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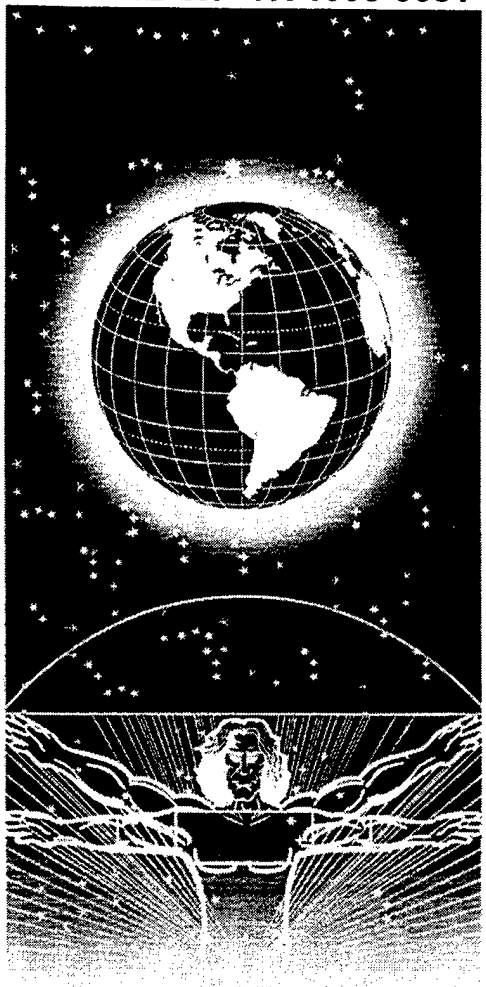

UNITED STATES AIR FORCE RESEARCH LABORATORY

A PRELIMINARY STUDY OF EXPOSURE TO PYRIDOSTIGMINE BROMIDE, DIETHYLTOLUAMIDE, JP-4 JET FUEL AND STRESS ON MALE SPRAGUE-DAWLEY RATS

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

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

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

FOR THE DIRECTOR

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This study investigated effects on male Sprague-Dawley rats of exposure to combinations of simulated stress and low doses of pyridostigmine bromide, diethyltoluamide, and JP-4 jet fuel vapor. Blood and tissue samples were analyzed for gross pathology, histopathology, and clinical chemistry effects. Serum and brain regions were analyzed for neurotransmitter levels. Body weights, gross pathology, histopathology, and clinical chemistry results revealed no significant differences between groups. Significant increases in serum 5-hydroxyindoleacetic acid and 5-hydroxytryptamine levels were present in the treated animals as compared to controls. Two-dimensional gel electrophoresis analysis of liver, kidney, and plasma samples from animals exposed to a combination of all chemicals and stress showed a statistically significant alteration in protein expression compared to controls. Further research is needed to assess the effect of the individual chemicals studied in combination here.

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PREFACE

This technical report describes the methods and results obtained through a collaborative effort between the Toxicology Division of Armstrong Laboratory, the Naval Medical Research Institute Detachment-Toxicology, and ManTech Geo-Centers Joint Venture. This document serves as a final report on the toxicity evaluation of exposure to low doses of pyridostigmine bromide, diethyltoluamide, JP-4 jet fuel vapor and stress on male Sprague-Dawley rats. This research was funded by the U.S. Army Medical Research and Materiel Command. The research described in this report began in October 1994 and was completed in December 1997 under Department of Defense Contract No. F41624-96-C-9010. Lt Col Terry A. Childress served as Contract Technical Monitor for the U.S. Air Force, Armstrong Laboratory. Darol E. Dodd, Ph.D., served as Program Manager for ManTech Geo-Centers Joint Venture. CDR (S) John Rossi served as the Principal Investigator for the U.S. Navy, Naval Medical Research Institute Detachment-Toxicology.

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*, Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1996.

The authors gratefully acknowledge Dr. Daniel Caldwell, currently of Exxon Biomedical Sciences Inc., NJ, for initiating and developing the protocol for this project. Invaluable technical assistance was provided by Ms. Marcia Feldmann, Mr. David Ellis, Mr. Richard Godfrey, Mr. Jerry Nicholson, Mr. Willie Malcomb, SSgt Frank Dessauer, Ms. Peggy Parish, SrA Stacie Trivunovic, and SSgt Gerry Miller. Also acknowledged are Mr. Carlyle D. Flemming for statistical analysis of the data, Mr. William Binole for software development, and Mr. William Sonntag and Mr. Rich Marker for equipment production.

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ABBREVIATIONS

Ag	Silver
AGP	Advanced gradient pump
ALT	Alanine transferase
AST	Aspartate transferase
BUN	Blood urea nitrogen
C	Carbon
°C	Degrees centigrade
cfm	Cubic feet per minute
CHAPS	Cholamidopropyldimethylamoniopropanesulfonate
Cl	Chloride
CO_2	Carbon dioxide
DA	Dopamine
DEET	N,N-diethyl-m-toluamide, diethyltoluamide
dL	Deciliter
DoD	Department of Defense
DOPAC	3,4-Dihydroxyphenylacetic acid
DTE	Dithioerythreitol
E	Epinephrine
°F	Degrees Fahrenheit
g	Gram
Hg	Mercury
HHS	Department of Health and Human Services
HPLC	High pressure liquid chromatography
hr	hour
HVA	Homovanillic acid
IR	Infrared
IU	International units
kg	Kilogram
L	Liter
m	meter
m^3	cubic meter
M	Molar
ma	Milliampere
mg	Milligram
mL	Milliliter
mm	Millimeter
mmol	Millimoles
mM	Millimolar
msec	Millisecond
N	Sample size
nA	Nanoamperes
NE	Norepinephrine
ng	Nanogram
NTAB	Neurobehavioral Toxicity Assessment Battery
PB	Pyridostigmine bromide
PED	Pulse electrochemical detector
psi	Pounds per square inch
SD	Standard deviation
µg	Microgram
µL	Microliter
USAF	United States Air Force
V	Volts
VA	Department of Veterans Affairs
V:V	volume per volume

SECTION I

INTRODUCTION

The investigation of health problems in Persian Gulf Veterans and their possible causes has been a major focus of the Department of Veterans Affairs (VA), the Department of Defense (DoD), and the Department of Health and Human Services (HHS). Military personnel were potentially exposed to a myriad of biological, chemical, and physiological stressors during their deployment in the Persian Gulf. Recent research has focused on the potential of a mixture of low-level stressors to elicit a physiological effect stronger than that predicted by each chemical alone. Two chemicals commonly implicated in exposure during the Persian Gulf War are pyridostigmine bromide (PB) and diethyltoluamide (DEET). JP-4 jet fuel was in use during the Persian Gulf War and some troops may have been exposed to it. Stress is a factor which may exacerbate the effects of chemicals. Development of an animal model that investigates the physiological effects of these chemicals individually and in combination with stress is important not only in investigating the possible causes of Persian Gulf Veterans' health complaints but also to ensuring the continued health of personnel in war-time deployments. With an increasing number of biological and chemical exposures anticipated for deployed personnel in the future, such as the anthrax vaccine, it is important to understand the potential toxicological and physiological effects of chemicals currently in use in order to continue to protect the health and readiness of military personnel world-wide.

The purpose of this study was to make a preliminary investigation into the potential adverse effects of low doses of pyridostigmine bromide (PB), diethyltoluamide (DEET), and JP-4 jet fuel in combination with stress in a Sprague-Dawley rat model. This combination of chemicals and stress was selected due to the self-reported exposure history of Gulf War Veterans whose respondents reported exposure to diesel/other fuels (85%), pyridostigmine bromide (70%), and personal insecticide use (64%). Animals were exposed to varying combinations of these chemicals and stress daily for a fourteen day exposure period. Half of the animals from each exposure group were observed for 14 days, subjected to a battery of neurobehavioral tests on postexposure days 15-36, and then sacrificed on postexposure days

38-40. The other half of the animals from each exposure group were rested and observed for 60 days postexposure. Following this observation period they were subjected to the same battery of neurobehavioral tests on postexposure days 61-82 and then sacrificed on postexposure days 86-88. The two different lengths of holding and observation periods were selected in order to investigate effects of the chemicals 14 days or 60 days postexposure. There was an interest in investigating the subchronic effects of these chemicals since many Persian Gulf Veterans did not have health complaints until they had been home from the Persian Gulf arena for an extended period of time.

