5-1988

The Immunological and Neurochemical Toxicity of Benzene and its Interaction with Toluene in Mice

Gin-Chang Hsieh
Utah State University

THE IMMUNOLOGICAL AND NEUROCHEMICAL TOXICITY OF BENZENE AND ITS INTERACTION WITH TOLUENE IN MICE

by

Gin-Chang Hsieh

A dissertation submitted in partial fulfillment

of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Toxicology

Approved:

UTAH STATE UNIVERSITY

Logan, Utah

1988

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my major professor Dr. Robert D. R. Parker, and my dissertation director, Dr. Raghubir P. Sharma, for their professional guidance, encouragement, and kind and patient support all the times I needed it so badly in the completion of this study. They have been teachers, advisors as well as friends throughout, for which I am grateful.

Special thanks must be given to those who served as my supervisory committee: Dr. Roger A. Coulombe, Dr. R. Ryan Dupont, and Dr. Reed P. Warren. They have all provided great support when needed, and I would like to thank each of them for time, energy, and knowledge expended in my behalf.

Everyone associated with the Toxicology Program has in one way or another contributed time and talent to make the completion of this project possible, and I am grateful to each of them: Brian Hughes, Randy Keller, Michael Huie, Brian Howard, Jeff Hincks, Darrel Warner, Yasuhiko Hatori, and the former graduate students in our program Sunil Jayasekara, Benjamart Srisuchart, and Michael Taylor.

Lastly, I extend the deepest thanks to my wife June for her continued love, encouragement, support, understanding, and sacrifice during the course of study. This dissertation is dedicated to my wonderful parents, Mr. & Mrs. Tung H. Hsieh, I owe them much for the person I am today.

Gin-Chang Hsieh

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

ABSTRACT

The Immunological and Neurochemical Toxicity of Benzene
and Its Interaction with Toluene in Mice
by
Gin-Chang Hsieh, Doctor of Philosophy
Utah State University, 1988

Major Professor: Dr. R. D. R. Parker
Department: Biology/Toxicology Program

Benzene and toluene are known groundwater contaminants. Male CD-1 mice were continuously exposed to 0, 31, 166, and 790 mg/L benzene and 0, 17, 80, and 405 mg/L toluene, respectively, in drinking water for four weeks. Benzene caused a reduction of leukocytes, lymphocytes and erythrocytes, and resulted in a macrocytic anemia. Lymphocyte response to both B- and T-cell mitogens, mixed lymphocyte response to alloantigens, and the ability of cytotoxic lymphocytes to lyse tumor cells were enhanced at the lowest dose of benzene and depressed in the higher dosage animals. Benzene at doses of 166 and 790 mg/L decreased the number of sheep red blood cell (SRBC)-specific plaque-forming cells, the level of serum anti-SRBC antibody, and the activity of interleukin-2 (IL-2).

Benzene treatment increased endogenous concentrations of the brain biogenic amines norepinephrine (NE), dopamine (DA) and serotonin (5-HT), and concomitantly, elevated the levels of their respective major metabolites vanillymandelic acid (VMA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA), in several brain regions. In most cases, the changes were dose-

related; in several instances, maximum effects occurred at the 166 mg/L benzene dose.

Toluene did not adversely affect the hematological parameters. Depression of immune function was evident at the highest dose (405 mg/L), except for mitogeneses. Increased neurochemical concentrations caused by toluene displayed a dose-dependent biphasic manner which began at a dose of 17 mg/L, peaked at 80 mg/L, and decreased at 405 mg/L. Toluene treatment had more selective effects on NE, 5-HT, VMA and 5-HIAA, than DA, DOPAC and HVA.

Both compounds, by increasing concentrations of the hypothalamic NE and its major metabolite VMA, stimulated the hypothalamic-pituitary-adrenocortical axis activity, resulting in an elevated plasma adrenocorticotropic hormone and serum corticosterone which had an additive adverse effect on IL-2 synthesis.

Toluene, 325 mg/L, completely inhibited benzene-induced cytopenia and immunosuppression when it was coadministered with benzene (166 mg/L). The low dose of toluene (80 mg/L) did not antagonize benzene immunotoxicity. Mice given the combined exposures exhibited raised levels of regional neurochemicals when compared to the untreated controls. Increased levels of monoamine metabolites in several brain regions were greater in the combined treatments of benzene and toluene than when either chemical was used alone. The results of the interaction studies support the known metabolic interaction mechanisms of benzene and toluene.

(226 pages)

CHAPTER I

INTRODUCTION

Benzene, the simplest aromatic hydrocarbon, is ubiquitous in occurrence. Its solubility and volatile nature indicate possible environmental mobility. This chemical has a long history of extensive use in industry. At present it is produced primarily by petrochemical industries and is used as a starting material for the manufacture of many chemicals. Benzene is also employed as a essential constituent in motor fuel (Fishbein, 1984; Brief et al., 1980). It has been identified in ambient water (U.S. Environmental Protection Agency, 1980a; Cotruvo, 1985) at concentrations up to 10 mg/L (U.S. National Toxicology Program, 1981).

Due to the high lipophilic nature of benzene which causes it to accumulate in lipid rich tissue, particularly the bone marrow and brain (Snyder et al., 1981a; Rickert et al., 1979), and because of the production of biologically reactive intermediates through metabolic biotransformations (Snyder et al., 1987; Irons, 1985; Sawahata et al., 1985; Bolcsak and Nerland, 1983), benzene is considered to be a potential health hazard. Like most organic solvents, benzene is a central nervous system depressant at high concentrations (Brief et al., 1980). Accumulation of this chemical in the nervous system may lead to impairment of the neuronal function. It has been shown that the major toxic effect of benzene for human beings or laboratory animals is primarily disturbance of the hematopoietic system, an effect unique to benzene and which can affect every subsequent cell line resulting in a variety of blood dyscrasias (Fishbein, 1984). The mechanism of benzene-

induced hematopoietoxic effects has not been well elucidated, however, most researchers believe oxidative benzene metabolites rather than benzene itself, are required to mediate these hematopoietic effects (Sawahata et al., 1985; Irons, 1985; Bolcsak and Nerland, 1983; Greenlee et al., 1981b). A number of recent studies on benzene carcinogenesis and mutagenesis have been reviewed by Dean (1985) who also suggests that benzene could be regarded as a classical initiator or a promotor to explain its leukaemogenesis.

Toluene is also considered a major aromatic chemical with environmental and occupational significance (Fishbein, 1985). It is produced in enormous quantities mainly from petroleum or petrochemical processes, and is employed as a solvent (for which it is increasingly used as a "safe" replacement for benzene) and as a starting compound for other chemicals and a component in gasoline (U.S. Environmental Protection Agency, 1980b). The neurotoxic property of this chemical represents its main health hazard (Benignus, 1981a,b). It has been detected in concentrations up to 6.4 mg/L in finished water (Tardiff and Youngren, 1986). Furthermore, it should be noted that commercial toluene contains varying amounts of benzene and it is frequently employed in industry with benzene (U.S. National Academy of Sciences, 1982). Toluene, in sufficient amounts, appears to have the potential to significantly alter the metabolism and resulting bioactivity of certain other chemicals, thereby possessing a potential for alteration of the toxicity of numerous chemical agents (Hayden et al., 1977). For example, it has been documented that toluene is a competitive inhibitor of the biotransformation, and antagonizes myelotoxic and genotoxic effects of benzene (Ikeda et al., 1972; Andrews et al., 1977; Sato and

Nakajima, 1979; Tunek et al., 1981; Gad-El-Karim et al., 1984; 1986).

Although there is a large amount of information available detailing the effects of benzene on the hematopoietic system, studies concerning the immunotoxic evaluations of this chemical are scant. Also, little information is available regarding the effects of toluene exposure on immune systems. Meanwhile, despite several published studies about the positive effects of benzene and toluene on behavior changes, research conducted on the neurotoxic potential e.g., alterations in brain neurochemicals), is still limited. Therefore the objective of this study was to investigate basic immunotoxic and neurotoxic mechanisms by utilizing various immunological and neuro-endocrinobiochemical parameters to better understand the mode of toxic actions of these two chemicals and their interactions in experimental animals. Because benzene and toluene are known contaminants of groundwater, which is a primary source of drinking water for approximately half the population of the United States (U.S. Office of Technology Assessment, 1984), oral administration via drinking water in animals was employed. Consequently, the information gathered may contribute to support of the development of groundwater quality standards and protection efforts.

CHAPTER II

LITERATURE REVIEW

IMMUNE SYSTEM AS A TARGET ORGAN FOR TOXICITY

The immune system is comprised of a complex but well-defined network of specialized cells that interact to mount a response following recognition of foreign materials termed antigens. The potential responses of the system range from the production of antibody or cell-mediated reactions to the induction and modulation of allergic and inflammatory responses (Bick et al., 1985). The response to antigens and the mechanisms of many cellular interactions is under genetic control. The use of the immune system as a target organ for detecting toxicity of drugs, chemicals or environmental toxicants recently has received considerable attention (Bick, 1982; Dean et al., 1985a). It has many sensitive parameters whose alterations, as a result of experimental procedures, are easily determined (Dean et al., 1982; 1986; Thomas et al., 1985).

Introduction of a chemical into a host may elicit a variety of reactions. Many chemicals, or their metabolites, may be recognized as antigens. This response may be directed at the native molecule or metabolite or may result from the chemical group binding to host protein creating new antigenic determinants. Foreign chemicals may enhance or suppress immune competence. Enhancement of immune potential may lead to increased host resistance, or to autoimmunity. Decreased immune function may lead to the lack of an appropriate response. It is possible for these interactions to occur at the same time in various combinations. Lastly, a chemical may have no effect on the immune

system at doses that produce toxicity in other organ systems.

In order to be able detect slight modifications in immune responsiveness, sensitive assay systems (Dean et al., 1986; Thomas et al., 1985a; Sharma and Zeeman, 1980) must be employed and require better standardization so little variation in data occurs. Table II-1 summarizes a comprehensive panel of *in vivo* and *in vitro* assays used to measure host resistance and immunity, and the end points measured. A number of industrial and environmental chemicals have been shown to affect the immune response in human beings and animals (Dean et al., 1985a; Sharma, 1981; Faith et al., 1980). Risk assessment scientists have begun to recognize that immunological information gained from cellular and molecular approaches for the safety assessment of a drug or chemical should facilitate improved human risk assessment.

NEUROBIOCHEMICAL APPROACHES TO TOXICOLOGY

As technology has become more refined in recent years, useful biochemical methods are available to understand the mechanisms involved in normal and altered nervous system function (Narahashi, 1984; Bondy, 1986). It is through the determination of such mechanisms that neurochemistry can best contribute to the overall assessment of neurotoxicity. Neurotoxic agents can affect the nervous system in a number of diverse ways (Bondy, 1985; Norton, 1986; Cooper et al., 1986; Mitchell, 1982). Some agents act directly on the nervous system, whereas others disturb non-neuronal processes which affect the nervous system indirectly. In most instances, biochemical systems are affected through general or specific mechanisms. A neurotoxin can disturb the blood-brain barrier; interfere with energy metabolism or the

TABLE II-1

IMMUNE FUNCTION ASSAYS FOLLOWING CHEMICAL OR DRUG EXPOSURE*

Parameter	Endpoint measured	Immune correlate
Immunopathology		Overall immune system
Hematology	Complete blood count, WBC differentials	
Weights	Body, spleen, thymus, kidney, adrenal	
Histology	Spleen, thymus, bone marrow, lymphonode, adrenal	
Cellularity	Spleen, bone marrow, B-cell progenitor, pluripotent stem cell, granulocyte/macrophage progenitor, cell surface marker profile (% T, B, macrophage; T-cell subsets)	
Mitogenesis		
LPS	Incorporation of radiolabled nucleic acid precursors into DNA of dividing lymphocytes	B-cells
PHA, Con A		T-cells
PWM		B- and T-cells
Mixed lymphocyte response	Incorporation of radiolabled nucleic acid precursors into DNA of dividing responder cells	CMI
Lymphokines	Quantitated by incorporation of radiolabled nucleic acid precursors into DNA of dividing lymphokine-dependent cells	CMI
Cytotoxic cells		
CTL	% cytotoxicity of ^{51}Cr-labeled tumor target cells (determined by ^{51}Cr-release assay)	CMI
K		Natural or innate immunity
NK		"

TABLE II-1 (CONT.)

IMMUNE FUNCTION ASSAYS FOLLOWING CHEMICAL OR DRUG EXPOSURE

Parameter	Endpoint measured	Immune correlate
Antibody response		
T-dependent antigen (SRBC) T-independent antigen (LPS)	Specific serum antibody level or quantitation of antibody plaque forming cells (PFC)/10^6 spleen cells 4 days after antigen immunization	AMI, macrophage
Delayed hypersensitivity response	Radiometric assay with T cell-dependent antigen	CMI
Host resistance		
B16F10 melanoma	Measurement of lung tumor cell growth 21 days post-challenge by quantitation of uptake of [^{125}I] iododeoxyuridine	CMI, macrophage NK
PYB6 sacroma	Lethal tumor frequency 100 exposure days post-challenge	CMI, macrophage NK
Listeria monocytogenes	Cumulative % mortality 14 days post-challenge, mean survival time	CMI, macrophage
Streptococcus zooepidemicus	Cumulative % mortality 14 days post-challenge, mean survival time	AMI, macrophage polymorphonuclear leukocyte, non-specific immunity
Influenza A2/Taiwan/64	Cumulative % mortality 14 days post-challenge, mean survival time	CMI, AMI, interferon, macrophage, non-specific immunity
Host resistance		
Herpes simplex virus/ type 1 and 2	Cumulative % mortality 21 days post-change, mean survival time	CMI, AMI, macrophage, interferon, NK

TABLE II-1 (CONT.)

