-25 Apr.

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Kinkead, Wolfe, Freedman, Flemming, Caldwell, Miller, Marit, Reproductive Toxicity Screen of Nitrate Containing Explosives and Propellants, 1995 JANNAF Meeting, 4 Dec, Tampa, FL.

Latendresse, Marit, Still, Oncogenic Potential of Inhaled Hydrazine in the Nose of Rats After One or Ten One-Hour Exposures, 1995 JANNAF Meeting, Tampa, FL, 4-8 Dec.

Latendresse, Pereira, Dissimilar Characteristics of N-Methl-N-Mitrosourea (MNU) Initiated Hepatic Foci and Tumors Promoted by Dichloroacetic Acid (DCA) or Trichloroacetic Acid (TCA) in Mice, American College of Veterinary Pathology Annual Conference (ACVP), Seattle, WA, 1-6 Dec 1995.

Lipscomb, Lash, Silvers, Mahle, Garrett, Confer, Cytochrome P-450 and Glutathione S-Transferase Dependent Trichloroethylene Metabolism in Human Hepatocytes, Conference on Advances in Tox and App to Risk Assessment, WPAFB, 23-25 Apr.

Lipscomb, Silver, Mahle, Garrett, Confer, Trichloroethylene Metabolism by Human Hepatocytes, SOT 1996.

Mahle, McCafferty, Frazier, Gearhart, Barton, Tissue Distribution of Trichloroacetate in B6C3F1 Mice and Fischer 344 Rats, Conference on Advances in Tox and App to Risk Assessment, WPAFB, 23-25 Apr.

Mahle, McCafferty, Frazier, Gearhart, Barton, Tissue Distribution of Trichloroacetate in B6C3F1 Mice and Fischer 344 Rats, SOT 1996, Anaheim, CA, Mar 10-14.

Mattie, Marit, Flemming, Sterner, Cooper, The Effects of JP-8 Jet Fuel on Female Sprague-Dawley Rats after a 21-Week Exposure by Oral Gavage, SOT 1996.

Randall, Geiss, Drerup, Kidney, Channel, Liver mRNA Levels Following Trichloroethylene Gavage in Male B6C3F1 Mice, American Chemical Society Central Regional Meeting, Dayton, OH, 10 June.

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Seckel, Byczkowski, Experimental Parameters to Support a Pharmacodynamic Model for Ethane Exhalation, Conference on Advances in Tox and App to Risk Assessment, WPAFB, 23-25 Apr.

Seckel, Byczkowski, Experimental Parameters to Support a Pharmacodynamic Model for Ethane Exhalation, SOT 1996, Anaheim, CA.

Serve, Bombick, Baughman, Jarnot, Ketcha, Mattie, The Metabolism of n-Nonane in Male Fischer 344 Rats, 1996 SOT, Anaheim, CA.

Steel-Goodwin, Schmidt, Miller, Byczkowski, Carmichael, Quantitation of Free Radicals in B6C3F1 Mouse Liver Slices on Exposure to Four Chemical Carcinogens: An EPR/Spin Trapping Study, Conference on Advances in Tox and App to Risk Assessment, WPAFB, 23-25 Apr.

Steel-Goodwin, Schmidt, Miller, Byczkowski, Carmichael, Quantitation of Free Radicals in B6C3F1 Mouse Liver Slices on Exposure to Four Chemical Carcinogens: An EPR/Spin Trapping Study, American Chemical Society Mid-West Regional Meeting, Dayton, OH, Jun 9-12.

Steel-Goodwin, Schmidt, Miller, Byczkowski, Carmichael, Quantitation of Free Radicals in B6C3F1 Mouse Liver Slices on Exposure to Four Chemical Carcinogens: An EPR/Spin Trapping Study, SOT 1996.

Sterner, Barton, Oral Bioavailability of TPH and Other Chemical in Soil: Literature Review of Experimental Issues in Risk Assessment, Conference on Advances in Toxicology and Applications to Risk Assessment, WPAFB, OH, Apr 23-25.

Tao, Kramer, Latendresse, Pereira, Expression of Protooncogenes, TGF- α and β in the Liver of B6C3F1 Mice Treated with Trichloroethylene, SOT 1996.

Tao, Kramer, Latendresse, Pereira, Expression of Protooncogenes, TGF- α and β in the Liver of B6C3F1 Mice Treated with Trichloroethylene, Conference on Advances in Toxicology and App to Risk Assessment, WPAFB, 23-25 Apr.