Pyridostimine bromide (PB) is a quarternary ammonium carbamate cholinesterase inhibitor which does not penetrate the blood-brain barrier under normal conditions and dosages. It undergoes hydrolysis by cholinesterase and is also metabolized by microsomal enzymes in the liver. At usual doses PB is distributed to most tissues in the rat but not to the brain, intestinal wall, fat, or thymus; it does cross the placenta. The acute oral median lethal dose of PB in the rat has been reported as 61.6 mg/kg (McCain,1995) and 80 mg/kg (Hane,1977). Doses greater than 24 mg/kg produced overt signs of peripheral cholinergic intoxication as observed by a suppressed conditioned operant performance. A three month toxicological study of PB in Sprague-Dawley rats found significant red blood cell acetylcholinesterase inhibition at 15 mg/kg. PB dosed at 1 mg/kg will inhibit cholinesterase activity in rodents by 40 percent, the target inhibition rate in military troops (Kerenyi et al., 1988). Friedman et al. (1996) found increased penetration of PB into the brain under conditions of increased stress (swim test); in stressed mice the peripherally administered dose of PB needed to inhibit brain acetylcholinesterase by 50 percent was one hundredth that required in nonstressed mice. Hubert and Lison (1995) demonstrated that under normal conditions no muscle damage occurred in rats treated with PB; when the same PB-treated animals were exercised strenuously extensive muscle damage occurred. Based on literature data available the daily dose of PB selected for use in this preliminary study was 1.0 mg/kg body weight by oral gavage. Oral gavage was selected in order to most closely mimic the potential exposure route of military personnel.

The insecticide DEET has a low order of mammalian toxicity: the rat oral LD_{50} value is 2-3 g/kg; the dermal LD_{50} is about 5 g/kg. A single 500 mg/kg oral dose of DEET

demonstrates short-term (1 hr) effects on measures of thermal response and motor activity (Schoenig et al., 1993). DEET crosses the blood-brain barrier. Spongiform myelinopathy has been reported in the brainstem of rats exposed to near-lethal doses of DEET (Verschoyle et al., 1990). Spongiform myelinopathy occurs primarily in the cerebellar roof nuclei (Verschoyle et al., 1992). DEET has a potential for cardiovascular toxicity; hypotension and bradycardia due to DEET toxicity has been demonstrated in the rat (Leach, 1988). Rats in this study were dosed dermally 300 µl 33% DEET in ethanol daily. A formulation of 33% DEET in ethanol (its standard solvent) was selected for this study since this concentration was at the lower end of the dose range available to personnel in the Persian Gulf. Dermal application of DEET was used to most closely mimic the potential exposure route of military personnel.

JP-4 is an aircraft fuel made by refining crude petroleum oil or shale oil. It is a liquid at room temperature but can be vaporized. It is a complex mixture of hydrocarbons including paraffins, cycloparaffins, aromatics, and olefins. A government review of the literature summarizing the health effects of JP-4 jet fuel exposure has been published (ATSDR, 1995). Exposure to jet fuel may occur during manufacturing, refueling, fuel storage, and as a result of normal aircraft operations. Under some conditions jet fuel may be released to the environment during the jettisoning of excess fuel from aircraft. A minimal risk level for intermediate-duration inhalation exposure (15-364 days) to JP-4 has been determined to be 9 mg/m^3; for a chronic-duration inhalation exposure the minimal risk level is 0.3 mg/m^3 (U.S. Dept. Health and Human Services, 1995). Acute exposure of Sprague-Dawley rats to concentrations as high as 5,000 mg/m^3 JP-4 for four hours did not result in any toxic signs (Clark et al., 1989). A 90 day nearly continuous exposure of male and female Fischer 344 rats to 500 and 100 mg/m^3 JP-4 resulted in treatment-related $\alpha_{2\mu}$-globulin nephropathy but no respiratory toxicity was observed (Kinkead et al., 1995). A twelve month intermittent exposure of Fischer 344 rats to 1000 and 5000 mg/m^3 JP-4 vapor produced similar results (Bruner et al., 1993). For the current study, a JP-4 vapor concentration of 2 mg/L was selected for daily exposure. This concentration was selected both because of its low potential for toxicity and its ability to be comparable to the desired vapor concentrations of other jet fuels and diesel fuel that may be studied in follow-on research.

Stress is a factor that is important not only because of its pervasiveness in military operations, but also because of recent evidence that it may affect the toxicology of chemicals. Friedman et al. (1996) demonstrated that in stressed mice the peripherally administered dose of PB needed to inhibit brain acetylcholinesterase by fifty percent was one hundredth of that required in nonstressed mice. This change was attributed to alterations in the permeability of the blood-brain barrier. The ability of stress to affect the permeability of the blood-brain barrier has been demonstrated in several different animals models including rats exposed to acute immobilization stress (Belova and Jonsson, 1982; Dvorska et al,1992), rats exposed to summer heat (Sharma,1992), and cold and isolation exposure in mice (Ben-Nathan,1991). The form of stress which was used in this study was a low-level, random, intermittent foot shock during daily exposure to JP-4 jet fuel vapor.

In addition to the standard endpoints of toxicity testing including body weight, hematology, blood chemistries, gross necropsy and histopathology, the results of neuro-behavioral testing, neurotransmitter levels, and two-dimensional polyacrylamide gel electrophoresis were determined in this study. A battery of neurobehavioral tests was performed in order to determine any subtle effects of exposure to these chemicals and stress on nervous system integrity and motor system integrity. Brain neurotransmitter levels were evaluated in order to determine any subtle biochemical effects of these chemicals and stress. Two-dimensional electrophoresis was used to detect the toxic effect, if any, of these stressors on target tissues. Two-dimensional electrophoresis of stress proteins has been used to detect chemical toxicity and provide information regarding toxic mechanisms for single chemical agents (Witzmann et al., 1994). In this study it was used to determine similar changes in stress proteins from animals exposed to this multiple chemical and stress exposure.

Recent Persian Gulf research has focused on the potential of a mixture of chemicals and/or stress to elicit a physiological effect stronger than that predicted by the effect of each chemical alone. McCain et al. (1995) demonstrated that at lethal doses in the rat pyridostigmine bromide (PB), permethrin and DEET may become more toxic when given in combination than when given separately. Abou-Donia et al. (1996) found that PB, DEET, and permethrin in combination resulted in greater neurotoxicity in the hen than that predicted by the minimal toxic effects of each individual chemical at the same dosage levels. The purpose

of the current study was to investigate the effects of low doses of pyridostigmine bromide, DEET, and JP-4 jet fuel vapor in combination with stress on male Sprague-Dawley rats. Due to a physical constraint on the number of animals that could be exposed to this combination of chemicals and stress, and due to the screening nature of the investigation, male rats only were investigated. This preliminary study provided information on the effects of these chemical combinations with and without stress on male rats, and provided preliminary data on which to base further investigations.

SECTION II

MATERIALS AND METHODS

Test 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-hr inhalation exposures. Animal rooms were maintained on a 12 hr light/dark cycle (fluorescent light) and targeted at a temperature of 23 ± 2 °C and a relative humidity of 55 ± 15%. All animals were gently handled for 5 minutes per day for 5 days prior to the first day of exposure.

Test Agents

JP-4 Jet Fuel. The fuel-exposed animals received 2 mg/L JP-4 jet fuel vapor via the inhalation route for six continuous hours each day of the fourteen day exposure. The JP-4 jet fuel was supplied by the Propulsion Directorate of Wright Laboratory, Wright-Patterson Air Force Base, Ohio. Pertinent physical and chemical properties are below.

JP-4 Jet Fuel	Source:	U.S. Air Force
	Appearance:	Clear, colorless liquid
	Flash Point:	100 °F
	Specific gravity:	0.760 g/mL
	Vapor Pressure:	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 exposure system consisted of four Hinners-type 690-Liter inhalation chambers. The fuel mass concentration in each chamber during the 14-day exposure 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 two percent 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). The DEET-exposed animals received 300 μL 33% DEET in ethanol (approximately 40 mg DEET/kg bodyweight) applied topically to a shaved area of the back each day of the fourteen day JP-4 jet fuel vapor exposure. DEET was applied a maximum of one-half hour prior to the initiation of the JP-4 jet fuel vapor exposure and a maximum of ten minutes prior to the PB oral gavage. The back of the animals was shaved once per week to ensure accurate dermal application. Pertinent physical and chemical properties of DEET are listed below.

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

Pyridostigmine bromide (PB). Animals exposed to pyridostigmine bromide were orally gavaged with one mg PB/kg body weight each day of the fourteen day exposure period a maximum of one-half hour prior to the initiation of the JP-4 jet fuel vapor inhalation exposure. PB dosing occurred a maximum of ten minutes after DEET application. 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.