IMMUNE FUNCTION ASSAYS FOLLOWING CHEMICAL OR DRUG EXPOSURE

Parameter	Endpoint measured	Immune correlate
Macrophage** function		CMI, AMI, non-specific immunity
Peritoneal macrophage	Cellularity quantitation	
Phagocytosis ability	% phagocytosis of ^{51}Cr-labeled chicken or sheep erythrocytes	
Cytolysis of tumor cell	^{51}Cr-release assay	
Cytostasis	Inhibition of MBL-2 murine leukemia cell growth (measured by uptake of radiolebeled DNA precursors)	
Bactericidal activity	Ratio of the viable bacterial (^{32}S-labeled *Klebsiella pneumoniae*) counts	
Ectoenzyme levels	Quantitation	
Granulocyte function test	Nitroblue terazolium (NBT) dye	Non-specific immunity

*Abbreviations used in this table: AMI, antibody-mediated immunity; CMI, cell-mediated immunity; Con A, concanavalin A; CTL, cytotoxic T-lymphocyte; K, killer cell; LPS, lipopolysaccharide; NK, natural killer cell; PHA, phytohemagglutinin; PWM, pokeweed mitogen; SRBC, sheep red blood cell; WBC, white blood cell

**Both resident and activated peritoneal macrophages

biosynthetic pathways of neuronal macromolecules; alter axoplasmic flow or cellular permeability; affect neurotransmitter synthesis, storage, release, and uptake; or interfere with neurosecretory processes (Bondy, 1985; Mailman and Dehaven, 1984). Such changes can occur in localized brain regions, in specific cell types, or in subcellular organelles. Most of the studies have focused mainly on the levels of monoaminergic neurotransmitters, namely, catecholamines (dopamine and norepinephrine) and indoleamine (5-hydroxytryptamine). Functional significance of monoamine alterations has now been well documented (Rogawski and Baker, 1985). A number of neurological and psychiatric syndromes are caused by an excess or deficiency of these specific neurotransmitters (Willis and Smith, 1985; Poirier and Bedard, 1984). However, assays of steady state levels of neurotransmitters are difficult to interpret particularly when they are measured in whole brain homogenates or in large brain regions. Therefore monoamine metabolite levels would be a reflection of monoaminergic neuronal activity and yield more useful information (Commissiong, 1985; Bondy, 1986). A number of environmental chemicals, either of natural origin or products of industrial processes, have been shown to cause a variety of effects on neurochemical transmitters (O'Donoghue, 1985; Sharma et al., 1986; Coulombe and Sharma, 1985; 1986).

The hypothalamus is an area where around 40,000 nerve fibers mediate between central nervous system and the rest of the body, by way of release factors, trophic hormones, and endocrine hormones. In view of the incompleteness of the blood-brain barrier, it is more exposed to systemically circulating materials such as neurotoxins (Bondy, 1985). Thus, the functional changes in this rather small area can have major

reverberations. Classically, stress responses (as determined by increased circulating corticosteroid hormone levels) were thought to be mediated and regulated entirely by the hypothalamic-pituitary-adrenocortical (HPA) axis through the following cascade (Axelrod and Reisine, 1984; Besedovsky et al., 1985). The central or peripheral nervous system recognizes "stressors" (e.g. infections, chemical intoxications, trauma, pain, psychologic stress) and leads to increase of hypothalamic noradrenergic neural activity, which in turn increases the secretion of corticotropin-releasing factor (CRF) into the hypophyseal portal circulation. As a consequence of the actions of CRF at the corticotrope in the anterior pituitary, adrenocorticotropic hormone (ACTH) is released into the systemic circulation. ACTH acts on the adrenal cortex to stimulate the synthesis and secretion of corticosteroids. Corticosteroids, the end products of HPA axis, are well known to impair various aspects of immunocompetence (Besedovsky et al., 1985; Cupps et al., 1985) and have been shown to inhibit interleukin-2 (IL-2) as well as interleukin-1 (IL-1) production (Gillis et al., 1979a,b; Snyder and Unanue, 1982; Goodwin et al., 1986).

ENVIRONMENTAL ASPECTS OF BENZENE

Chemical and Physical Properties

Benzene (C_6H_6), an aromatic hydrocarbon with a molecular weight of 78.1, is a volatile, colorless, clear, highly flammable liquid hydrocarbon. Pure benzene has a boiling point of 80.1^{o}C and a melting point of 5.5^{o}C. Benzene has a density less than that of water (0.879 at 20^{o}C) and is soluble in water at concentrations which have been shown to be toxic to aquatic organisms. Benzene is also readily soluble in

natural fats and fat-soluble substances. It may accumulate in living organisms and appears to accumulate in animal tissues that exhibit a high lipid content or that represent major metabolic sites such as liver and brain. The fairly volatile nature (high vapor pressure of 74.6 mm Hg at 20^{o}C) and a relatively high solubility (1,780 mg/L at 25^{o}C) indicate that benzene could be washed out of the atmosphere with rainfall and then be evaporated back into the atmosphere, causing a continuous recycling between the two media. It is miscible with acetone, alcohol, carbon disulfide, carbon tetrachloride, chloroform, ether, glacial acetic acid and oils. Benzene undergoes the typical reactions of aromatic hydrocarbons including: chlorination, nitration, oxidation, and sulfonation (U.S. Environmental Protection Agency, 1980a; Maltoni, 1983; Fishbein, 1984). Benzene is also expected to be photooxidized in air and otherwise biodegraded in the environment (Korte and Klein, 1982).

Occurrence and Usage

Benzene has been produced industrially from coal tar distillation since 1849, and from petroleum by catalytic reforming of light naphthas from which it is isolated by distillation or solvent extraction since 1941. At present the major source of benzene is petroleum. It is one of the largest produced compounds and has been detected at various concentrations in the atmosphere and ambient water. Benzene is reported to occur in fruits, fish, vegetables, nuts, dairy products, beverages, eggs, cooked chicken and heat-treated or canned beef. Benzene has also been identified in cigarette smoke at levels of 150-204 mg/m^{3}. Its major use in the past was in blends with gasoline. Although this use has been reduced in the United States, benzene is still largely employed in

many countries for the production of commercial gasoline. The benzene content in gasoline varies from country to country and its range is estimated to be from 1 to 15%. Presently, benzene is largely employed as a chemical intermediate for the production of many important industrial compounds, such as ethylbenzene, styrene, phenol, cyclohexane, maleic anhydride, aniline, dichlorobenzenes, etc. In the past, benzene was used as a solvent for paints and rubber. It has also been used in medicine, in the treatment of hemoblastosis (leukemias, polycythemia and malignant lymphomas), and in the veterinary field for disinfestating wounds (Brief et al., 1980; U.S. Environmental Protection Agency, 1980a; Fishbein, 1984).

Populations at Risk and Allowed Levels

The following population groups may be exposed to benzene: workers engaged in its production; workers in chemical industries using benzene as an intermediate; workers in industries producing material containing benzene; workers utilizing or handling compounds containing benzene; people living in industrialized towns near factories producing or employing benzene, or compounds containing it; the general overall population since benzene is contained in gasoline and can also be found as a contaminant in drinking water. The levels of benzene allowed in workplaces vary from country to country. Until 1978, in the United States the Occupational Safety and Health Administration (OSHA) standard for benzene prescribed an 8-hour time-weighted average (TWA) of 10 ppm with an acceptable ceiling concentration of 25 ppm in air. In addition, this standard allowed excursions above the ceiling to a maximum peak concentration not exceeding 50 ppm, provided that such exposure occurred for no more than 10 minutes in any 8-hour work period. In 1978, OSHA

stated that: "1 ppm TWA, with 5 ppm ceiling limit for 15 minutes during the 8-hour day, is the level which most adequately assures, to the extent feasible, the protection of workers exposed to benzene." However, that rule was annulled by the U.S. Supreme Court (Maltoni, 1983; Brief et al., 1980; Holmberg and Lundberg, 1985; Fishbein, 1984).

For the maximum protection of human health from the potential carcinogenic effects of exposure to benzene through ingestion of contaminated water and contaminated aquatic organisms, the ambient water concentrations should be zero, based on the non-threshold assumption for this chemical. However, zero level may not be attainable at the present time. Therefore, if the incremental increase of cancer risk over a lifetime is estimated at 10^{-5}, 10^{-6}, and 10^{-7} (one additional case of cancer in populations of 100,000, 1,000,000, and 10,000,000), the corresponding recommended criteria are 6.6 µg/L, 0.66 µg/L, and 0.066 µg/L, respectively. If these estimates are made for consumption of aquatic organisms only, excluding consumption of water, the levels are 400 µg/L, 40.0 µg/L, and 4.0 µg/L, respectively (U.S. Environmental Protection Agency, 1986a).

Routes of Exposure

The respiratory route is believed to be the major source of human exposure to benzene. Benzene diffuses rapidly through the lungs and is quickly absorbed into the blood, with the rate of absorption being greatest at the beginning of exposure. Inhalation of benzene vapors may be supplemented by percutaneous absorption, although benzene is poorly absorbed through intack skin. In the general population, much of the benzene exposure is from gasoline vapors and automotive emissions (Fishbein, 1984). The dermal route is only a minor source of human

exposure since skin contact is infrequent (Franz, 1983). Exposure to benzene through oral ingestion is also considered to be a problem for the general population (Lee et al., 1983; U.S. Environmental Protection Agency, 1980a). It has been reported that chromosomal damage is higher with orally administered benzene than other exposure routes in mice (Gad-El-Karim et al., 1986).

BENZENE METABOLISM

Metabolism and Toxicity of Benzene

Benzene metabolism is required for its toxicity (Snyder et al., 1983). Exposure to benzene has long been associated with toxicity to blood and bone marrow, including lymphocytopenia, pancytopenia, thrombocytopenia, and aplastic anemia (Brief et al., 1980; Goldstein, 1983). Benzene is also a carcinogen and mutagen (Aksoy, 1985; Dean, 1985; Ereson et al., 1985). It is associated with an increased incidence of acute myelogenous leukemia and some of its variants in humans (Infante and White, 1985; Rinsky et al., 1987), an increased incidence of several solid tumors (Maltoni et al., 1985; Dean, 1985), and possibly leukemia/lymphoma in rodents (Snyder et al., 1980; Cronkite et al., 1984). Several studies are also in agreement that benzene possesses embryotoxic/teratogenic potential in experimental animals (Green et al., 1978; Kuna and Kapp, 1981; Ungvary and Tatrai, 1985).

The metabolic fate of benzene has interested biochemists and toxicologist throughout this century. A variety of studies have established that benzene itself is not the toxic species but requires metabolism to reactive intermediates (Snyder, 1984; Irons, 1985),

through complex bioactivation mechanisms (Pellack-Walker et al., 1985). *In vivo* metabolic studies following benzene exposure have identified phenol as the primary metabolite of benzene with hydroquinone, catechol, *trans,trans*-muconic acid and 1,2,4-trihydroxybenzene as significant secondary metabolites (Snyder et al, 1981; Gad-El-Karim et al., 1985b). The semiquinone radicals and/or *p*-benzoquinone derived from hydroquinone and/or catechol are generally considered to be the toxic metabolites (Tunek et al., 1980). Recently, it has been shown that *trans,trans*-mucondialdehyde, an open-ring product of catechol, is a possible toxic reactive intermediate (Witz et al., 1985; Latriano et al., 1986). Ethanol potentiates benzene toxicity *in vivo* by accelerating the hydroxylation of benzene and the conversion of phenol into toxic metabolites, thus, increasing benzene hematotoxicity (Nakajima et al., 1985). The major sites of benzene metabolism are liver and bone marrow. Benzene is converted by the liver to (a) metabolite(s) which travel(s) to the bone marrow and exerts its toxic effects.

Metabolism of Benzene in Liver

In the liver, the major site of benzene metabolism, benzene is converted via a cytochrome P-450-mediated pathway (Gonasun et al., 1973) to benzene oxide. The majority of benzene oxide formed spontaneously rearranges to yield phenol (Tunek et al., 1978), with a small portion of the oxide undergoing hydrolysis (catalyzed by epoxide hydrolase) to benzene-1,2-trans-dihydrodiol. The dihydrodiol can be further converted to catechol by cytosolic dehydrogenase (Jerina et al., 1968). This is believed to be the major metabolic pathway from benzene to catechol (Tomaszewski et al., 1975). Phenol can be further hydroxylated by the cytochrome P-450-dependent monoxygenase system to hydroquinone and

catechol, the former being the major product (Tunek et al., 1980; Sawahata and Neal, 1983). Different isozymes of cytochrome P-450 may be involved in the formations of hydroquinone and catechol (Sawahata and Neal, 1983).

Metabolism of Benzene in Bone Marrow

The bone marrow is the target organ of benzene. However, since the concentration of cytochrome P-450 measured in bone marrow is low compared with that measured in liver or lung (Andrews et al., 1979; Sawahata et al., 1985), it possesses a limited capacity to metabolize benzene (Andrews et al., 1977; 1979; Rickert et al., 1979). This suggests that the metabolism of benzene by bone marrow cannot account for the concentration of metabolites reported in this tissue *in vivo*. Andrews et al. (1979) also found the metabolism of benzene by bone marrow microsomes required NADPH and was inhibited by carbon monoxide.