Vinegar, Jepson, Cardiac Sensitization Thresholds of Halon Replacement Chemicals Predicted by Human Physiologically Based Pharmacokinetic Modeling, AL/OE-TR-1995-0132, 1996 SOT, Anaheim, CA.

Vinegar, Jepson, Cardiac Sensitization Thresholds of Halon Replacement Chemicals Predicted by Human Physiologically Based Pharmacokinetic Modeling, AL/OE-TR-1995-0132, 1996 Toxicology Conference, WPAFB, Apr 96.

Wolfe, Kinkead, Feldmann, Caldwell, Dose (and Time Dependent) Blockade of Pregnancy in Sprague-Dawley Rats Administered Ammonium Dinitramide in Drinking Water, Conference on Advances in Tox and App to Risk Assessment, WPAFB, 23-25 Apr.

Wolfe, Kinkead, Feldmann, Caldwell, Dose (and Time Dependent) Blockade of Pregnancy in Sprague-Dawley Rats Administered Ammonium Dinitramide in Drinking Water, SOT 1996, Anaheim, CA.

Wyman, Eggers, Steel-Goodwin, Flemming, Caldwell, Preparation of Bovine Testicular Slices: Evaluation of Trinitrobenzene Toxicity, Conference on Advances in Toxicology and Applications to Risk Assessment, WPAFB, 23-25 Apr.

Yu, Tillitt, Channel, Drerup, McCafferty, Burton, Fisher, *In Vivo/In Vitro* Comparison of Pharmacokinetics and Pharmacodynamics of 3,3', 4,4'-Tetrachlorobiphenyl (PCB77), Society of Environmental Toxicology & Chemistry, Vancouver, B.C., 5-9 Nov.

Yu, Byczkowski, Drerup, McCafferty, Fisher, Pharmacokinetic and Pharmacodynamics of 3,3', 4,4',-Tetrachlorobiphenyl in the Rats: PBPK Model Development, Conference on Advances in Toxicology and Applications to Risk Assessment, WPAFB, 23-25 Apr.

Yu, Byczkowski, Drerup, McCafferty, Fisher, Pharmacokinetic and Pharmacodynamics of 3,3', 4,4',-Tetrachlorobiphenyl in the Rats: PBPK Model Development, SOT 1996.

Zhang, Pollard, Tsui, Brashear, Application of Supercritical Fluid Chromatographic Methods to the Analysis of Meta Environmental Hazards in Biological Matrices, 28th Regional ACS Meeting, Dayton, OH, 9-12 June.

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AWARDS and HONORS

Brashear, Recipient of the ManTech Environmental's On-the-Spot Award in May, 1996, for receiving a team award presented by the Air Force for participating in the trichloroethylene project that was considered the "Best for the Last Half of CY95" for Armstrong Laboratory.

Byczkowski, "Service Recognition Achievement Award," 1996. ManTech Environmental Technology, Inc.

Byczkowski, "Excellence Award for Science and Technology Achievement in support of the Trichloroethylene Team," 1996. Department of the Air Force USA.

Dodd, Selected to serve on the editorial board for the journal, *Inhalation Toxicology*; elected President-elect of the Ohio Valley Regional Chapter of the Society of Toxicology.

Greenberg, Recipient of ManTech Environmental's On-the-Spot Award, Dec 8, 1995, "Honorable Mention" for poster presentation, Use of Bioenergetics-based Toxicokinetic Modeling to Predict Tissue Burdens of Benzo[a]pyrene in a Vertebrate Species, at the Ohio Valley Society of Toxicology Annual Meeting in Cincinnati, OH.

Greenberg, Received "Outstanding Graduate Student Award" in the Biomedical Sciences, May 1996, at Wright State University for 1995-1996.

Latendresse, Served as the chairperson for the Respiratory Pathology Specialty Group Session of the 1996 Annual Meeting of the American College of Veterinary Pathologists in Seattle, WA.

Vinegar, "President's Award for Excellence," December 1995. ManTech Environmental Technology, Inc.

Wyman, Eggers, Steel-Goodwin, Flemming, Caldwell, Preparation of Bovine Testicular Slices: Evaluation of Trinitrobenzene Toxicity, Conference on Advances in Toxicology and Applications to Risk Assessment, WPAFB, 23-25 Apr.