Stress Simulation

Throughout the six hour JP-4 jet fuel vapor exposures, half of the animals were exposed to a randomly generated mild electrical shock. Foot shock was delivered through two Master Shockers with Scramblers and Interrupters (Model 82404SS, Lafayette Instruments, Lafayette, IN) to the gridbar flooring of the exposure cages. A custom designed (Wright-Patterson Air Force Base Electronics Engineering Shop, WPAFB, OH) Voltage Switching and Current Limiting Neon Bulb Router ensured the equal delivery of voltage to each of the eight housing cages within the two exposure chambers that received voltage. The foot shock equipment was controlled by a central computer to activate randomly six times per hour (total of 36 shocks per exposure day) at a voltage of no more than 0.5 ma for 300 msec. A custom designed software program (William Binole, Dayton, OH) ensured stimulus randomization and synchronicity. The shock delivery was scrambled so that alternating gridbars became positive or grounded many times per second so that animals could not avoid the stimulus. The computer simultaneously

delivered shock to each of the eight shock cages within the two shock chambers. The shock level selected was barely perceptible when applied to the finger of a human subject.

14-Day Repeat Exposure Regimen

Eight groups of 16 male rats (Table 1) were placed into four Hinners-type 690-L inhalation chambers and exposed for six hours daily for fourteen days to either air only (Groups 5-8: AIR/STRESS/DEET/PB, AIR/STRESS, AIR/DEET/PB, AIR) or JP-4 jet fuel vapor (Groups 1-4: JP4/STRESS/DEET/PB, JP4/STRESS, JP4/DEET/PB, JP4). One air only chamber and one JP-4 jet fuel vapor chamber were connected to the shock apparatus so that two groups from each inhalation regimen (air only or JP-4 jet fuel vapor) were subjected to a mild intermittent random shock during each inhalation exposure (Groups 1, 2, 5, and 6; JP4/STRESS/PB/DEET, JP4/STRESS, AIR/STRESS/PB/DEET, and AIR/STRESS, respectively). Two groups from each inhalation regimen were orally gavaged with PB and dermally treated with DEET prior to each inhalation exposure (Groups 1, 3, 5, and 7; JP4/STRESS/DEET/PB, JP4/DEET/PB, AIR/STRESS/DEET/PB, and AIR/DEET/PB, respectively). 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.

Each exposure chamber held four cage units. Each of these cage units 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 chamber. The exposure cage units were rotated clockwise (moving one position) within each inhalation chamber every exposure day. The rats were observed for signs of toxic stress prior to each day's exposure, during exposure, and again postexposure. Rat body weights were measured prior to study initiation, and then weekly throughout the study.

Table 1. Exposure Treatment Groups

Group Number	Group Name	Inhalation	Stress	Dermal	Oral
1	JP4/STRESS/PB/DEET	JP-4	Shock	DEET	PB
2	JP4/STRESS	JP-4	Shock	-----	-----
3	JP4/PB/DEET	JP-4	-----	DEET	PB
4	JP4	JP-4	-----	-----	-----
5	AIR/STRESS/PB/DEET	Air	Shock	DEET	PB
6	AIR/STRESS	Air	Shock	-----	-----
7	AIR/PB/DEET	Air	-----	DEET	PB
8	AIR	Air	-----	-----	-----

Neurobehavioral Evaluation

After the 14-day exposure period, eight randomly selected rats from each exposure group were allowed to recover for 14 days; the remaining eight rats from each exposure group were allowed to recover for 60 days. Following these observations periods the rats were evaluated for performance on tests selected from the Neurobehavioral Toxicity Assessment Battery (NTAB). The NTAB was developed to identify specific areas of neurobehavioral deficit (motivational, sensory, motor, and cognitive) from complex changes in performance induced by toxic exposures, as well as to provide a mechanism to evaluate recovery of neurobehavioral integrity (Ritchie et al., 1995). The NTAB was performed on eight rats from each exposure group 15-36 days postexposure and in the remaining eight rats from each exposure group 61-82 days postexposure. The battery of tests included evaluation of forelimb grip strength, photosensitivity, appetitive reinforcer approach sensitization, acoustic startle-prepulse inhibition/habituation, general locomotor activity, tail flick nociception, treadmill physical fatigue, and one-trial passive avoidance learning. The neurobehavioral evaluation was performed by personnel of the US Navy Medical Research Institute, Toxciology Divison, Wright-Patterson AFB. Results of these tests and their significance will be reported separately.

Necropsy Evaluations

Following neurobehavioral testing, eight animals from each group were necropsied at one of two timepoints, 38 to 40 days post-exposure and 86 to 88 days post-exposure.. At each timepoint, three animals from each group were euthanized on days 1 and 2, and two animals from each group on day 3. Animals were fasted twelve hours prior to necropsy. Following euthanasia, the brain was immediately harvested and dissected into five regions, frozen on dry ice, and stored at -70°C for future neurotransmitter analysis. The nasal turbinates, lungs, liver, kidneys, spleen, stomach, duodenum, jejunum, ileum, colon, testes, thymus, bone marrow (from the femur), sciatic nerve, skeletal muscle, skin, cervical lymph nodes, thyroids, parathyroids, adrenals, pituitary, cervical and lumbar vertebrae with spinal cord, and any gross lesions were collected at necropsy and preserved in 10% buffered formalin solution. Tissues weighed prior to fixation included liver, kidneys, testes, brain, spleen, adrenals, and lungs. Kidney and liver samples were frozen in liquid nitrogen and stored at -70°C for two-dimensional electrophoresis analysis. Histologic examination was performed on all collected tissues from eight animals from the AIR control and JP4/STRESS/PB/DEET groups (Groups 1 and 8; four animals chosen randomly from each group), and on the spinal cord, sciatic nerve, and skeletal muscle from all sixteen animals in the same two groups. Processed tissues were embedded in paraffin and stained with hematoxylin and eosin.

Clinical Pathology Measurements

At each scheduled necropsy, blood samples were taken via the posterior vena cava from each animal for complete hematology and serum chemistry assays. Additional sera samples were frozen at -70°C for neurotransmitter analysis. Plasma samples were frozen in liquid nitrogen and stored at -70°C for two-dimensional electrophoresis (0.5 ml). 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.

Neurotransmitter Analysis

Neurotransmitter analysis was performed on the brain and sera of six animals selected randomly from each group. The five brain regions dissected at necropsy and analyzed were the brain stem, cerebral cortex, caudate nucleus, hippocampus, and cerebellum. Frozen tissue and sera samples were thawed and homogenized (Polytron homogenizer, GLAS-COL, Terre Haute, IN) for 30 seconds in 0.17M perchloric acid (90 mg/ml). Samples were centrifuged at 31,500G for 30 minutes at 4°C. The supernatants were separated and analyzed. Each sample was analyzed for levels of norepinephrine (NE), epinephrine (E), dopamine (DA), 5-hydroxytryptamine (5-HT), homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC), and 5-hydroxyindoleacetic acid (5-HIAA).

Neurotransmitter analysis was performed following the methods described in Kim et al., 1987. High Pressure Liquid Chromatography (HPLC) determinations were performed with a Dionex Model, DX-300 isocratic liquid chormatography coupled with a pulse electrochemical detector (PED-2). An advanced gradient pump (AGP-Standard size) was used. A glassy-carbon working electrode was set at 0.8 V versus a Ag/AgCl reference electrode. The sensitivity of the detector was maintained between 0.5 and 1.0 nA depending on the concentration of the neurotransmitters. Separation by isocratic elution was performed on a C18 reverse phase column preceded by a guard column (Guard-Pak, C18, Waters Association, Milford, MA). The mobile phase was 15% (v/v) methanol in a solution of 32 mM citric acid (pH 4.2), 12.5 mM disodium hydrogen orthophosphate, 0.5 mM octyl sodium sulfate and 0.05 mM EDTA. The mobile phase was filtered through a 0.45 mm filter (Millipore, Bedford, MA) and degassed under a vacuum before use. A flow rate of 1.2 mL/min (2200 p.s.i.) at ambient temperature was employed.

Known amounts of NE, DA, E, 5-HT, DOPAC, HVA, and 5-HIAA in the range 0.2-20 ng were injected into the HPLC system. 3,4-Dihydroxybenzylamine hydrobromide (DHBA, 2.5 ng) was used as an internal standard in the homogenate. Its recovery efficiency was 98-100%.

All compounds were easily oxidized at 0.8 V versus a Ag/AgCl reference electrode. Each compound gave a linear response in the 0.2-20 ng range.