Phenol, hydroquinone, and catechol are more toxic to bone marrow cultures than benzene. After benzene exposure, phenol concentrations within the bone marrow rise and decrease relatively quickly, whereas hydroquinone and catechol concentrations, although moderately low initially, rise with time and persist in relatively high concentrations (Rickert et al., 1979; Greenlee et al., 1981a,b). The retention of phenol, hydroquinone and catechol in bone marrow could result from the formation of these compounds in the liver and their subsequent uptake by bone marrow cells. The importance of hepatic metabolism of benzene in production of benzene metabolites found in the bone marrow is supported by the experiments performed by Sammett et al. (1979). They found that partial hepatectomy resulted not only in a reduction in amounts of benzene metabolites excreted in urine, but also in a decrease in

concentrations of benzene metabolites detected in bone marrow.

Microsomal Metabolism of Benzene

Benzene induces the enzymes required for its metabolism. Benzene is converted to phenol by rat liver microsome (Gilmour et al., 1986; Sawahata and Neal, 1983). Small amounts of hydroquinone and catechol are produced from both benzene and phenol in a reaction mediated by cytochrome P-450 and stimulated by benzene pretreatment. Two distinct forms of mixed-function oxidase activity in rat liver microsomes appear to metabolize benzene (Post and Snyder, 1983). Benzene pretreatment increases enzyme activity without affecting total cytochrome P-450 content (Post and Snyder, 1983; Pathiratne et al., 1986a). The addition of methyl groups to the aromatic ring influences the inductive pattern of benzene hydroxylase (Pathiratine et al., 1986b). Gilmour et al. (1986) also found that metabolism of benzene and phenol was competitively inhibited by toluene, and benzene and phenol reciprocally inhibited the metabolism of each other. The rate of conversion of phenol to hydroquinone by mouse liver microsomes is significantly higher than the rate of metabolism of benzene (Lunte and Kissinger, 1983).

The mechanism of the microsomal cytochrome P-450-dependent oxidation of benzene has been studied with rabbit liver microsomes and reconstituted membrane vesicles containing cytochrome P-450 LM2 by Johansson and Ingelman-Sunberg (1983), who concluded that the reaction is mediated by free hydroxyl radicals generated from hydrogen peroxide and superoxide anion in a modified Haber-Weiss reaction. However, the hydroxyl radicals do not contribute significantly to the metabolism of benzene to phenol when the concentration of benzene is close to the *Km* value for P-450 LM2. At a benzene concentration below the *Km* values,

the free radical-mediated pathway of phenol formation becomes increasingly predominant (Gorsky and Coon, 1985). Involvement of free radicals in benzene metabolism is also suggested by Khan et al. (1984) and Latriano et al. (1985). The microsomal cytochrome P-450 conversion of benzene to phenol via an arene oxide intermediate is demonstrably followed by an NIH shift (Hinson et al., 1984; 1985). Hinson et al. (1985) studied the role of the NIH shift in the formation of phenol from the deuterated benzene derivative, 1,3,5-[$^2H_3$] benzene, and found the expected products, 2,3,5-[$^2H_3$] phenol and 2,4-[$^2H_2$] phenol, which indicated that the shift had occurred.

Peroxidase-Dependent Metabolism of Benzene Metabolites

The isolation of phenol, hydroquinone, and catechol in the bone marrow following benzene exposure suggests the possibility that the critical metabolic events may involve an interaction of these phenolic compounds during localized metabolism within the bone marrow (Sawahata et al., 1985; Eastmond et al., 1987b). Since bone marrow contains relatively low concentrations of cytochrome P-450-dependent-monooxygenases, the metabolism of hydroxylated benzene metabolites, i.e. phenol, hydroquinone and catechol, in bone marrow by enzyme systems other than the cytochrome P-450-mediated system has been reported. Bone marrow contains appreciable levels of myeloperoxidase for which phenol is a known substrate and hydroquinone and catechol are probable substrates (Eastmond et al., 1986). Myeloperoxidases are capable of converting phenol to reactive protein-binding species (Eastmond et al., 1986; 1987a,b). A secondary peroxidase-dependent metabolism of phenol, hydroquinone, and catechol may be occurring within the bone marrow. This

mechanism may be responsible for benzene-induced myelotoxicity. It has been suggested that phenol metabolized to hydroquinone and catechol, and hydroquinone oxidized to *p*-benzoquinone is mediated by the myeloperoxidase-dependent pathway (Pellack-Walker et al., 1985).

Covalent Binding of Reactive Benzene Metabolites

Several studies have indicated that benzene and certain benzene metabolites are converted in nuclei and mitochondria to biologically reactive intermediates that covalently bind to macromolecules and cause DNA damage in the form of strand breaks and covalently bound adducts in liver and bone barrow *in vivo* or *in vitro* (Lutz and Schlatter, 1977; Irons et al., 1980; Gill and Ahmed, 1981; Rushmore et al., 1984; Artellinoi et al., 1985; Kalf et al., 1985; Wallin et al., 1985; Snyder et al., 1987). The level of DNA binding is highest in bone marrow. Either benzene-induced aplastic anemia or leukemia might be related to an interaction of the biologically reactive intermediates derived from benzene with nuclei acids. It has been shown that covalent binding was greatly enhanced by H_2O_2 and horseradish peroxidase or myeloperoxidase, and prevented by ascorbate (Smart and Zannoni, 1984; 1985). Peroxidase appears to be responsible for the oxidation of phenol, as ascorbate blocks the oxidation of phenol, and H_2O_2 is required for the activation of phenol to covalently binding species. The myelotoxicity of benzene may result from the high ratio of peroxidase activity to quinone reductases, the enzymes which reduce quinone to hydroquinone or catechol via a two-electron transfer, activity in bone marrow (Smart and Zannoni, 1984). Lack of benzene toxicity in the liver, as compared to the bone marrow, may thus result from the high quinone reductase

activity in the liver (Wermuth et al., 1986). DNA synthesis is inhibited in mouse lymphoma cells after exposure to the benzene metabolites, but not to benzene (Pellack-Walker et al., 1985; Pellack-Walker and Blumer, 1986). Benzoquinone is the most potent inhibitor, followed by hydroquinone, 1,2,4-benzenetriol, catechol, and phenol. These findings suggest that inhibition of DNA synthesis correlates with the ease of oxidation to reactive metabolite(s). Decreased synthesis of DNA or RNA *in vitro* by benzene metabolites is also observed in rat liver and rabbit bone marrow mitochondria (Schwartz et al., 1985; Kalf et al., 1982), mouse lymphocytes and macrophages (Post et al., 1985; 1986), and rabbit bone marrow nuclei (Post et al., 1984). Rat bone marrow mitoplasts, prepared by stripping the outer membrane of mitochondria to avoid microsomal contamination, *in vitro* incubated with benzene metabolites show concentration-dependent inhibition of mitochondrial RNA synthesis (Kalf et al., 1985).

HEMATOTOXICITY AND BENZENE

Benzene-Induced Hematotoxicity

Benzene is a proven hematoxin and is well known to exert its greatest toxicity on hematopoietic cells both in the peripheral blood and in the hematopoietic organs (Fishbein, 1984; Bolcsak and Nerland, 1983). Repeated benzene exposure in humans and laboratory animals results in the induction of pancytopenia, a condition characterized by markedly decreased numbers of circulating erythrocytes, leukocytes and thrombocytes. The resultant aplastic anemia, i.e., pancytopenia associated with fatty replacement of functional bone marrow, is a potentially fatal disorder which in its severe form has greater than a

fifty percent mortality rate (Green et al., 1981a,b; U.S. Environmental Protection Agency, 1980a; Brief et al., 1980). In certain individuals, continued exposure to benzene leads to both pancytopenia and bone marrow hyperplasia, a condition that may indicate a preleukemic state (Snyder et al., 1977). It was suggested that chronic benzene poisoning is most easily detected by using erythrocyte reduction and increased mean corpuscular volumes (MCV) but other hematologic parameters increase the diagnostic accuracy (Haley, 1977). In mice Baarson et al. (1984) reported that low-level repeated exposure to benzene (10 ppm) via inhalation could markedly depress the number of erythrocytes.

A number of observations have demonstrated that lymphocytes are particularly sensitive to benzene toxicity in animals (Aoyama, 1986; Rozen and Snyder, 1985; Dempster et al., 1984; Baarson et al., 1984; Snyder et al., 1982; Green et al., 1981a), or in humans who are inadvertently exposed to this chemical in the workplace (Moszczynsky and Lisiewicz, 1984; Brief et al., 1980). Mice exposed to benzene via inhalation showed significant depressions in the numbers of B-lymphocytes (s-IgM+ cells) in blood, bone marrow and spleen, and in the numbers of T-lymphocytes (Thy1,2+ cells) in blood, thymus and spleen (Aoyama, 1986; Rozen and Snyder, 1985). In addition, benzene has been shown to increase the permeability of lymphocyte lysosomal membranes and to release lysosomal enzyme into the cytoplasm, resulting in decrease of lymphocytes (Moszczynski and Lisiewicz, 1984).

The mechanism for benzene-induced hematopoietoxic effects has been studied for many years. Studies have shown that oxidative benzene metabolites, rather than benzene itself, are required to mediate these hematopoietic effects of benzene (Sawahata et al., 1985; Irons, 1985;

Bolcsak and Nerland, 1983; Snyder et al., 1981c; Greenlee et al., 1981a,b). The increases in leukocyte alkaline phosphatase activity were reported both in humans occupationally exposed to benzene (Moszczynski, 1980; Yin et al., 1982) and in experimental animals (Li et al., 1986; Yin et al., 1982). The validity of the increase in activity of alkaline phosphatase in leukocytes as an early indicator of benzene-induced leukopenia has been demonstrated (Li et al., 1986). Hematopoietic toxicity of benzene would arise from the toxic damage to one or more of the components of the hematopoietic system: stem cells, transit cells (progenitor cells in various degrees of differentiation), and the bone marrow stromal microenvironment (Kalf et al., 1987).

Effect of Benzene on Stem Cell

Several studies have shown that benzene causes reductions in the numbers of pluripotent stem cells (CFU-S) in mice following exposure to benzene (Uyeki et al., 1977; Green et al., 1981b; Snyder et al., 1981b; Tunek et al., 1981; 1982), whereas other workers have reported no change (Frash et al., 1976). This discrepancy has been explained, in part, by a toxic effect of benzene on the transit cells, which signals stem-cell proliferation and differentiation, and thus depletes the stem-cell pool (Snyder et al., 1977; Gill et al., 1980; Cronkite et al., 1982).

Effect of Benzene on Progenitor Cell

Benzene affects committed progenitor cells by a decrease in the numbers of differentiating erythroid and myeloid progenitors in benzene-exposed rats, without an effect on the number of progenitor cells or mature cells in the bone marrow (Irons et al., 1979). Studies on the effects of benzene on the kinetics of ^{59}Fe uptake into maturing mouse

erythrocytes indicated that the pronormoblast was the progenitor most sensitive to benzene, whereas stem cells and nondividing reticulocytes were not affected (Lee et al., 1974). The metabolites phenol, catechol, and hydroquinone have also been reported to reduce ^{59}Fe incorporation into developing erythrocytes. The erythropoietic toxicity of benzene and phenol is completely alleviated by the mixed-function oxidase inhibitor, but not of hydroquinone or catechol (Bolcsak and Nerland, 1983). *Trans,trans*-mucondialdehyde, a six-carbon alpha, beta-diene aldehyde and an open-ring metabolite of benzene, is a potent bone marrow toxin in CD-1 mice in a manner similar to benzene (Witz et al., 1985; Latriano et al., 1986). Other studies have shown that the number of erythroid (CFU-E) and granulocyte/macrophage (CFU-GM) progenitor cells was depressed in benzene-exposed mice (Tunek et al., 1981; Baarson et al., 1984; Keller and Snyder, 1986). A reduction of lymphocyte progenitor cells was also observed in the spleen and bone marrow of hydroquinone or catechol-treated animals (Wierda and Irons, 1982; King et al., 1986).

Effect of Benzene on Marrow Stromal Cells

Bone marrow stromal cells act as an essential component for hemopoiesis by forming a supporting matrix for developing precursor cells and releasing soluble factors involved in the regulation of hemopoiesis (Lichtman, 1981; Tavassoli and Friedenstein, 1983; Allen and Dexter, 1984). Stromal cells produce colony-stimulating factors (CSF), growth factors, which induce myelopoietic cell proliferation, and maturation. Extracellular matrix components produced by bone marrow stromal cells may also regulate myelopoiesis. Several studies have indicated that administration of benzene, phenol, or hydroquinone to animals altered cellular characteristics of the bone marrow stromal

cells and inhibited the ability of stromal cells to support hemopoiesis in culture (Harigaya et al., 1981; Garnett et al., 1983; Gaido and Wierda, 1985; 1987). Gaido and Wierda (1984) showed that phenol, hydroquinone, and *p*-benzoquinone were most effective in decreasing the ability of stromal cells to support the colony formation granulocyte/macrophage progenitor cells (CFU-GM) in a coculture system, whereas catechol only inhibited stromal cells at high concentrations.