Two-Dimensional Electrophoresis

Two-dimensional electrophoresis (2DE) analysis was performed on six randomly selected AIR only control animals and six randomly selected JP4/STRESS/PB/DEET animals following the 30 day holding period. Each tissue sample (0.5 g) and plasma sample (0.5 mL) was homogenized in 4 mL of a lysis buffer containing 9M urea, 4% CHAPS (3-[(3-cholamidopropyl)-dimethylamonio]-1-propanesulfonate), 2% dithioerythreitol (DTE) and 2% ampholytes (pH 8-10.5) for ISO-DALT electrophoresis. Solubilized protein samples (150-200 μg) were separated via 2DE as described by Anderson, 1988. Samples were stained for 96 hrs with Coomassie brilliant blue G-250. Stained gels were digitized at 125 micron resolution via Charge-Coupled Device scanner. Images were processed using the KEPLER® software system to generate a spotlist giving x,y position, shape, and density information for each detected spot. Groupwise statistical comparisons were made to screen for protein alterations (Student's t-Test). Corresponding spot volume information was presented graphically.

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 body weights (Barcikowski, 1983). Tissue lesion severity data were analyzed using the Kruskal-Wallis analysis of variance (Rosner, 1990).

SECTION III

RESULTS

Clinical Measurements

No clinical signs of toxicity were noted during or after the exposure period for any animals. Body weights, absolute organ weights and organ-to-body weight ratios were not statistically significantly different between all eight groups of males at each sacrifice (Tables 2-7). No exposure-related differences between groups were noted in clinical chemistry or hematological parameters measured at both the 38-40 day and 86-88 day postexposure sacrifices (Tables 8-15).

Necropsy

At each necropsy, all rats utilized in this study were in good general condition. One animal from the AIR/STRESS/DEET/PB group euthanatized during the exposure had an esophageal perforation due to oral gavage trauma. No other treatment-related lesions were observed.

Histopathology

There were no significant differences at the 0.05 level in the incidence or average severity scores for histopathology between the AIR control and JP4/STRESS/PB/DEET animals analyzed using the Kolmogorov-Smirnov two-tailed test of statistical analysis. In both groups minimal (early) nephrophathy changes were a common finding. The presence of multifocal dilated myelin sheaths(vacuolation) was frequently observed in spinal cord sections from both groups. This finding was interpreted as a processing artifact and was not significantly different between control and treatment groups.

Table 2. Body Weights[a] (g) of Male Sprague-Dawley Rats Before, During, and After 14 Day Exposure[b]

Study Day	Air[c,d]	Air/PB/DEET[cd]	Air/Stress[c,d]	Air/Stress PB/DEET[e]
-4	293.3 ± 10.4	293.1 ± 9.8	293.2 ± 9.8	293.5 ± 9.3
0	326.1 ± 14.5	322.2 ± 12.8	326.4 ± 16.1	322.6 ± 11.2
7	371.3 ± 17.9	368.1 ± 16.6	370.6 ± 21.5	361.9 ± 26.2
14	407.2 ± 21.6	402.4 ± 22.2	408.4 ± 30.3	398.2 ± 20.0
21	438.9 ± 26.8	434.7 ± 24.7	441.9 ± 35.0	431.9 ± 23.3
28	474.2 ± 31.3	470.3 ± 30.2	475.7 ± 42.4	467.6 ± 26.8
35	503.6 ± 35.7	502.3 ± 34.7	498.2 ± 48.4	492.2 ± 27.0
42	533.0 ± 39.8	532.5 ± 37.5	521.1 ± 51.2	515.8 ± 34.4
49	547.3 ± 42.3	553.5 ± 39.9	541.7 ± 53.4	541.8 ± 35.4
56	601.0 ± 44.3	577.3 ± 42.4	565.7 ± 44.9	572.7 ± 51.2
63	614.3 ± 46.7	587.3 ± 46.3	573.2 ± 48.9	585.1 ± 58.2
70	633.4 ± 49.6	604.3 ± 51.5	583.7 ± 53.0	594.4 ± 59.1
77	652.3 ± 52.9	626.0 ± 51.4	605.9 ± 51.0	614.6 ± 61.1
84	666.6 ± 55.6	636.9 ± 57.0	616.4 ± 53.6	630.3 ± 62.6
91	669.4 ± 54.3	643.7 ± 61.9	616.2 ± 54.3	634.8 ± 70.2
98	675.5 ± 54.7	654.8 ± 65.2	627.4 ± 55.5	645.5 ± 71.4

[a]Mean ± Standard Deviation (SD).
[b]Animals exposed Days 0 through 14.
[c]N = 16, Days -4 through 49.
[d]N = 8, Days 56 through 98.
[e]N = 16, Days -4 through 14; N = 15, Days 21 through 49; N = 7, Days 56 through 98.

Table 3. Body Weights[a] (g) of Male Sprague-Dawley Rats Before, During and After 14 Day Exposure[b]

Study Day	JP4	JP4/PB/DEET	JP4/Stress	JP4/Stress PB/DEET
-4[c]	294.0 ± 10.2	293.5 ± 11.0	293.4 ± 10.6	293.3 ± 9.6
0	327.1 ± 14.8	321.6 ± 15.4	329.6 ± 14.2	328.3 ± 9.9
7	368.8 ± 18.1	365.2 ± 21.2	371.3 ± 20.7	370.7 ± 15.0
14	405.4 ± 25.0	396.9 ± 28.1	406.2 ± 27.1	408.6 ± 19.1
21	437.0 ± 26.7	427.7 ± 30.6	442.7 ± 32.6	446.6 ± 24.2
28	469.9 ± 31.1	464.6 ± 35.2	476.5 ± 37.3	480.5 ± 29.0
35	495.0 ± 38.5	488.0 ± 43.9	499.0 ± 37.5	501.7 ± 32.8
42	518.3 ± 38.3	508.5 ± 47.1	526.1 ± 42.3	537.7 ± 40.8
49	540.7 ± 40.0	534.7 ± 52.9	545.9 ± 43.3	555.2 ± 44.1
56[d]	553.5 ± 35.3	579.6 ± 28.5	562.7 ± 48.0	588.0 ± 55.7
63	565.5 ± 37.8	573.1 ± 48.4	570.9 ± 53.8	602.7 ± 59.9
70	576.1 ± 40.0	600.4 ± 23.9	581.8 ± 54.5	617.6 ± 64.9
77	594.5 ± 43.7	621.3 ± 23.1	597.1 ± 50.6	636.8 ± 60.4
84	609.9 ± 45.4	630.1 ± 39.1	607.9 ± 48.5	650.1 ± 65.4
91	614.1 ± 51.1	643.7 ± 25.3	612.4 ± 51.6	655.4 ± 66.8
98	616.8 ± 52.1	655.9 ± 21.9	622.7 ± 56.0	673.0 ± 71.1

[a]Mean ± SD.
[b]Animals exposed Days 0 through 14.
[c]N = 16, Days -4 through 49.
[d]N = 8, Days 56 through 98.

Table 4. Absolute Organ Weights[a](g) and Organ to Body Weight Ratios[b] of Male Sprague-Dawley Rats 38-40 Days Postexposure

Organ	Air	Air/PB/DEET	Air/Stress	Air/Stress PB/DEET
Liver	15.42 ± 2.50	16.07 ± 2.03	15.44 ± 2.59	15.44 ± 0.89
Ratio	3.03 ± 0.41	3.01 ± 0.23	3.01 ± 0.37	3.02 ± 0.15
Kidneys	3.44 ± 0.36	3.65 ± 0.37	3.53 ± 0.57	3.80 ± 0.17
Ratio	0.68 ± 0.07	0.69 ± 0.06	0.69 ± 0.07	0.74 ± 0.04
Testes	3.31 ± 0.63	3.18 ± 0.85	3.28 ± 0.29	3.27 ± 0.28
Ratio	0.65 ± 0.13	0.61 ± 0.18	0.65 ± 0.07	0.64 ± 0.06
Brain	2.13 ± 0.05	2.13 ± 0.05	2.13 ± 0.13	2.06 ± 0.08
Ratio	0.42 ± 0.03	0.40 ± 0.04	0.42 ± 0.04	0.40 ± 0.02
Spleen	0.86 ± 0.08	0.85 ± 0.07	0.86 ± 0.09	0.85 ± 0.05
Ratio	0.17 ± 0.02	0.16 ± 0.02	0.17 ± 0.02	0.17 ± 0.01
Adrenals	0.061±0.016	0.073±0.012	0.074±0.017	0.064±0.006
Ratio	0.012±0.003	0.014±0.002	0.014±0.002	0.012±0.001
Lungs	2.37 ± 0.36	2.44 ± 0.37	2.44 ± 0.26	2.46 ± 0.31
Ratio	0.47 ± 0.07	0.46 ± 0.05	0.48 ± 0.07	0.48 ± 0.06
Body Wts	508 ± 31.7	534 ± 45.7	512 ± 61.7	512 ± 17.9

[a]Mean ± SD, N = 8.
[b]Organ weight ÷ body weight X 100.