Benzene administration *in vivo* induced elevated prostaglandin E_2 (PGE_2) in bone marrow samples (Gaido and Wierda, 1987). These authors suggested that increases in PGE_2 may be involved in myelosuppression by benzene. Prostaglandin E_2 can act as a negative regulator of hemopoiesis. *In vivo* administration of PGE_2 can block the ability of myelopoietic cells to respond to colony-stimulating factor (CSF) (Gentile and Pelus, 1987; Tavassoli and Friedenstein, 1983) and can inhibit CSF production by peritoneal macrophages and bone marrow stromal cells (Kriegler et al., 1984). Thus, bone marrow stromal cells, by producing both an inducer, CSF, and an inhibitor, PGE_2, can act to regulate myelopoiesis via a feedback control system (Kurland and Moore, 1977; Dayer et al., 1985).

Phenol and hydroquinone also alter prostaglandin production (Polsky-Cynkin et al., 1976; Hemler and Lands, 1980; Gollmer et al., 1984; Gaido and Wierda, 1987). One possible mechanism may be via cooxidation with prostaglandin synthetase (Hirafuji and Ogura, 1985). Through cooxidation, phenol and hydroquinone can be activated to more reactive intermediates, such as semiquinone, which bind to macromolecules and inhibit cellular enzymes (Tunek et al., 1982). The tendency of these agents to cooxidize with prostaglandin synthetase and

alter PGE_2 synthesis may contribute to benzene hematoxicity (Gollmer et al., 1984). However, benzene toxicity to stromal cells is not due solely to increased prostaglandin synthetase activity (Gaido and Wierda, 1987).

Benzene and Leukemia

There is no longer any real doubt that benzene is considered to be a leukemogenic agent in man (Aksoy, 1985). The evidence is derived from a number of different approaches including different types of epidemiological studies and a series of case reports beginning more than 50 years ago (Aksoy, 1985; Infante and White, 1985; Rinsky et al., 1987). These observations were corroborated recently by carcinogenesis bioassays in mice and rats (Snyder et al., 1980; Maltoni et al., 1983; 1985; Cronkite et al., 1984; 1985).

The type of leukemia most commonly associated with chronic benzene exposure is acute myelogenous leukemia and its variants, including acute myelomonocytic leukemia, acute erythroleukemia and acute myelocytic leukemia (Goldstein, 1983; Aksoy, 1985). Acute myelogenous leukemia is the adult form of acute leukemia, and is a rapidly fatal disease. The other major acute form of leukemia, acute lymphocytic leukemia, has been reported to be associated with benzene exposure. There are several reports (Goldstein, 1983) describing the frequency of occurrence of chronic myelogenous leukemia or even chronic lymphoid leukemia following benzene exposure. Other hematological disorders possibly associated with benzene exposure include Hodgkin's disease, lymphoma, myeloid metaplasia or myeloma. Leukemia associated with benzene exposure frequently develops following a period of bone marrow depression, i.e., benzene-induced pancytopenia has been followed through a preleukemic phase into

the development of acute myelogenous leukemia (Vigliani, 1976).

IMMUNOTOXICITY OF BENZENE AND ITS METABOLITES

Immunotoxicity of Benzene

The sensitivity of lymphocytes to benzene indicates an immunotoxic potential for this compound since lymphocytes play a principal role in alterations of the immune function. Evidence has been presented in several reports that the short-term administration of benzene by inhalation or intraperitoneal injection to experimental animals results in abnormalities of immune-associated parameters including a suppression in mitogenic response of B-and T-lymphocyte proliferations (Rozen and Snyder, 1985; Rozen et al., 1984; Wierda et al., 1981), an impairment in humoral antibody response as measured by plaque-forming cells to sheep erythrocytes (Aoyama, 1986; Wierda et al., 1981), and an increased susceptibility to pathogenic microorganisms, such as *Listeria monocytogenes* (Rosenthal and Snyder, 1985) and *Klebsiella pneumoniae* (Aranyi et al., 1986). Pandya et al. (1986) also showed that serum hemaglutinating SRBC antibody titers and interferon levels were reduced in rats exposed to benzene via intraperitoneal administration.

Recently, it has been demonstrated that exposure to 100 ppm benzene (5 days/week x 4 weeks) via inhalation in mice depressed the mixed lymphocyte culture (MLC) responses and also reduced the cytolytic abilities of cytotoxic T lymphocytes, and ruled out the possibilities that depressions in immune function are due to an induction of suppressor cell activity (Rosenthal and Snyder, 1987). As MLC responsiveness is generally thought to represent the initial phases in the induction of cytotoxic T cells (CTL), the benzene-induced loss of

CTL lytic ability to tumor cells might be explained by an impaired ability of T cells to recognize foreign tissue or tumor-associated antigens during T-helper-cell-mediated induction of CTL cells. More importantly, short-term exposure of mice to benzene concentrations at the industrial standard-exposure level (10 ppm) significantly depresses mitogen-induced lymphoproliferation of both B- and T-lymphocytes (Rozen et al., 1984).

Immunotoxicity of Benzene Metabolites

In vitro. Benzene metabolites have been shown to produce a variety of alterations in immune function *in vitro*. The ability of phenol, hydroquinone, and catechol to suppress lymphocyte growth and function *in vitro* correlates with their capacity to undergo oxidation and with their concentration in the bone marrow or lymphoid organs (Irons et al., 1981; Pfeifer and Irons, 1981; 1982; 1983). Hydroquinone and its oxidation product, *p*-benzoquinone, inhibit proliferation and differentiation in lectin-stimulated lymphocytes in culture at concentrations that are not cytotoxic, while phenol or catechol suppress lymphocyte growth or function only at concentrations that result in cell death. Suppression of lymphocyte blastogenesis by hydroquinone is postulated to be mediated by the interaction of *p*-benzoquinone with sulfhydryl (SH) groups on tubulin (Irons et al., 1981; 1984; Pfeifer and Irons, 1983). Tubulin has previously been shown to possess highly nucleophilic SH groups that are required for guanosine triphosphate (GTP) binding during the process of microtubule assembly (Mann et al., 1974). This binding interferes with microtubular integrity, which is required not only for cell division via spindle formation but also for regulation of surface receptor movement and normal signal transduction

at the cell membrane.

In vivo. Benzene metabolites are also immunotoxic *in vivo.* Post et al. (1985) demonstrated that exposure to *p*-benzoquinone in mice completely inhibits the proliferation and production of the T-cell lymphokine, interleukin-2, by concanavalin A (Con A)-stimulated T-lymphocytes. Proliferation and maturation of lymphocyte cells are regulated by this polypeptide lymphokine (Dinarello and Mier, 1986). Hydroquinone and catechol have been shown to reduce the frequency of spleen and bone marrow progenitor B-lymphocyte and inhibit polyclonal plaque-forming cells (Wierda and Irons, 1982).

NEUROTOXICITY OF BENZENE

Effect of Benzene on Neurobehavior

Organic solvents have long been associated with neurophysiological and psychological disorders (Grasso et al., 1984; Savolainen, 1977). The critical acute effects associated with inhalation of high concentrations of benzene, namely, depression of central nervous system function and narcosis, most likely ensues directly from benzene itself rather than from its metabolites (Dempster et al., 1984; Brief et al., 1980; Haley, 1977). Exposure of human beings to lower concentrations (250-500 ppm) of benzene has been reported to cause the following clinical symptoms: vertigo, drowsiness, giddiness, euphoria, headache, and nausea (Brief et al., 1980; Haley, 1977). Chronic industrial exposure to benzene also cause neurological abnormalities (Baslo and Aksoy, 1982). The behavioral endpoints including locomotor activity, appetitive indices and hind-limb grip strength were also demonstrated to be altered by benzene in experimental animals (Dempster et al., 1984).

In another study, inhalation of 300 or 900 ppm benzene increased the occurrence of eating and grooming, and reduced the number of mice that were sleeping or resting (Evans et al., 1981).

Effect of Benzene on Neurochemicals

Despite numerous positive investigations on neurobehavior effects, few published data are available on the neurochemical effects of benzene. Recently, Paradowski et al. (1985) showed that a four week prolonged subcutaneous administration of benzene to rats increases the contents of norepinephrine (NE) and dopamine (DA) in brain or other internal organs such as spleen, liver and kidney. Under the same conditions, increases of the indoleamine serotonin (5-HT) and its metabolite, 5-hydroxyindole acetic acid (5-HIAA) were also observed in brain tissues, particularly in the striatum (Paradowski et al., 1984). The mechanism for the benzene-induced changes in levels of monoamines and their metabolites is not yet known. It appears that the effects of benzene on the major enzyme systems in the monoaminergic biosynthetic pathway have never been clearly shown. Intraperitoneal administration of benzene to mice increased striatal levels of tryptamine, 5-HT, and 5-HIAA, and produced an induction aromatic-L-amino acid decarboxylase (AADC) but ruled out the possibility of inhibition of monoamine oxidase (MAO) (Juorio and Yu, 1985a). Benzene also produced marked increases in mouse and rat striatal concentrations of beta-phenylethylamine, *p*-tyramine and, to a lesser extent, *m*-tyramine (Juorio and Yu, 1985b).

ENVIRONMENTAL AND TOXICOLOGICAL ASPECTS OF TOLUENE

Chemical and Physical Properties

Toluene ($C_6H_5CH_3$), also referred to as toluol, methylbenzene, and

phenylmethane, is a colorless liquid with both volatile and flammable properties. It has a molecular weight of 92.13, boiling point of 110.63°C, freezing point of 94.9°C, specific gravity of 0.867 at 20°C, and a vapor pressure of 30 mm Hg at 26°C. This chemical is only slightly soluble in water, 535 mg/L in fresh water and 379 mg/L in sea water (U.S. Environmental Protection Agency, 1980b). It is miscible with alcohol, chloroform, ether, acetone, glacial acetic acid, carbon disulfide, and other organic solvents. Toluene is soluble in natural fats and fat-soluble substances, and accumulates readily in animal tissues that exhibit a high lipid content or represent major metabolic sites such as liver and brain. The nucleus of toluene, like that of benzene, undergoes substitution reactions. Substitution occurs almost exclusively in the *orthro* and *para* positions and occurs faster with toluene than benzene. The presence of a methyl group offers additional possibilities for reaction; the most important is dealkylation to benzene (U.S. National Academy of Sciences, 1982).

Occurrence and Usage

Toluene is produced primarily from catalytic reformating processes in refineries and from petroleum-derived pyrolysis gasoline as a by-product of olefin manufacture during the cracking of hydrocarbons (96%). Small amounts (4%) of toluene are also produced in coal-derived coke oven light oil and as a by-product in the manufacture of styrene (Merian and Zander, 1982). Toluene is extensively employed in a wide spectrum of applications. Approximately 70% of toluene is converted to benzene, another 15% is used as a starting agent for other chemicals (e.g., benzoic acid, nitrotoluene, toluene diisocyanates, as well as dyes, pharmaceuticals, food additives, plastics, etc.), and the remainder

(15%) is used as a solvent (which is increasingly being used as a "safe" replacement for benzene) for consumer products and as an essential gasoline component. Additionally, toluene has been widely abused by "glue sniffers". Hence, there is a broad potential for exposure both for industrial workers and the general public (Fishbein, 1985). It has been detected at various concentrations in the atmosphere and ambient water. There is a paucity of data available on levels of toluene in food (Fishbein, 1985).

Population at Risk and Allowed Levels

It is known that workers and segments of the general public are exposed to toluene from chemical manufacturing processes, coke ovens, petroleum refineries, solvent operations, the storage and distribution of gasoline, urban automobile emission, urban gasoline service stations, the use of self-service gasoline, and from consumer products containing toluene as well as from drinking water contaminated with this compound (Fishbein, 1985).

The occupational exposure limits for toluene vary from country to country. In the United States, the National Institute for Occupational Safety and Health (1973) has recommended a workplace limit of 100 ppm (8-hr TWA) with a ceiling of 200 ppm (750 mg/m^3) for a 10-minute sampling period. Occupational exposure levels are generally lower than this standard, although short exposures to higher vapor concentrations occur (Fishbein, 1985).

Toluene is also a major organic chemical contaminant in drinking water. For the protection of human health from the toxic properties of toluene following ingestion of contaminated water and aquatic organisms, the ambient water criterion is set at 14.3 mg/L (U.S. Environmental

Protection Agency, 1986a). This permissible concentration is basically extrapolated from a general toxicity evaluation of an oral dose study in rats (Wolf et al., 1956), which at the time was thought to be well below any toxicity-based criterion. A level up to 6.4 mg/L has been detected in drinking water (Tardiff and Youngren, 1986).

Routes of Exposure

Toluene may enter the human body by inhalation of vapor, percutaneous absorption of liquid, ingestion, and eye contact. Most actual exposures have occurred in relation to its use as a solvent, vapor inhalation being the mode of entry of major concern. Skin contact with toluene is of far less significance (U.S. National Academy of Sciences, 1982). Exposure to toluene through oral ingestion is considered to be a problem for the general population (U.S. Environmental Protection Agency, 1980b). After toluene is absorbed, it is rapidly distributed to the higher vascular tissues including the brain and eventually accumulates in adipose tissue (Savolainen, 1978).

Metabolism of Toluene

In vivo studies have shown that toluene exposure is associated with the induction of microsomal cytochrome P-450 dependent enzymes and conjugation enzymes (Pyykko, 1980; Toftgard et al., 1982; Pathiratne et al., 1986b). The time-course of effects of toluene on several microsomal monooxygenases in the liver or some non-hepatic tissues of rats exposed to toluene have been reported (Pyykko, 1983).

An important pathway of toluene metabolism proceeds initially through the oxidation of its methyl group by microsomal monooxygenase to benzyl alcohol in the endoplasmic reticulum of the liver. In turn, the

benzyl alcohol is oxidized to benzaldehyde and, subsequently, to benzoic acid by alcohol dehydrogenase. The benzoic acid is then conjugated with glycine to form so-called hippuric acid which is excreted via urine in man and animals, (Ikeda et al., 1972; U.S. National Academy of Sciences, 1982; Toftgard et al., 1982). Conjugation of benzoic acid with glucuronic acid to produce benzoyl glucuronide apparently occurs when the glycine conjugation reaction is saturated after heavy toluene absorption (Fishbein, 1985).

Toluene is also metabolized via (a) chemically reactive intermediate(s). The formation of *o*-cresol and *p*-cresol from aromatic oxidation of the absorbed toluene is mediated by cytochrome P-450 enzymes in the liver. These metabolites are synthesized through an arene oxide intermediate which is a reactive metabolite and is responsible for the binding of toluene to biomacromolecules (Bakke and Scheline, 1970; Pathiratne et al., 1986b). In the formation of *p*-cresol, a part of the hydrogen in the *para* position migrates to the *meta* position. Superoxide anions are also formed during the formation of secondary metabolites, such as semiquinones and quinones, from the oxidation of cresol (Pathiratne et al., 1986b); covalent binding of toluene to biological macromolecules involves more than one reactive metabolite.

Toxicity of Toluene

Exposure to toluene is reported to produce a variety of pathologic states in humans or experimental animals, including cardiac sensitization, kidney and liver damage, and various neurological disorders (Benignus, 1981a,b; Hayden et al., 1977). The neurotoxic properties of toluene represent the main health hazards (Fishbein, 1985). Although there is general agreement that toluene does not have

the hematotoxic properties of benzene, alterations of lymphocyte functions, host resistance to infectious microorganisms, and clastogenic activity by toluene were reported (Mohtashamipur et al., 1985; 1987; Suleiman, 1987; Schmid et al., 1985; Dean, 1985; Aranyi et al., 1985; Fishbein, 1985). Toluene was also found to be embryotoxic in rats, mice, and rabbits (Courtney et al., 1986; Ungvary and Tatrai, 1985; Nawrot and Staples, 1979).

Toluene appears to have a pronounced effect on behavior; schedule-controlled behaviors have been reported to produce inverted U-shaped concentration effect curves on response rates, i.e. initially increasing rates and at higher levels decreasing rates of response (Dyer et al., 1984; Benignus, 1981b). Several studies have indicated that concentrations of various brain biogenic monoamines are affected by acute or subchronic administration injection to rats. Increases in steady-state levels of NE, DA, and 5-HT have been reported in whole-brain homogenates or discrete brain areas of rats who continuously inhaled 100, 300, or 1,000 ppm toluene for 8 hr (Rea et al., 1984). Subchronic repeated exposure of rats to toluene vapor also increased concentrations of catecholamines and their turnover rates in hypothalamus or median eminence (Andersson et al., 1980; 1983). After a single ip administration of 200 mg toluene/kg body weight to rats, Arito et al. (1984) reported an increase in the regional concentrations of NE, 5-HT and their respective metabolites, 3-methoxy-4-hydroxyphenyl ethyleneglycol (MHPG) and 5-HIAA; alterations in DA and its metabolites 3-4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were not detected. In their follow up study, however, 14 consecutive days of ip injection of toluene in rats increased NE and MHPG only (Arito et

al., 1985). Pronounced increases in the concentrations of serum corticosterone, growth hormone, follicle stimulating hormone, and prolactin were increased in rats under the influence of toluene exposure via inhalation (Andersson et al., 1980; 1983).

Evaluations of the myelotoxicity of toluene in laboratory animals have generally indicated that the chemical is nontoxic. Wolf et al. (1956) conducted a long-term toxicity study in which toluene was given orally to rats and indicated there were no adverse effects on the cell counts of circulating blood and bone marrow. Andrews et al. (1977) noted that benzene inhibited the incorporation of ^{59}Fe into erythrocytes of mice but that toluene did not. A marked decrease in leukocyte counts were reported in dogs exposed to 200-700 ppm toluene, but these changes were found to be temporary (Hobara et al., 1984). After exposure to toluene vapor at concentrations ranging from 2.5 to 500 ppm, mice exhibited increased susceptibility to respiratory infection with *Streptococcus zooepidemicus* and decreased pulmonary bactericidal activity to *Klebisella pneumoniae* (Aranyi et al., 1985). Several reports have appeared in the literature which link exposure of toluene to altered immunocompetence in animals and humans (U.S. National Toxicology Program, 1987; U.S. Environmental Protection Agency, 1980b).

BENZENE-TOLUENE INTERACTION

One possible mode by which xenobiotics modify the toxicity of their own and also that of other compounds is alteration of their metabolism by induction of drug-metabolizing enzymes (Sipes and Gandolfi, 1986). Toluene is frequently employed in industry with other chemicals (U.S. National Academy of Sciences, 1982). In sufficient amounts, it is known

to possess the potential for significantly altering metabolism and resulting bioactivity of certain chemical agents (Hayden et al., 1977; Fishbein, 1985). Toluene exposure can stimulate the activity of many microsomal monoxygenases and increase the concentration of cytochrome P-450 (Pyykko, 1980; Toftgard et al., 1982; Pathiratne et al., 1986a), thereby enhancing metabolism of the second chemical. The time of exposure to toluene relative to the time of exposure to a second chemical could be quite important (Sipes and Gandolfi, 1986). Time-course of effects of toluene on several microsomal enzymes in the liver and some non-hepatic tissues of rats exposed to toluene has been demonstrated (Pyykko, 1983). Should concurrent exposure occur, toluene, which is readily hydroxylated by the microsomal monooxygenease system, would be expected to inhibit the metabolism of other compounds which are acted upon by this same enzyme system. This phenomenon would be expected to result in a prolonged half-life of both toluene and the other compound. Inhibition of metabolism of a second compound may be beneficial or detrimental, depending upon the toxicity of the parent compound versus its metabolite(s). Toluene also undergoes alcoholic oxidation and conjugation reactions subsequent to the initial hydroxylation reaction. Therefore, a substantial dose of toluene could conceivably interfere with the metabolism of compounds that undergo oxidation and glycine conjugation (U.S. National Academy of Sciences, 1982; Ungvary et al., 1983; Takahashi et al., 1987).

Several *in vitro* or *in vivo* studies indicate that toluene is a competitive inhibitor of the biotransformation of benzene and can significantly influence the myelotoxic and genotoxic effects of benzene. The conversion of benzene to phenols in rats, mice or *in vitro* has been

reported to be suppressed when benzene was given in combination with toluene (Ikeda et al., 1972; Andrews et al., 1977; Sato and Nakajima, 1979; Gilmour et al., 1986). Andrews et al. (1977) coadministered benzene and toluene to mice or rabbits and observed a marked reduction in levels of benzene metabolites in bone marrow, coupled with a compensatory increase in pulmonary excretion of unmetabolized benzene. It was also demonstrated using liver microsomes *in vitro* that toluene is a competitive inhibitor of benzene metabolism. When benzene and toluene were administered in combination to rats intraperitoneally, their disappearance rates from blood and the rates of urinary excretion of their metabolites were delayed compared with those observed when they were given separately. Their metabolic interaction was found to be dose-dependent (Sato and Nakajima, 1979).

Toluene protects against benzene-induced depression of red cell ^{59}Fe uptake in bone marrow of mice (Andrews et al., 1977). Toluene not only alleviates the benzene-induced leukopenia but also the benzene-induced increase in leukocyte alkaline phosphatase activity (Li et al., 1986). Toluene significantly decreases the adverse effects of benzene on granulopoietic stem cells (CFU per tibia) and on tibial bone marrow cellularity in mice (Tunek et al., 1981; 1982). When benzene-exposed mice were simultaneously given an ip injection of toluene, the sister chromatid exchange (SCE) incidence was reduced by up to 90% compared with animals exposed to benzene alone (Tice et al., 1982). Gad-El-Karim et al. (1984; 1985a; 1986) also found that toluene antagonized benzene's myeloclastogenicity and metabolism when mixtures of both chemicals were administered orally to CD-1 mice.

CHAPTER III

ALTERATION OF HUMORAL AND CELLULAR IMMUNITY BY BENZENE IN CD-1 MICE

INTRODUCTION

Concern for the safety of pollutants present in ground water is attracting attention, since ground water is a major source of drinking water supplies. The major concern is the possible subclinical effects after long-term exposure. The immune system is being studied as a target organ for detecting toxicity since its sensitive parameters are easily evaluated (Bick et al., 1985; Dean et al., 1982). A number of industrial and environmental chemicals induce immunosuppression (Faith et al., 1980). Benzene, a ubiquitous environmental pollulant, exerts its greatest toxicity on hematopoietic cells, including erythroid, myeloid and lymphoid lineages both in the peripheral blood and in the hematopoietic organs (Fishbein, 1984; Bolcsak and Nerland, 1983). Repeated benzene exposure in human and laboratory animals induces various forms of cytopenia, aplastic anemia, leukaemia and the development of chromosomal abnormalities (Dean, 1985; Green et al., 1981a; U.S. Environmental Protection Agency, 1980a; Brief et al., 1980). The mechanism for benzene-induced hematopoietoxic effects has not been well elucidated, however, most researchers believe oxidative benzene metabolites, rather than benzene itself, are required to mediate these hematopoietic effects of benzene (Sawahata et al., 1985; Irons, 1985; Bolcsak and Nerland, 1983; Greenlee et al., 1981b).

Lymphocytes in animals are sensitive to benzene toxicity (Rozen and Snyder, 1985; Dempster et al., 1984; Snyder et al., 1982; Green et al.,

1981a; Aoyama, 1986), as are those from humans who are inadvertently exposed to this chemical (Moszczynsky and Lisiewicz, 1984; Brief et al., 1980). Benzene-induced depressions in lymphocyte counts may impair immunocompetence, since lymphocytes play a principal role in immune function. The short-term administration of benzene by inhalation or intraperitoneal injection to experimental animals has caused abnormalities of immune-associated parameters, including a suppression in mitogenic response of B-and T-lymphocyte proliferations (Rozen and Snyder, 1985; Rozen et al., 1984; Wierda et al., 1981; Aoyama 1986), impaired humoral antibody response as measured by plaque-forming cells to sheep erythrocytes (Wierda et al., 1981; Aoyama, 1986), and an increased susceptibility to pathogenic microorganisms (Rosenthal and Snyder, 1985; Aoyama, 1986). Similarly, treatment with benzene metabolites has also impaired immune responses (Pfeifer and Irons, 1982; Wierda and Irons, 1982).

The inbred C-57BL mice used in experiments described above may harbor an endogeneous lymphoma virus (Kaplan, 1967); activation of this latent virus after benzene treatment may influence the toxicity of benzene (Snyder et al., 1980; Longacre et al., 1981). The outbred CD-1 mice were used in this study. There are relatively few reports on effects of benzene after oral ingestion. Because some water supplies may be contaminated with benzene (U.S. Environmental Protection Agency, 1980a), oral administration was employed via drinking water for a four-week period.

MATERIALS AND METHODS

Animals

Male, adult CD-1 mice (Charles River Breeding Laboratory Inc., Wilmington, MA) were procured at 5 to 6 weeks of age (approximately 18 g) and were acclimatized to the AAALAC-accredited animal care facility for 1 week before use. Mice were randomly assigned to control and treatment groups and housed five per group in plastic cages with hardwood-chip bedding. They were maintained on laboratory rodent chow and tap water *ad libitum*. Room conditions were maintained at an ambient temperature of 21 $\pm$ 1^{o}C and a relative humidity of 50 $\pm$ 10%. The light/dark cycle was maintained at 12-hr intervals.

Exposure

Analytical reagent grade of benzene (99.99% purity, J.T. Baker chemical Co. Phillipsburg, NJ), was dissolved in normal tap water to make nominal concentrations of 40, 200, and 1000 mg/L. Benzene has a solubility of 1,780 mg/L in water at 25^{o}C (U.S. Environmental Protection Agency, 1980a). The benzene-treated water was administered to mice continuously for 28 days via drinking water; the control group received untreated tap water. To minimize decomposition and to maintain the concentration of benzene, drinking water was provided in glass water bottles, was shaken frequently during treatment and was changed every three days. Feed and water consumption was monitored continuously, and animals were weighed once a week. Benzene concentrations in drinking water were confirmed on different days by a gas chromatography (U.S. Environmental Protection Agency, 1986a).

Gross Observations and Hematology

Twenty-eight days after the exposure to benzene, mice were killed by decapitation, and gross pathological examinations were performed on all mice. Major organs, i.e., liver, spleen, thymus and kidney were removed, trimmed and weighed. Blood samples were collected in siliconized test tubes coated with potassium ethylene-diaminetetraacetate. Leukocytes and erythrocytes were counted with an automated electronic cell counter (Model ZBI, Coulter Electronics, Inc., Hialeah, FL). Hematocrits were performed with microhaematocrit equipment. Differential leukocyte counts were evaluated by Wright's-Giemsa stained thin smears.