Table 5. Absolute Organ Weights[a](g) and Organ to Body Weight Ratios[b] of Male Sprague-Dawley Rats 38-40 Days Postexposure

Organ	JP4	JP4/PB/DEET	JP4/Stress	JP4/Stress PB/DEET
Liver	15.97 ± 2.84	15.06 ± 2.28	15.94 ± 2.29	15.41 ± 1.86
Ratio	3.06 ± 0.28	3.09 ± 0.20	3.05 ± 0.31	2.98 ± 0.22
Kidneys	3.94 ± 0.41	3.70 ± 0.49	3.73 ± 0.35	3.60 ± 0.25
Ratio	0.76 ± 0.07	0.76 ± 0.08	0.72 ± 0.06	0.70 ± 0.08
Testes	3.25 ± 0.32	3.34 ± 0.17	3.38 ± 0.27	3.29 ± 0.28
Ratio	0.63 ± 0.04	0.70 ± 0.10	0.65 ± 0.08	0.64 ± 0.06
Brain	2.16 ± 0.08	2.13 ± 0.10	2.10 ± 0.11	2.16 ± 0.07
Ratio	0.42 ± 0.04	0.44 ± 0.05	0.41 ± 0.05	0.42 ± 0.03
Spleen	0.94 ± 0.36	0.81 ± 0.15	0.87 ± 0.08	0.80 ± 0.11
Ratio	0.18 ± 0.05	0.17 ± 0.02	0.17 ± 0.02	0.15 ± 0.02
Adrenals	0.073 ±0.014	0.066 ±0.011	0.061 ±0.015	0.067 ±0.011
Ratio	0.014 ±0.002	0.014 ±0.002	0.012 ±0.002	0.013 ±0.002
Lungs	2.43 ± 0.15	2.25 ± 0.39	2.45 ± 0.37	2.36 ± 0.29
Ratio	0.47 ± 0.05	0.46 ± 0.07	0.47 ± 0.06	0.46 ± 0.04
Body Wts	519 ± 47.4	487 ± 56.1	522 ± 44.7	516 ± 36.5

[a]Mean ± SD, N = 8.

[b]Organ weight ÷ body weight X 100.

Table 6. Absolute Organ Weights[a](g) and Organ-to-Body Weight Ratios[b] of Male Sprague-Dawley Rats Sacrificed 86-88 Days Postexposure

Organ	Air	Air/PB/DEET	Air/Stress	Air/Stress PB/DEET[c]
Liver	19.84 ± 2.26	16.90 ± 2.25	17.89 ± 1.58	17.94 ± 3.62
Ratio	3.08 ± 0.28	2.71 ± 0.17	3.01 ± 0.22	2.88 ± 0.31
Kidneys	4.29 ± 0.38	4.12 ± 0.47	4.14 ± 0.41	4.15 ± 0.81
Ratio	0.67 ± 0.06	0.66 ± 0.07	0.70 ± 0.10	0.67 ± 0.06
Testes	3.62 ± 0.27	3.51 ± 0.31	3.55 ± 0.31	3.49 ± 0.37
Ratio	0.57 ± 0.07	0.57 ± 0.06	0.60 ± 0.09	0.57 ± 0.04
Brain	2.26 ± 0.12	2.26 ± 0.15	2.23 ± 0.10	2.25 ± 0.04
Ratio	0.35 ± 0.03	0.37 ± 0.04	0.38 ± 0.04	0.37 ± 0.04
Spleen	0.94 ± 0.19	0.84 ± 0.10	0.88 ± 0.07	0.87 ± 0.22
Ratio	0.15 ± 0.03	0.14 ± 0.02	0.15 ± 0.02	0.14 ± 0.02
Adrenals	0.070 ±0.011	0.057 ±0.009	0.061 ±0.009	0.067 ±0.010
Ratio	0.011 ±0.002	0.009 ±0.002	0.010 ±0.002	0.011 ±0.002
Lungs	2.88 ± 0.20	2.48 ± 0.15	2.49 ± 0.32	2.66 ± 0.42
Ratio	0.45 ± 0.04	0.40 ± 0.03	0.42 ± 0.03	0.43 ± 0.05
Body wts	645 ± 50.2	623 ± 66.3	596 ± 53.1	617 ± 71.6

[a]Mean ± SD, N = 8.
[b]Organ weight ÷ body weight X 100.
[c]N = 7.

Table 7. Absolute Organ Weights[a](g) and Organ-to-Body Weight Ratios[b] of Male Sprague-Dawley Rats Sacrificed 86-88 Days Postexposure

Organ	JP4	JP4/PB/DEET	JP4/Stress	JP4/Stress PB/DEET
Liver	16.67 ± 2.02	18.46 ± 2.04	16.55 ± 1.59	18.18 ± 2.71
Ratio	2.84 ± 0.22	2.95 ± 0.25	2.81 ± 0.26	2.85 ± 0.19
Kidneys	4.01 ± 0.23	4.34 ± 0.54	3.84 ± 0.47	4.14 ± 0.55
Ratio	0.69 ± 0.05	0.69 ± 0.07	0.65 ± 0.07	0.65 ± 0.07
Testes	3.20 ± 0.78	3.45 ± 0.24	3.36 ± 0.25	3.45 ± 0.14
Ratio	0.54 ± 0.12	0.55 ± 0.03	0.57 ± 0.06	0.55 ± 0.07
Brain	2.25 ± 0.07	2.23 ± 0.14	2.25 ± 0.16	2.25 ± 0.13
Ratio	0.39 ± 0.04	0.36 ± 0.02	0.38 ± 0.02	0.36 ± 0.05
Spleen	0.75 ± 0.05	0.84 ± 0.11	0.82 ± 0.11	0.87 ± 0.09
Ratio	0.13 ± 0.01	0.14 ± 0.02	0.14 ± 0.02	0.14 ± 0.02
Adrenals	0.070 ± 0.010	0.196 ± 0.377	0.061 ± 0.015	0.066 ± 0.009
Ratio	0.012 ± 0.002	0.031 ± 0.058	0.010 ± 0.003	0.011 ± 0.002
Lungs	2.64 ± 0.34	2.61 ± 0.32	2.93 ± 0.41	2.60 ± 0.18
Ratio	0.45 ± 0.04	0.42 ± 0.04	0.50 ± 0.06	0.41 ± 0.04
Body wts	586 ± 49.4	624 ± 21.1	590 ± 48.4	637 ± 66.2

[a]Mean ± SD, N = 8.
[b]Organ weight ÷ body weight X 100.

Table 8. Blood Hematology Values[a] for Male Sprague-Dawley Rats Sacrificed 38-40 Days Postexposure

	Air	Air/DEET/PB	Air/STRESS	Air/Stress DEET/PB
WBC (10^3)	13.9 ± 5.5	12.5 ± 2.0	14.5 ± 4.0	13.3 ± 2.1
RBC (10^6)	8.5 ± 0.5	8.5 ± 0.4	8.5 ± 0.5	8.4 ± 0.2
HGB (g/dL)	15.2 ± 0.6	15.4 ± 0.4	15.3 ± 0.5	15.0 ± 0.5
HCT (%)	47.4 ± 2.2	48.2 ± 1.3	47.4 ± 1.5	47.0 ± 1.3
MCV (fL)	55.6 ± 1.4	56.5 ± 2.5	56.1 ± 2.0	55.8 ± 0.8
MCH (g/dL)	17.8 ± 0.5	18.2 ± 0.9	18.1 ± 0.7	17.8 ± 0.4
MCHC(g/dL)	32.1 ± 0.3	32.1 ± 0.3	32.1 ± 0.4	31.9 ± 0.4
Platelets (10^3)	1284.9 ± 123	1277.8 ± 117	1321.1 ± 201	1261.1 ± 91
Neutrophil (%)	15.0 ± 4.3	15.0 ± 3.6	13.5 ± 3.8	13.5 ± 4.3
Lymphocytes (%)	75.0 ± 5.2	74.8 ± 4.8	77.8 ± 3.4	77.0 ± 5.0
Monocytes (%)	8.1 ± 1.9	8.5 ± 1.9	7.2 ± 2.0	7.9 ± 2.1
Eosinophils (%)	1.4 ± 0.5	1.2 ± 0.3	1.2 ± 0.3	1.1 ± 0.2
Basophils (%)	0.5 ± 0.3	0.5 ± 0.3	0.4 ± 0.2	0.5 ± 0.4

[a]Mean ± SD, N=8.