Isolation and Culture of Splenic Lymphocytes

After sacrifice, the spleen was aseptically removed from each animal, rinsed in ice-cold sterile isotonic saline and single cell suspensions were prepared and cultured according to Sharma and Gehring (1979). Cells were suspended in RPMI 1640 media (Gibco Laboratories, Grand Island, NY) supplemented with 5% heat-inactivated fetal calf serum (Hyclone Sterile Systems, Inc., Logan, UT) and 100 units penicillin and 100 μL streptomycin per mL. Cell counts were made by using the Coulter counter.

Lymphocyte Proliferation to Mitogens

Splenic lymphocytes were plated in triplicate cultures (5×10^5 cells/culture well) in 96-well flat-bottom microtiter plates (Microtest II, Falcon Plastics, Oxnard, CA) to assay their responses to *E. coli* lipopolysaccharide (LPS; Sigma Chemical Co., St. Louis, MO), pokeweed mitogen (PWM; Gibco Laboratories, Grand Island, NJ), Concanavalin A (Con

A; Sigma Chemical Co., St. Louis, MO), or phytohemagglutinin (PHA; Wellcome Reagents Ltd., Beckenham, England). Culture media with or without mitogen was added; total well volume was adjusted to 0.15 mL. The optimized concentrations of mitogens were 20 μg/mL, 30 μg/mL, 2.5 μg/mL, and 6.0 μg/mL for LPS, PWM, Con A and PHA, respectively. Cultures were incubated at 37°C in a humidified atmosphere with 3.5-4% CO_2 for 48 hours and harvested after an additional 6-hr pulse with 0.5 μCi of [methyl-^{3}H]-thymidine (^{3}H-TdR; New England Nuclear, Boston, MA) per well. The cells were collected on a glass fiber filter using an automated multiple sample cell harvester (Model M12, Biomedical Research and Development Laboratory, Rockville, MD). The amount of radioactivity (dpm) incorporated was measured with a scintillation counter (Model 2660, Packard Instrument Co., Drowners Grove, IL).

Mixed Lymphocyte Culture (MLC) Response

The proliferation of splenic lymphocytes (responders) from benzene-treated or control animals in response to challenge with allergenic YAC-1 tumor cells (stimulators) was evaluated. The exponential growth phase YAC-1 murine lymphoma cells (H-2^a) of A/Sn origin previously maintained in culture medium (> 90% viability by trypan blue exclusion) at a concentration of 2 x 10^7 cells/mL were treated with 50 μg/mL mitomycin C (Sigma) for 1 hr at 37°C, then washed three times immediately before putting into culture. Cultures were set up in triplicate in 96-well microtiter plates. Responder cells from the spleen of CD-1 mice were cocultured alone or with stimulator cells at a responder-stimulator ratio of 2:1. The complete RPMI culture medium was supplemented with 5 x 10^{-5} M 2-mercaptoethanol (2-ME). The proliferative response was assessed by [methyl-^{3}H]-thymidine incorporation during the last 24 hr of

a 3-day culture. The cultures were harvested and counted as described for the mitogenesis assays above.

Cell-Mediated Cytolysis (CMC) Response

Cytotoxic activities of splenic cytotoxic T-lymphocyte (CTL) were generated *in vitro* against YAC-1 tumor cells as described by Grabstein (1980) with modifications. The splenic lymphocytes (responder cells) were incubated with mitomycin C-treated YAC-1 cells (stimulator cells) in RPMI complete medium supplemented with 5×10^{-5} M 2-ME for 5 days at 37°C in a 3.5-4% CO_2 atmosphere. The exponential growth phase YAC-1 cells were labeled with 0.25 mCi of sodium chromate-51 (New England Nuclear) by incubation at 37°C for 1 hr under gentle agitation and then washed, counted, and resuspended at 2×10^5 cells/mL. A constant number (2×10^4 in 0.1 mL) of ^{51}Cr-labeled YAC-1 cells (target cells) were added to each well of a plastic-well microtiter plate followed by the addition of aliquots of the incubated splenic cells (effector cells) in a total volume of 200 μL. The desired effector-to-target cell ratios were 50:1 and 25:1 which gave optimal lysis of target cells. Effector and target mixtures were spun for 1 min at 1000 g, and then incubated for another 4 hr at 37°C. After incubation, cells were pelleted by centrifugation for 10 min at 1000 g, and the supernatants were decanted and counted in a gamma counter (Model 3320, Packard) The labeled target cells in the presence of complete medium or 0.5% sterilized saponin served as the spontaneous ^{51}Cr released and total ^{51}Cr released, respectively. The triplicate mean was used to calculate the percentage of specific lysis of target cells as described earlier (Grabstein, 1980).

Plaque Forming Assay

Numbers of IgM plaque-forming cells (PFC) were determined by the method of Jerne and Nordin (1963) as modified by Cunningham and Szenberg (1968). Each mouse from control and test groups was sensitized by ip injection with 0.25 mL of a 20% sheep red blood cell SRBC suspension 4 days before the end of the benzene exposure. Splenic cell suspensions were prepared (1×10^7 cells/mL) as described above. A mixture containing 45% splenic cells suspension, 45% of a 10% SRBC suspension, and 10% guinea pig complement (Cappel Laboratories, Cochranville, PA) was delivered to counting chambers, three replicates per animal. Cultures were incubated at 37°C for 1 hr and PFC were calculated and expressed as plaques/10^6 splenic cells or plaques/total splenic cells.

Enzyme-Linked Immunosorbent Assay (ELISA)

Double sandwich enzyme-linked immunosorbent assay (ELISA) was used to analyze anti-SRBC (α-SRBC) antibodies (Voller et al., 1979). Serum samples were collected from mice utilized in the plaque-forming assay. Plates (Gilford polystyrene EIA cuvettes Medical Instruments Laboratories, Inc., Oberlin, OH) were coated with 200 µL sheep red blood cell ghosts (prepared as described by Hanahan and Ekholm, 1974) containing 3.6 µg/mL protein solubilized in 0.1% sodium dodecyl sulfate, and kept at 4°C for 24 hr. After several rinses, 200 µL aliquots of sera (dilution 1:150) were added to the plate wells and incubated for 2 hr at 37°C, and rinsed. Subsequently, 250 µL peroxidase-conjugated goat anti-mouse immunoglobulins (Hyclone Sterile Systems, Inc., Logan, UT) diluted 1:2000 were pipeted to each well to determine α-SRBC antibody levels, and incubated another 3 hr at 37°C. The enzyme reaction was performed by adding the phosphate-citrate

buffered substrate solution containing 0.4 mg/mL o-phenylenediamine (Sigma) and 0.4 μL/mL H_2O_2, prepared immediately before use. The results were expressed as change in absorbance (ΔOD) 15 min after the addition of substrate solution. Non-sensitized mice pooled sera were used as a negative control. The optical density was determined by using an automated spectrophotometer (Gilford, Model EIA 50, Oberlin, OH); readings were made at 490 nm.

Statistical Analysis

The results are expressed as the means ± their standard errors. Statistical significances for differences between benzene-exposed and corresponding normal water control data were determined by a one-way analysis of variance (ANOVA) design followed by multiple comparison procedures of Fisher's least significant differences (LSD) test (Dowdy and Wearden, 1983), when F ratios indicated significant difference. A level of $p<0.05$ was considered statistically significant.

RESULTS

The average concentration of benzene in drinking water as determined by gas chromatographic analysis on several days is indicated in Table III-1. There was a 10% loss of benzene on the first day, an additional 10% loss per day followed on the second and third day, when the water was changed. Based on observed average intake of benzene-treated water, the daily dose of benzene in each animal was estimated to be 0, 8, 40, and 180 mg/kg, respectively.

Body and Organ Weights

Continuous exposure of adult, CD-1 male mice to various levels of

TABLE III-1

ORGAN AND BODY WEIGHTS OF MICE FOLLOWING 4 WEEKS OF BENZENE EXPOSURE[a]

Concentration in water, mg/L		Doses mg/kg/day	Body Weight (g)[b]		Organ weight(g/100 body weight)[b]			
Nominal	Observed[c]		Day 0	Day 28	Spleen	Liver	Kidney	Thymus
0	0	Control	23.20 ± 0.37	31.22 ± 0.62	0.33 ± 0.02	5.57 ± 0.13	1.55 ± 0.06	0.16 ± 0.02
40	31	8	22.68 ± 0.33	29.94 ± 0.98	0.30 ± 0.01	5.46 ± 0.29	1.58 ± 0.08	0.12 ± 0.01
200	166	40	23.74 ± 0.22	32.98 ± 0.65	0.28 ± 0.01	5.86 ± 0.07	1.65 ± 0.10	0.12 ± 0.01
1000	790	180	23.14 ± 0.29	32.26 ± 0.86	0.26 ± 0.01*	5.98 ± 0.20	1.87 ± 0.05*	0.11 ± 0.01

[a] Benzene was administered continuously via drinking water

[b] Values are given as mean ± SE (n=5)

[c] The observed overall ranges of benzene concentration were 28 to 36, 147 to 178, and 734 to 855 mg/L, in the three treatment groups, respectively

* Significantly ($p<0.05$) different from control values

benzene via drinking water over a 4-week period did not produce overt clinical symptoms of toxicity. Overall, there was no change in food and water consumption. No gross lesions were observed on any of the organs of mice in all treatment groups. There was no apparent alteration in body weight gain at termination of the study. Although the two groups receiving higher doses (40 and 180 mg/kg/day) had a slight increase in growth rate, growth was not significantly different than the controls (Table III-1). The weights of selected organs are also presented in Table III-1. Weights of spleen, a secondary lymphatic tissue, decreased significantly in animals treated with benzene at the 180 mg/kg/day dose. Kidney weights correspondingly increased. Thymus weight was reduced in all treated groups but not significantly.

Hematological Parameters

The effects of benzene exposure on erythrocyte counts, hematocrits, mean corpuscular volumes (MCV), leukocyte counts, and leukocyte differential counts are presented in Table III-2. Numbers of erythrocytes decreased significantly as dose increased. Hematocrits were depressed significantly at the 40 and 180 mg/kg/day of benzene. However, all of the MCV values of treated animals, when calculated from the hematocrits and erythrocyte counts, exhibited a significant increase. There was a significant dose-related reduction in number of total circulating white cells after oral ingestion of benzene. Lymphocyte population decreased significantly when leukocyte differentials were expressed on an absolute basis. The number of neutrophils and other differential white blood cells (monocytes, eosinophils and basophils) were not altered greatly by the benzene exposure.

TABLE III-2

EFFECTS OF BENZENE EXPOSURE ON SELECTED BLOOD PARAMETERS

Dose[a] mg/kg/day	Erythrocytes ($10^6/mm^3$)	Hematocrit (%)	MCV[b] (fL)	Leukocytes ($10^3/mm^3$)	Leukocyte absolute differentials		
					Lymphocytes ($10^3/mm^3$)	Neutrophils ($10^3/mm^3$)	Others[c] ($10^3/mm^3$)
Control	7.01 ± 0.65[d]	50.30 ± 0.58	74.01 ± 6.03	5.96 ± 0.60	4.38 ± 0.52	1.01 ± 0.13	0.45 ± 0.08
8	4.57 ± 0.28*	48.80 ± 0.46	108.30 ± 6.49*	4.23 ± 0.07*	2.99 ± 0.07*	0.85 ± 0.10	0.38 ± 0.05
40	3.68 ± 0.65*	46.20 ± 0.46*	125.18 ± 15.68*	4.22 ± 0.57*	2.35 ± 0.30*	1.46 ± 0.24	0.40 ± 0.09
180	3.43 ± 0.39*	45.00 ± 1.22*	136.76 ± 12.57*	2.87 ± 0.16*	1.61 ± 0.10*	1.02 ± 0.07	0.25 ± 0.09

[a] Benzene was administered continuously to CD-1 mice via drinking water for 4 weeks

[b] Mean corpuscular volume

[c] Including monocytes, eosinophils and basophils

[d] Values are given as mean ± SE (n=5)

* Significantly ($p<0.05$) different from control values

Mitogen-Induced Lymphocytic Proliferations

Following 4 weeks of benzene treatment, the proliferative response of either mitogen stimulated or nonstimulated splenic lymphocytes were elevated in low dose group and depressed in higher dose groups (Table III-3). At the optimal concentration of LPS (20 ug/mL), ^{3}H-TdR uptake increased significantly in the group receiving 8 mg/kg/day of benzene. In contrast, the benzene-treated groups receiving 40 and 180 mg/kg/day manifested a significant suppression of ^{3}H-TdR incorporation of splenocytes upon LPS stimulation. Similarly, there were significant biphasic alterations of lymphoproliferation in T cell mitogen-treated cells; both the Con A and PHA-induced responses were enhanced in the lowest treatment group and inhibited at the two highest levels. The PWM or nonmitogen-stimulated responses increased significantly in the animals treated with the lowest dose of benzene but were insignificantly depressed in groups receiving higher amounts. Total spleen cellularity decreased significantly at the highest dose of benzene treatment.