Table 9. Blood Hematology Values[a] for Male Sprague-Dawley Rats Sacrificed 38-40 Days Postexposure

	JP4	JP4/ DEET/PB	JP4/Stress	JP4/Stress/ DEET/PB
WBC (10^3)	13.6 ± 3.7	12.4 ± 2.3	15.0 ± 3.3	12.1 ± 2.3
RBC (10^6)	8.5 ± 0.3	8.4 ± 0.5	8.3 ± 0.5	8.5 ± 0.4
HGB (g/dL)	15.3 ± 0.5	15.0 ± 0.7	15.2 ± 0.8	15.0 ± 0.5
HCT (%)	47.9 ± 1.4	46.7 ± 2.2	47.4 ± 2.6	47.1 ± 1.5
MCV (fL)	56.2 ± 1.3	56.0 ± 2.2	57.2 ± 1.4	55.7 ± 1.3
MCH (g/dL)	17.9 ± 0.6	18.0 ± 0.8	18.2 ± 0.4	17.8 ± 0.5
MCHC (g/dL)	31.8 ± 0.4	32.2 ± 0.3	32.0 ± 0.2	31.9 ± 0.6
Platelets (10^3)	1239.9 ± 128	1334.8 ± 163	1268.5 ± 63	1345.1 ± 102
Neutrophils (%)	11.3 ± 2.8	16.2 ± 5.2	14.6 ± 10.3	14.7 ± 3.0
Lymphocytes (%)	80.6 ± 5.1	73.8 ± 6.7	76.7 ± 10.8	75.5 ± 3.5
Monocytes (%)	6.7 ± 2.2	8.3 ± 2.8	7.3 ± 2.4	8.1 ± 1.9
Eosinophils (%)	1.2 ± 0.4	1.2 ± 0.3	1.0 ± 0.4	1.0 ± 0.3
Basophils (%)	0.4 ± 0.2	0.6 ± 0.3	0.3 ± 0.2	0.6 ± 0.2

[a]Mean ± SD, N=8.

Table 10. Blood Hematology Values[a] for Male Sprague-Dawley Rats Sacrificed 86-88 Days Postexposure

	Air	Air/ DEET/PB	Air/Stress	Air/Stress/ DEET/PB[b]
WBC (10^3)	14.6 ± 6.0	12.9 ± 3.9	11.1 ± 2.3	12.5 ± 2.5
RBC (10^6)	9.2 ± 0.4	9.1 ± 0.5	9.2 ± 0.6	9.0 ± 0.6
HGB (g/dL)	15.5 ± 0.6	15.4 ± 0.6	15.5 ± 0.8	15.2 ± 1.0
HCT (%)	48.8 ± 2.1	48.7 ± 2.1	48.8 ± 2.6	47.9 ± 3.5
MCV (fL)	52.8 ± 1.7	53.3 ± 2.1	53.2 ± 1.6	53.2 ± 1.5
MCH (g/dL)	16.8 ± 0.5	16.9 ± 0.7	16.8 ± 0.5	16.9 ± 0.5
MCHC (g/dL)	31.7 ± 0.3	31.8 ± 0.4	31.7 ± 0.4	31.8 ± 0.4
Platelets (10^3)	1254.4 ± 120	1214.1 ± 203	1164.8 ± 148	1248.1 ± 169
Neutrophils (%)	14.9 ± 5.8	17.6 ± 17.3	18.7 ± 14.0	12.0 ± 2.7
Lymphocytes (%)	75.2 ± 7.8	72.1 ± 18.3	69.6 ± 12.6	78.5 ± 7.6
Monocytes (%)	8.1 ± 1.9	8.7 ± 3.6	9.8 ± 2.1	7.7 ± 5.1
Eosinophils (%)	1.5 ± 0.7	1.5 ± 0.7	1.6 ± 0.7	1.5 ± 0.4
Basophils (%)	0.4 ± 0.2	0.3 ± 0.2	0.4 ± 0.3	0.3 ± 0.2

[a]Mean ± SD, N=8.
[b]N=7.

Table 11. Blood Hematology Values[a] for Male Sprague-Dawley Rats Sacrificed 86-88 Days Postexposure

	JP4	JP4/ DEET/PB	JP4/Stress	JP4/Stress/ DEET/PB
WBC (10^3)	10.7 ± 5.2	11.9 ± 2.0	12.2 ± 3.0	11.4 ± 1.8
RBC (10^6)	9.0 ± 0.5	8.9 ± 0.5	8.9 ± 0.5	9.3 ± 0.6
HGB (g/dL)	15.4 ± 0.6	15.3 ± 0.8	15.2 ± 0.6	15.6 ± 0.4
HCT (%)	48.2 ± 2.2	47.7 ± 2.2	48.1 ± 1.9	48.9 ± 1.4
MCV (fL)	53.5 ± 1.7	53.8 ± 1.9	54.1 ± 2.3	52.8 ± 2.9
MCH (g/dL)	17.1 ± 0.7	17.2 ± 0.7	17.1 ± 1.0	16.8 ± 0.9
MCHC (g/dL)	32.0 ± 0.6	32.0 ± 0.6	31.7 ± 0.6	32.0 ± 0.5
Platelets (10^3)	1196.9 ± 157	1189.9 ± 115	1229.5 ± 187	1386.8 ± 179
Neutrophils (%)	12.5 ± 3.9	11.4 ± 6.1	15.4 ± 11.2	13.1 ± 3.1
Lymphocytes (%)	76.2 ± 6.5	76.3 ± 7.4	75.7 ± 10.8	77.5 ± 3.4
Monocytes (%)	9.4 ± 2.6	10.3 ± 2.0	7.7 ± 0.9	7.8 ± 1.6
Eosinophils (%)	1.5 ± 0.4	1.5 ± 0.4	1.1 ± 0.3	1.2 ± 0.5
Basophils (%)	0.4 ± 0.2	0.4 ± 0.1	0.3 ± 0.2	0.4 ± 0.2

[a]Mean ± SD, N=8.

Table 12. Mean Values[a] of Serum Chemistry Parameters for Male Sprague-Dawley Rats Sacrificed 38-40 Days Postexposure

	Air	Air/ DEET/PB	Air/Stress	Air/Stress/ DEET/PB
BUN	13.6 ± 2.3	15.4 ± 1.9	14.5 ± 3.2	14.8 ± 2.7
Creatinine (mg/dL)	0.6 ± 0.1	0.6 ± 0.1	0.5 ± <0.0	0.6 ± 0.1
Chloride (mmol/L)	100.8 ± 1.4	100.0 ± 1.8	100.4 ± 2.3	100.3 ± 2.7
Calcium (mg/dL)	11.0 ± 0.4	11.1 ± 0.3	11.1 ± 0.6	10.9 ± 0.5
AST (IU/L)	101.5 ±46.0	111.3 ±53.9	92.5 ±39.0	98.1 ± 45.2
ALT (IU/L)	48.3 ±11.2	57.4 ± 5.9	50.5 ± 7.7	52.1 ± 5.7
Alkaline phosphatase (IU/L)	138.3 ±34.5	117.1 ±17.0	133.5 ±24.6	134.0 ± 32.9
Glucose (mg/dL)	201.3 ±27.2	197.9 ±19.8	207.8 ±38.1	198.5 ± 28.1
Sodium (mmol/L)	148.3 ± 1.2	147.9 ± 1.6	148.9 ± 1.2	147.8 ± 2.3
Magnesium (mg/dL)	3.0 ± 0.2	3.0 ± 0.3	3.1 ± 0.5	2.9 ± 0.5
Potassium (mmol/L)	6.0 ± 1.0	6.0 ± 0.7	5.2 ± 0.6	6.1 ± 1.1
CO_2 (IU/L)	33.0 ± 4.3	34.1 ± 4.4	33.4 ± 2.1	32.6 ± 4.8

[a]Mean ± SD, N=8.

Table 13. Mean Values[a] of Serum Chemistry Parameters for Male Sprague-Dawley Rats Sacrificed 38-40 Days Postexposure

	JP4	JP4/ DEET/PB	JP4/Stress	JP4/Stress/ DEET/PB
BUN	13.5 ± 2.1	14.6 ± 2.6	16.1 ± 4.9	15.3 ± 3.1
Creatinine (mg/dL)	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
Chloride (mmol/L)	100.4 ± 1.1	100.9 ± 1.5	101.0 ± 2.1	100.9 ± 1.4
Calcium (mg/dL)	11.1 ± 0.4	10.9 ± 0.6	11.2 ± 0.5	10.9 ± 0.4
AST (IU/L)	89.3 ± 33.1	117.8 ± 63.3	96.4 ± 34.1	103.6 ± 41.0
ALT (IU/L)	54.6 ± 12.6	53.4 ± 5.7	52.0 ± 6.4	53.6 ± 13.0
Alkaline phosphatase (IU/L)	124.4 ± 35.5	128.0 ± 18.6	137.5 ± 46.8	131.8 ± 43.1
Glucose (mg/dL)	177.4 ± 27.9	193.8 ± 28.7	205.6 ± 26.6	178.9 ± 26.7
Sodium (mmol/L)	149.1 ± 1.7	148.9 ± 1.5	147.8 ± 1.8	147.9 ± 1.4
Magnesium (mg/dL)	3.0 ± 0.5	2.8 ± 0.4	3.0 ± 0.4	3.0 ± 0.4
Potassium (mmol/L)	5.8 ± 0.4	5.9 ± 0.7	5.9 ± 1.1	6.1 ± 0.6
CO_2 (IU/L)	34.8 ± 2.9	32.5 ± 3.4	32.8 ± 3.9	33.4 ± 4.1

[a]Mean ± SD, N=8.

Table 14. Mean Values[a] of Serum Chemistry Parameters for Male Sprague-Dawley Rats Sacrificed 86-88 Days Postexposure

	Air	Air/ DEET/PB	Air/Stress	Air/Stress/ DEET/PB[b]
BUN	13.5 ± 1.5	14.0 ± 1.6	14.4 ± 1.7	13.1 ± 2.3
Creatinine (mg/dL)	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Chloride (mmol/L)	98.5 ± 1.4	98.9 ± 1.6	99.0 ± 0.9	98.1 ± 1.9
Calcium (mg/dL)	11.2 ± 0.4	11.4 ± 0.5	10.9 ± 0.2	11.3 ± 0.4
AST (IU/L)	102.3±25.4	87.1 ±24.6	90.9 ± 30.0	132.1 ± 37.6
ALT (IU/L)	43.6 ± 5.0	48.6 ±10.0	48.8 ± 12.9	50.3 ± 7.8
Alkaline phosphatase (IU/L)	82.3 ± 10.0	103.6 ± 29.4	99.1 ± 22.8	104.9 ± 14.0
Glucose (mg/dL)	238.5±77.3	231.0±29.8	207.3 ±27.7	233.7 ± 42.4
Sodium (mmol/L)	149.0 ±2.1	147.9 ±1.2	147.1 ± 1.4	148.3 ± 1.4
Magnesium (mg/dL)	2.8 ± 0.5	2.8 ± 0.3	2.5 ± 0.1	2.7 ± 0.5
Potassium (mmol/L)	6.4 ± 0.6	6.6 ± 0.9	6.4 ± 1.0	6.5 ± 1.1
CO_2 (IU/L)	33.5 ± 2.3	32.0 ± 1.7	33.1 ± 1.4	33.3 ± 2.4

[a]Mean ± SD, N=8.
[b]N=7.

Table 15. Mean Values[a] of Serum Chemistry Parameters for Male Sprague-Dawley Rats Sacrificed 86-88 Days Postexposure

	JP4	JP4/ DEET/PB	JP4/Stress	JP4/Stress/ DEET/PB
BUN	13.8 ± 1.7	13.9 ± 1.7	15.4 ± 1.8	15.8 ± 2.5
Creatinine (mg/dL)	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.8 ± 0.1
Chloride (mmol/L)	100.0 ± 1.1	99.0 ± 1.2	99.0 ± 1.3	98.1 ± 1.7
Calcium (mg/dL)	11.2 ± 0.3	11.3 ± 0.3	11.1 ± 0.2	11.7 ± 0.4
AST (IU/L)	100.5 ± 43.9	89.6 ± 30.7	97.3 ± 15.1	128.4 ± 65.3
ALT (IU/L)	47.9 ± 8.8	48.5 ± 4.5	51.6 ± 11.8	51.5 ± 6.4
Alkaline phosphatase (IU/L)	99.3 ± 27.5	91.1 ± 27.2	118.6 ± 26.0	103.0 ± 28.2
Glucose (mg/dL)	234.9 ± 67.5	225.6 ± 43.6	192.1 ± 9.2	242.6 ± 50.7
Sodium (mmol/L)	148.5 ± 1.2	147.4 ± 0.7	148.0 ± 0.9	148.9 ± 1.6
Magnesium (mg/dL)	6.3 ± 9.6	2.7 ± 0.3	2.7 ± 0.3	3.0 ± 0.4
Potassium (mmol/L)	6.7 ± 0.6	6.3 ± 0.6	6.2 ± 0.7	6.4 ± 0.7
CO_2 (IU/L)	33.3 ± 2.3	33.1 ± 1.4	34.1 ± 1.2	33.9 ± 3.4

[a]Mean ± SD, N=8.

Neurotransmitter Analysis

The levels of epinephrine, norepinephrine, dopamine, homovanillic acid, and 3,4-dihydroxyphenylacetic acid in the brain regions studied of treated rats (Groups 1-7) were not statistically significant from those of AIR-only controls (Group 8). Animals exposed to JP-4 jet fuel vapor, stress, and/or PB and DEET had significantly ($p<0.05$) increased levels of 5-hydroxy-indoleacetic acid and 5-hydroxytryptamine in the brainstem, cerebellum, cerebral cortex, caudate nucleus, hippocampus, and serum (Tables 16-19) compared to AIR-only controls at 38-40 and/or 86-88 days postexposure.

Table 16. Concentration[a] of 5-Hydroxytryptamine in Male Sprague-Dawley Rats 38-40 Days Postexposure

Sample	Air	Air/ Stress	Air/PB/ DEET	Air/Stress/ PB/DEET	JP4	JP4/ Stress	JP4/PB/ DEET	JP4/Stress/ PB/DEET
Brain-stem	0.65 ± 0.05	1.07 ± 0.07	1.04 ± 0.05	1.16 ± 0.09	0.92 ± 0.05	1.15 ± 0.08	0.95 ± 0.05	1.24 ± 0.11
Cere-bellum	0.73 ± 0.04	1.08 ± 0.10	0.84 ± 0.03	1.15 ± 0.09	0.84 ± 0.06	1.19 ± 0.09	0.93 ± 0.05	1.56 ± 0.10
Cerebral cortex	0.78 ± 0.05	1.20 ± 0.13	0.96 ± 0.03	1.42 ± 0.14	0.85 ± 0.04	1.26 ± 0.14	1.08 ± 0.08	1.42 ± 0.22
Caudate nucleus	0.78 ± 0.08	0.93 ± 0.05	0.73 ± 0.05	1.09 ± 0.16	1.10 ± 0.07	1.19 ± 0.11	1.17 ± 0.08	1.47 ± 0.11
Hippo-campus	0.77 ± 0.07	1.13 ± 0.05	1.08 ± 0.04	1.12 ± 0.09	0.90 ± 0.06	1.07 ± 0.02	1.00 ± 0.04	1.41 ± 0.08
Serum	0.05 ± 0.01	0.08 ± 0.003	0.06 ± 0.01	0.10 ± 0.01	0.06 ± 0.003	0.10 ± 0.01	0.07 ± 0.004	0.12 ± 0.01

[a]µg 5-Hydroxytryptamine/g wet weight, mean ± SD; N = 6.

Table 17. Concentration[a] of 5-Hydroxytryptamine in Male Sprague-Dawley Rats 86-88 Days Postexposure

Sample	Air	Air/ Stress	Air/PB/ DEET	Air/Stress PB/DEET	JP4	JP4/ Stress	JP4/PB/ DEET	Fuel/JP4/ PB/DEET
Brainstem	0.75 ± 0.05	1.23 ± 0.12	0.92 ± 0.05	1.25 ± 0.08	0.90 ± 0.01	1.17 ± 0.10	1.02 ± 0.06	1.28 ± 0.24
Cere-bellum	0.83 ± 0.03	1.22 ± 0.06	1.00 ± 0.07	1.25 ± 0.13	0.93 ± 0.04	1.12 ± 0.06	1.08 ± 0.08	1.48 ± 0.14
Cerebral cortex	0.81 ± 0.04	1.45 ± 0.20	0.93 ± 0.04	1.26 ± 0.14	0.92 ± 0.07	1.27 ± 0.11	1.00 ± 0.05	1.39 ± 0.22
Caudate nucleus	0.89 ± 0.03	1.24 ± 0.13	1.06 ± 0.07	1.12 ± 0.14	0.90 ± 0.04	1.15 ± 0.14	1.00 ± 0.06	1.28 ± 0.13
Hippo-campus	0.78 ± 0.04	1.26 ± 0.15	0.92 ± 0.03	1.26 ± 0.10	0.94 ± 0.08	1.27 ± 0.11	0.98 ± 0.05	1.31 ± 0.11
Serum	0.06 ± 0.002	0.10 ± 0.004	0.07 ± 0.01	0.10 ± 0.01	0.06 ± 0.004	0.10 ± 0.01	0.06 ± 0.003	0.13 ± 0.01

[a]µg 5-Hydroxytryptamine/g wet weight , mean ± SD; N=6.