MLC and CMC Responses

The results of MLC presented in Figure III-1 demonstrated a dose-related biphasic response, i.e., ^{3}H-TdR uptake was inhibited at the higher benzene doses but increased in lowest dose group. The effect of benzene exposure on CMC activity is shown in Table III-4. Cytotoxicity data was calculated as the % of total releasable counts, corrected for spontaneous release of radiolabel. Total release $\pm$ SE of the 2×10^{4} ^{51}Cr-labeled target cells was 3253 $\pm$ 324 cpm. Spontaneous release was less than 7% of total releasable counts for all assays. The results of ^{51}Cr-release assay revealed that benzene significantly impaired the capacity of cytotoxic lymphocyte to lyse the alloantigenic cells (target

TABLE III-3

EFFECTS OF BENZENE EXPOSURE ON SPLENIC LYMPHOCYTE PROLIFERATIVE RESPONSE TO MITOGENS

Dose[a] mg/kg/day	Spleen cellularity[b] ($x10^{-7}$)	Mitogenic responses[c]				
		None	LPS	PWM	Con A	PHA
Control	7.51 ± 0.40[d]	1.68 ± 0.31	102.04 ± 23.66	7.45 ± 1.90	154.69 ± 49.33	68.02 ± 18.57
8	7.91 ± 0.62	3.55 ± 0.89*	261.23 ± 25.62*	12.92 ± 2.19*	384.90 ± 108.61*	285.81 ± 88.01*
40	7.50 ± 0.41	1.13 ± 0.13	22.43 ± 3.48*	3.25 ± 0.71	29.30 ± 7.69*	16.43 ± 3.94*
180	5.17 ± 0.64*	0.91 ± 0.19	25.44 ± 6.20*	3.65 ± 0.72	14.61 ± 2.66*	8.87 ± 1.83*

[a] Benzene was administered continuously to CD-1 mice via drinking water for 4 weeks

[b] Recovered number of splenocytes per spleen $x10^{-7}$

[c] $dpm/10^{-3}/10^{6}$ splenic cells: response evaluated by incorporation of splenocyte cultures for 6 hours pulsing

[d] Values are given as mean ± SE (n=5).

* Significantly ($p<0.05$) different from control values

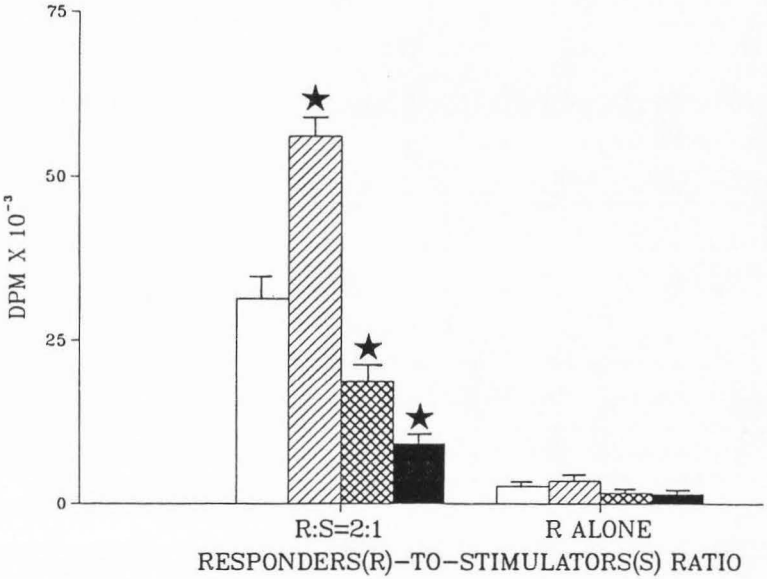

Fig. III-1. Effect of benzene exposure on mixed lymphocyte culture (MLC) response. Benzene was administered continuously to CD-1 mice via drinking water for 4 weeks. The bar (left to right), ▭, ▨, ▩, and ■, indicate benzene doses at 0 (control), 8, 40, and 180 mg/kg/day, respectively. Stars (*) denote significantly ($p<0.05$) different from control values. Responder cells were the syngeneic splenic cells of CD-1 mice and stimulator cells were the allogeneic YAC-1 cells. Several stimulator responder ratios were used, but data are presented only from a ratio of 1:2 stimulators to responders which gave maximal stimulation. The stimulator cells were treated with mitomycin-C before addition to the culture. MLC evaluated by incorporation of [methyl-3H]-thymidine into day 3 MLC cultures for 6 hours pulsing.

TABLE III-4

EFFECTS OF BENZENE EXPOSURE ON CYTOTOXIC LYMPHOCYTE FUNCTIONS

Dose[a] mg/kg/day	% Cytotoxicity	
	50:1[b]	25:1
Control	15.72 ± 1.16[c]	15.98 ± 2.98
8	17.94 ± 1.61	23.32 ± 2.05*
40	8.77 ± 1.87*	11.09 ± 1.71
180	5.82 ± 0.94*	9.17 ± 1.53*

[a] Benzene was administered continuously to CD-1 mice via drinking water for 4 weeks

[b] Effector (day 5 MLC lymphocytes)-to-target (^{51}Cr labelled YAC-1 cells) cell ratio

[c] Values are given as mean ± SE (n=5)

* Significantly ($p<0.05$) different from control values

cells) at 25:1 and 50:1 effector (E)-to-target (T) cell ratios after 4 weeks of oral ingestion. The lowest dose (8 mg/kg/day) of benzene also obviously enhanced the CTL responsiveness in 25:1 E:T ratio.

Antibody Production

The primary antibody response to SRBC was assessed four days later after sensitization with SRBC; the SRBC-specific plaque-forming cells (PFC) were counted, and the α-SRBC antibody titer was determined. As shown in Table III-5, ability of treated animals to produce SRBC-specific antibody changed. The number of PFC in the animals receiving 40 and 180 mg benzene/kg/day was significantly reduced, when expressed as either specific activity (PFC/10^6 spleen cells) or whole spleen basis. However, there were more PFC per 10^6 splenic cells in animals exposed to the lowest level of benzene. The total number of recovered splenocytes was significantly reduced in a dose-related manner. The titer of α-SRBC antibodies corresponded to the numbers of PFC, but significant depression was only found at the highest concentration of benzene (Table III-5).

DISCUSSION

The results demonstrate that oral administration of various dosages of benzene via drinking water *ad libitum* to CD-1 mice markedly altered the immune function. The alteration of immunocompetence was grossly evidenced by a decrease of thymic and splenic weights, which are useful indicators of immune dysfunction, and a loss of lymphocytes from peripheral blood and spleen. Benzene appeared to specifically affect lymphocytes since numbers of neutrophils and other leukocytes did not decrease significantly (Table III-2). Various studies have revealed

TABLE III-5

EFFECTS OF 4 WEEKS OF BENZENE EXPOSURE ON THE ANTIBODY RESPONSES TO THYMIC-DEPENDENT ANTIGEN SHEEP ERYTHROCYTE (SRBC)[a]

Dose mg/kg/day	Spleen cellularity ($\times 10^{-7}$)	PFC/10^6 Splenic cells	PFC/total spleen cells ($\times 10^{-3}$)	α-SRBC titer
Control	22.59 ± 3.02[c]	1,254 ± 171	295.72 ± 74.51	0.44 ± 0.06
8	16.80 ± 1.59*	1,576 ± 65*	268.80 ± 36.10	0.57 ± 0.11
40	15.36 ± 0.65*	643 ± 49*	99.76 ± 10.32*	0.31 ± 0.05
180	12.70 ± 1.51*	229 ± 40*	31.41 ± 9.52*	0.21 ± 0.01*

[a] Mice were sensitized with SRBC 4 days before the end of the benzene exposure. Splenic lymphocytes were analyzed for antibody forming cells (plaque-forming cell, PFC) and sera were detected for antibody titer (α-SRBC)

[b] Benzene was administered continuously via drinking water

[c] Values are given as mean ± SE (n=5)

* Significantly ($p<0.05$) different from control values

that lymphocytes are particularly sensitive to benzene toxicity via inhalation (Rozen and Snyder, 1985; Dempster et al., 1984; Snyder et al., 1982; Green et al., 1981a). Benzene increases the permeability of lymphocyte lysosomal membranes and releases lysosomal enzyme into the cytoplasm, resulting in a decrease in the number of lymphocytes (Moszczynsky and Lisiewicz, 1984). Mice exposed to 300 ppm benzene via inhalation showed significant depressions in the numbers of B-lymphocytes (s-IgM+ cells) in bone marrow and spleen and in the numbers of T-lymphocytes (Thy 1,2+ cells) in thymus and spleen (Rozen and Snyder, 1985). Similar effects on these two lymphocyte populations in blood and spleen were also reported in mice after 7 or 14-day inhalation exposure to 50 or 200 ppm benzene (Aoyama, 1986). Occupational exposure to benzene showed significantly decreased T-lymphocyte counts (Moszczynsky and Lisiewicz, 1984).

In our studies, mice exposed to benzene had significant fewer peripheral erythrocyte numbers and hematocrits. The increase in mean corpuscular volume (MCV) indicates that benzene ingestion induced a macrocytic anemia. In human beings chronic benzene poisoning is most easily detected by RBC reduction and increased MCV, but an accurate diagnostic also requires other hematologic parameters (Haley, 1977). Baarson et al. (1984) reported that repeated exposure to low levels of benzene (10 ppm) via inhalation in mice depressed the *in vitro* colony-forming ability of one of the erythroid progenitor cells, the colony-forming unit-erythroid (CFU-E), the numbers of splenic nucleated red cells and the numbers of circulating erythrocytes and lymphocytes.

Generally, the size of lymphatic organs associated with immunologic function decreased. The effect of benzene on immune parameters in the

present study was often a biphasic response, enhanced at lowest dose (8 mg/kg/day) and suppressed at the higher doses (40 and 180 mg/kg/day). Several workers have reported the *in vivo* immunosuppressive effect of benzene (Aoyama, 1986; Rozen and Snyder, 1985; Pandya et al., 1986). However, differences in animal models, doses and duration of exposure, and routes of administration make comparisons difficult (Longacre et al., 1981). Inhalation of benzene depressed host resistance to the infectious agents, *Listeria monocytogenes* and *Klebsiella pneumoniae* (Rosenthal and Snyder, 1985; Aranyi et al., 1986). Mitogen-induced lymphoproliferation was suppressed in mice treated ip with benzene metabolites, i.e., hydroquinone and catechol (Wierda and Irons, 1982). The cell renewal rate as well as the rate of DNA, RNA, and protein synthesis in cultured leukocytes and lymphoma cell lines exposed to benzene or its metabolites were inhibited (U.S. Environmental Protection Agency, 1980a; Pellack-Walker et al., 1985; Post et al., 1985; Schwartz et al., 1985). Hemopoiesis *in vitro* in stromal cells pretreated with low doses of oxidative metabolites of benzene, such as hydroquinone, was enhanced (Gaido and Wierda, 1984). Garnett et al. (1983) also obtained biphasic responses *in vitro* with CFUs (colony forming units-spleen) from mice that inhaled benzene. These results support the hypothesis that benzene or its metabolites may promote as well as suppress the hemopoietic system (Harigaya et al., 1981).

Recently, Post et al. (1985) indicated that exposure of T-lymphocytes to *p*-benzoquinone, one of the metabolites of benzene, inhibited the formation of lymphokine interleukin-2 (IL-2). IL-2, produced by T lymphocytes, is an important regulatory cytokine in lymphocyte proliferation since it is a growth factor for helper, and

cytotoxic T lymphocytes (Dinarello and Mier, 1986).

The data from this study show that ingestion of benzene via drinking water in CD-1 mice noticeably affected all aspects of the immune system, including cellular and humoral parameters. Of particular importance was the fact that the effects were evident at the lowest dose of treatment and a no-effect level could not be established. Further studies involving lower levels of exposure are necessary to fully ascertain the safety of this chemical in groundwater.

CHAPTER IV

ALTERATION OF REGIONAL BRAIN MONOAMINE NEUROTRANSMITTERS BY BENZENE IN CD-1 MICE

INTRODUCTION

Benzene, the simplest aromatic hydrocarbon, is a ubiquitous chemical. It occurs naturally in petroleum, trees, fruit, seeds, dairy products, eggs and meat (U.S. Environmental Protection Agency, 1980a). This chemical is also among the most widely distributed of the environmental pollutants. It is produced by the petrochemical industry, and is a basic starting material in the manufacture of many beneficial products (Fishbein, 1984). Due to its lipophilic nature and to the production of biologically reactive intermediates through metabolic biotransformation (Irons, 1985; Bolcsak, and Nerland, 1983; Greenlee et al., 1981a), benzene is considered to pose a significant health hazard. The major toxicity of benzene reported in humans and laboratory animals has mainly focused on the dysfunction of the hematopoietic system (Sawahata et al., 1985; Bolcsak and Nerland, 1983).

Organic solvents have been associated with neurophysiological and psychological disorders (Grasso et al., 1984; Savolainen, 1977). However, few studies have concerned the effects of benzene on the nervous system, particularly with neurotransmitters. Acute effects associated with inhalation of extremely high concentrations of benzene (about 25,000 ppm), namely, depression of the central nervous system (CNS) and narcosis, most likely are caused by benzene rather than its metabolite(s) (Dempster et al., 1984; Brief et al., 1980; Haley, 1977). Exposure of human beings to lower concentrations of benzene (250-500

ppm) has reportedly caused vertigo, drowsiness, giddiness, euphoria, headache, and nausea (Brief et al., 1980; Haley, 1977). Chronic industrial exposure to benzene also causes neurological abnormalities (Baslo and Aksoy, 1982). The behavioral endpoints including locomotor activity, appetitive indices, and hind-limb grip strength were demonstrated to be altered by benzene in animals (Dempster et al., 1984). Recently, Paradowski et al. (1985) showed that subcutaneous administration of benzene to rats for 4 weeks increased the concentrations of norepinephrine (NE) and dopamine (DA) in brain or other internal organs. Under the same conditions, the concentrations of the indoleamine serotonin (5-HT) and its metabolite, 5-hydroxyindole acetic acid (5-HIAA), also increased in brain tissues (Paradowski et al., 1984). Intraperitoneal administration of benzene to mice has been shown to increase brain levels of 5-HT and 5-HIAA and produce an induction of aromatic-L-amino acid decarboxylase, a major enzyme in the synthesis of biogenic amines (Juorio and Yu, 1985a).