Table 18. Concentration[a] of 5-Hydroxyindoleacetic Acid in Male Sprague-Dawley Rats 38-40 Days Postexposure

Sample	Air	Air Stress	Air/PB DEET	Air/Stress PB/DEET	Fuel	Fuel Stress	Fuel/PB DEET	Fuel/Stress PB/DEET
Brainstem	3.28 ± 0.19	4.90 ± 0.18	4.00 ± 0.28	5.00 ± 0.30	4.01 ± 0.33	5.63 ± 0.22	4.80 ± 0.16	5.72 ± 0.25
Cerebellum	3.38 ± 0.30	4.32 ± 0.63	4.19 ± 0.50	4.95 ± 0.56	4.12 ± 0.63	4.95 ± 0.50	4.95 ± 0.41	5.15 ± 0.55
Cerebral cortex	2.23 ± 0.24	5.49 ± 0.25	2.84 ± 0.13	6.55 ± 0.64	3.22 ± 0.29	5.96 ± 0.92	4.52 ± 0.45	6.62 ± 0.45
Caudate nucleus	3.07 ± 0.38	4.79 ± 0.48	3.90 ± 0.45	5.13 ± 0.72	3.77 ± 0.12	5.41 ± 0.18	4.59 ± 0.33	5.72 ± 0.24
Hippocampus	3.45 ± 0.42	5.92 ± 0.75	4.89 ± 0.42	5.75 ± 0.38	5.28 ± 0.51	5.71 ± 0.37	5.90 ± 0.55	7.18 ± 0.33
Serum	6.60 ± 0.42	9.75 ± 0.30	8.62 ± 0.82	10.46 ± 0.61	11.49 ± 0.82	16.66 ± 1.65	12.91 ± 1.24	21.25 ± 2.08

[a]µg 5-Hydroxyindoleacetic acid/g wet weight , mean ± SD; N=6.

Table 19. Concentration[a] of 5-Hydroxyindoleacetic Acid in Male Sprague-Dawley Rats 86-88 Days Post Exposure

Sample	Air	Air/ Stress	Air/PB DEET	Air/Stress/ PB/DEET	JP4	JP4/ Stress	JP4/PB/ DEET	JP4/Stress/ PB/DEET
Brainstem	1.78 ± 0.12	3.12 ± 0.20	2.51 ± 0.17	3.39 ± 0.19	2.27 ± 0.22	3.08 ± 0.47	2.97 ± 0.28	3.59 ± 0.43
Cerebellum	2.79 ± 0.23	4.30 ± 0.48	4.09 ± 0.45	4.46 ± 0.53	3.61 ± 0.12	4.86 ± 0.39	4.06 ± 0.46	5.47 ± 0.50
Cerebral cortex	1.69 ± 0.28	3.46 ± 0.16	2.59 ± 0.21	4.17 ± 0.45	2.24 ± 0.15	3.95 ± 0.27	2.96 ± 0.20	4.34 ± 0.17
Caudate nucleus	2.27 ± 0.16	3.39 ± 0.48	2.84 ± 0.41	3.26 ± 0.35	2.59 ± 0.30	3.70 ± 0.32	3.19 ± 0.39	3.47 ± 0.18
Hippocampus	2.68 ± 0.36	4.30 ± 0.41	3.53 ± 0.58	4.54 ± 0.89	2.78 ± 0.18	3.86 ± 0.20	3.03 ± 0.14	5.40 ± 0.63
Serum	11.44 ± 0.98	19.23 ± 2.72	15.66 ± 1.04	18.29 ± 3.48	15.55 ± 2.32	19.61 ± 4.48	17.28 ± 2.73	24.30 ± 5.62

[a]μg 5-Hydroxyindoleacetic acid/g wet weight, mean ± SD; N = 6.

Two-Dimensional Electrophoresis

Subtle but statistically significant alterations in protein expression were present in the JP4/STRESS/PB/DEET animals 38-40 days postexposure. The liver was the most susceptible of the three samples studied. Fifteen hepatic proteins were either upregulated or downregulated by the combination of treatments. Hsp60, a prominent mitochondrial stress protein was induced as was the tentatively identified D-dopachrome tautomerase. In the plasma samples eight proteins were significantly altered. In the kidney samples seven minor proteins were significantly altered. The identities of the altered plasma and kidney proteins are unknown.

Table 20. Two-dimensional Electrophoresis Results: Liver, Kidney, and Plasma Samples 38-40 Days Postexposure to PB, DEET, JP-4 Jet Fuel Vapor, and Stress

Sample	Liver		Kidney		Plasma	
	# of Spots	% of Total	# of Spots	% of Total	# of Spots	% of Total
Proteins Resolved	1060		942		639	
Spots used for scaling	385	36.3%	346	36.7%	216	33.8%
CV[a] < 15%	154	14.5%	122	13.0%	76	11.9%
CV < 20%	258	24.3%	206	21.9%	129	20.2%
P < .002 vs. control	5	0.5%	2	0.2%	4	0.6%
P < .003	8	0.8%	5	0.5%	4	0.6%
P < .004	11	1.0%	6	0.6%	6	0.9%
P < .005	15	1.4%	7	0.7%	8	1.3%

[a] CV = coefficient of variation.

SECTION IV

DISCUSSION

The fourteen day exposure of male rats to air or JP-4 jet fuel vapor, with or without additional exposure to stress and/or oral PB and dermal DEET, resulted in no mortality. The only mortality was one rat determined to have an esophageal perforation due to trauma after oral gavage. No clinical signs of toxicity were noted during exposure or postexposure. No treatment-related decreases in animal body weights were observed over the two-week exposure period, nor during the 38-40- or 86-88-day postexposure periods. No absolute organ or organ-to-body weight differences were noted for any of the eight study groups. There were no significant differences between control and treated groups in clinical chemistry values or hematology parameters. These results indicate that 14 day exposure to these chemicals and stress, either individually or in combination did not result in any overt signs or symptoms of toxicity.

Gross necropsy examination of all animals combined with histologic evaluation of selected animals from the control and JP4/STRESS/PB/DEET groups failed to identify any treatment-related lesions in these animals. In a previously reported study of exposure of chickens to pyridostigmine bromide, DEET, and permethrin (Abou-Donia et al. 1996) neurotoxic lesions characterized histologically by swollen axons in the spinal cord and sciatic nerve were observed. Axonal swelling was not seen in the animals analyzed from this study. Dilated myelin sheaths were observed routinely, and are a commonly encountered artifact of routine formalin fixation of these tissues. Each finding was graded for severity and statistically analyzed to ensure that a similar appearing treatment-related lesion was not present. No treatment effect was evident. No vacuolation, real or artifactual, was observed in any sections of peripheral sciatic nerve. The different results found in the two studies may be due to the use of differing chemicals, different doses, different routes of administration, or different species of test animals.

Most of the neurotransmitters analyzed, including epinephrine, norepinephrine, dopamine, homovanillic acid, and 3,4-dihydroxyphenylacetic acid, did not exhibit any significantly different levels when compared to the control group. Homovanillic acid and 3,4-dihydroxyphenylacetic acid are metabolites of dopamine. Significantly increased levels of 5-

hydroxyindoleacetic acid and 5-hydroxytryptamine were found in the brainstem, corpus callosum, hippocampus, cerebellum, caudate nucleus, and serum of animals exposed to stress, PB and DEET, and/or JP-4 jet fuel vapor at 38-40 or 86-88 days postexposure. Serotonin is the neurotransmitter most implicated in humans in the etiology or treatment of central nervous system disorders including anxiety and depression (Borne, 1994). 5-Hydroxy-indoleacetic acid (5-HIAA) is the major metabolite of 5-hydroxytryptamine (5-HT, serotonin). These significant changes in the levels of serotonin and its metabolite indicate the need for additional detailed investigation into the individual effects of these chemicals and stress on 5-HT and 5-HIAA levels at varying doses. Analysis of serum levels of these neurotransmitters in humans exposed to any of these chemicals and stress would be a valuable collaborative study. No synergistic effect of these chemicals and stress when presented in varying combinations was found on neurotransmitter levels. Due to the constraint on the number of exposure groups the individual effects of pyridostigmine bromide and DEET on neurotransmitter levels were not able to be determined. The greatest changes in neurotransmitter levels resulted from exposure to JP-4 jet fuel vapor or stress.

This screening study has presented some preliminary evidence that low doses of these chemicals, in combination with stress, have postexposure effects on neurotransmitter levels and neurobehavior (reported separately). Further acute range-finding studies, and/or subchronic and chronic testing protocols would provide valuable information on the effects of these chemical combinations on rat physiology and health. Continued development of this rat model to investigate chemical effects would provide data for human modelling studies and human risk assessment of exposure to these chemicals.

SECTION V

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