Since relatively little information is available concerning the effects of benzene on the neurochemical transmitters in animal brain, current studies were undertaken to evaluate the neurotoxicity of benzene in mice. The primary objective was to determine if subchronic administration of benzene to CD-1 mice via drinking water altered the concentrations of specific brain catecholamines, indoleamine, and their metabolites in discrete brain regions. These selected neurotransmitters are known to play an important role in physiology and behavior (Rogawski and Baker, 1985; Poirier and Bedard, 1984; Willis and Smith, 1985). The results of this investigation would help to understand the potential neurotoxic actions of this ubiquitous environmental contaminant, which

is also a major groundwater pollutant.

MATERIALS AND METHODS

Animals

Male, adult CD-1 mice (Charles River Breeding Laboratory Inc., Wilmington, MA) were quarantined and allowed to acclimatize to the animal care facility for one week prior to experiment (approximately 21-24 g initial body weight). Room conditions were kept at ambient temperature (21 $\pm$ 1^{o}C) with relative humidity ranging between 40 and 60%, and the light/dark cycle was maintained at 12-hour intervals. Commercial rodent chow (Wayne Products, Chicago. Ill) and tap water were given *ad libitum*. After acclimatization, the apparently healthy animals were randomly assigned to control and treatment groups and housed (five per cage) in plastic cages containing hardwood-chip bedding.

Exposure

Analytical reagent grade benzene (99.9% purity, JT Baker Chemical Co., Phillipsburg, NJ) was dissolved in normal tap water to provide the intended concentrations of 40, 200, and 1000 mg/L. Benzene has a solubility of 1,780 mg/L in water at 25^{o}C (U.S. Environmental Protection Agency, 1980a). Treatments were achieved by supplying the indicated concentrations of benzene in the drinking water to mice for 28 days; controls received tap water with no benzene. Treated water solutions were changed every three days to ensure freshness. Feed and water consumptions were measured continuously, and animals were weighed weekly. Drinking water samples were collected daily for chemical analysis after making the fresh benzene-water solution. Final benzene concentration in water was confirmed with a gas chromatographic method

described earlier (U.S. Environmental Protection Agency, 1986b).

Processing of Brain Tissues

Following four weeks of continuous exposure, the animals were killed by decapitation, and their brains were rapidly removed, placed on an ice-cooled glass plate and microdissected into the following discrete regions according to the methods described by Glowinski and Iversen (1966): hypothalamus, medulla oblongata, cerebellum, corpus striatum, cerebral cortex and midbrain. The midbrain included the hippocampus, thalamus and subthalamus (Coulombe and Sharma, 1986). Each brain section was weighed to the nearest milligram and immediately placed in tared vials containing various volumes (in relation to tissue weight) of ice-cold 0.05 M perchloric acid (containing 0.1% cysteine to prevent oxidation of monoamine neurotransmitters). To overcome possible diurnal alterations in regional brain neurochemicals, all animals were sampled between 10:00 and 12:00 AM on the same day. Brain tissues were homogenized individually, and the mixtures were centrifuged at 10,000 g (30 min at $4^{o}C$). The clear supernatant fractions were passed through 0.2 μm pore cellulose filter membrane by centrifugal filtration (MF-1 filters, Bioanalytical Systems Inc., W Lafayette, IN) at 1,000 g for 3 min at $4^{o}C$, then transferred to a 1.5 mL conical microcentrifuge tube. These filtrates were frozen at $-80^{o}C$ until analyzed within a few weeks.

Assay of Monoamines and Their Metabolites

The concentrations of the major brain catecholamines norepinephrine (NE), dopamine (DA), and their principal metabolites 3-methoxy-4-hydroxymandelic acid (vanillymandelic acid, VMA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA); the

indoleamine serotonin (5-hydroxytryptamine, 5-HT) and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA) in perchloric acid extracts of individual brain sections were determined by high performance liquid chromatography (HPLC) with a multiple-electrode electrochemical detector (Mayer and Shoup 1983). The HPLC system (Bioanalytic Systems Inc., W Lafayette, IN) included a Model LC 150 electrochemical analyzer, Model LC 3A amphoteric detector, Model LC 22A temperature controller, and a Biophase ODS reverse phase column. The mobile phase was prepared according to methods described by Mayer and Shoup (1983) and was allowed to equilibrate overnight at a flow rate of 1.4 mL/min before sample analysis. The column temperature was maintained at 30°C. Levels of brain monoamines or their metabolites were computed (using a 3390A reporting intergrator, Hewlett-Packard, Avondale, PA) from chromatograms derived from the responses of analytical amine standards (Sigma Chemical Co., St. Louis, MO) prepared by diluting amine in 0.05 M perchloric acid containing 0.1% cysteine to produce concentrations of 10, 25, 50, 100, 200, 500 ng/mL. Detector response provided a linear correlation coefficient for all standards($r^2 > 0.98$). The retention time (min) of VMA, NE, DOPAC, DA, 5-HIAA, HVA, and 5-HT is 2.84, 3.66, 5.85, 6.72, 9.67, 11.41, and 16.68, respectively.

Statistical Analyses

The levels of monoamines determined from brain regions were expressed as µg/g wet tissue weight. The effects on each regional neurochemical were analyzed by one-way analysis of variance (ANOVA), followed Fisher's least significant difference (LSD) test (Dowdy and Wearden, 1983). Statistically significant differences were considered at $p<0.05$.

RESULTS

Benzene concentration of drinking water mixtures was determined by gas chromatography on different days during the test period. Although the concentrations of benzene in the drinking water decreased approximately 27-30% over a 3-day period, the average concentration was 31 mg/L for the 40 mg/L nominal concentration (79% of the intended concentration), 166 mg/L (83% of the intended concentration) for the 200 mg/L group, and 790 mg/L (79% of the intended concentration) for the 1000 mg/L group. Based on the observed water consumption during the period of exposure, the daily estimated benzene doses corresponded to 0, 8, 40, 180 mg/kg, respectively.

Benzene levels used in this study did not significantly alter body weights, compared to the controls. Also, there were no apparent treatment-related behavioral alterations in test animals. Food and water consumptions were not significantly affected by benzene addition to water in any of the groups.

Administration of benzene via drinking water induced significant increases in monoamine neurotransmitters in several of the brain regions studied. In most cases, the changes were dose-related; in several instances maximum effects occurred at the benzene dose of 40 mg/kg/day, and did not increase in the highest dose group (180 mg/kg/day). An elevation in concentrations of NE and its acid metabolite VMA was observed in various brain regions after four weeks of benzene treatment (Figure IV-1 and IV-2, Table IV-1). Of particular interest, was a significant increase in the concentration of NE in the hypothalamus, the region containing the highest level of this monoamine, in the two higher dosed groups. The NE concentrations in this region increased by

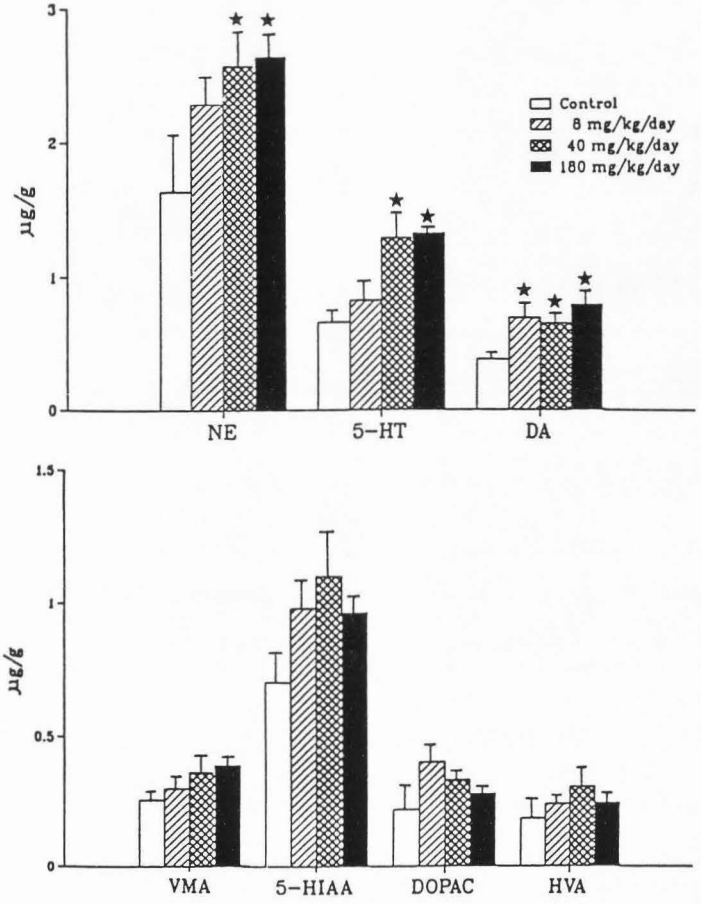

Fig. IV-1. Concentrations of the various monoamines and their metabolites in the hypothalamus of mice exposed to benzene in drinking water for 4 weeks. Stars (*) denote significantly different from untreated control ($p<0.05$). Values are given as mean ± SE of five animals per group. NE, norepinephrine; 5-HT, serotonin; DA, dopamine; VMA, vanillymandelic acid; 5-HIAA, 5-hydroxyindoleacetic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; and HVA, homovanillic acid.

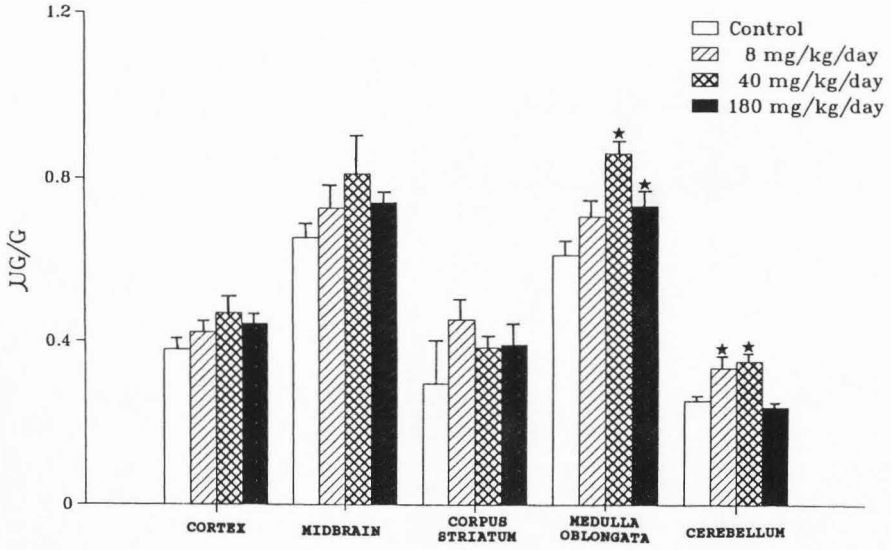

Fig. IV-2. Concentrations of norepinephrine in the various brain regions of mice exposed to benzene in drinking water for 4 weeks. Stars(*) denote significantly different from untreated control ($p<0.05$). Values are given as mean $\pm$ SE of five animals per gouup.

TABLE IV-1

EFFECTS OF BENZENE EXPOSURE ON REGIONAL CONCENTRATIONS OF BRAIN VANILLYMANDELIC ACID (VMA)

Doses[b] (mg/kg/day)	Concentrations (μg/g wet tissue)[a]				
	Medulla oblongata	Cerebellum	Midbrain	Corpus striatum	Cortex
Control	0.189 ± 0.034	0.194 ± 0.010	0.231 ± 0.031	0.204 ± 0.028	0.170 ± 0.017
8	0.238 ± 0.009	0.241 ± 0.016*	0.285 ± 0.017	0.282 ± 0.029*	0.287 ± 0.030*
40	0.240 ± 0.005	0.234 ± 0.006*	0.248 ± 0.015	0.291 ± 0.013*	0.350 ± 0.040*
180	0.242 ± 0.008	0.281 ± 0.014*	0.266 ± 0.012	0.295 ± 0.019*	0.293 ± 0.006*

[a] Values are given as mean ± SE (n=5)

[b] Benzene was administered continuously via drinking water for 4 weeks

* Significantly ($p < 0.05$) different from control values

40, 58 and 61% in groups receiving 8, 40 and 180 mg/kg/day, respectively. Similar effects on NE were produced by benzene in medulla oblongata and cerebellum at various dose levels. As indicated in Table IV-1, VMA was induced concomitantly with increases of its parent chemical, NE. Significant elevation of VMA was observed in the corpus striatum, cerebellum and cerebral cortex in all treatment groups.

Increases in concentrations of DA and its major metabolites, DOPAC and HVA, in the various brain regions were also observed. Concentrations of DA increased significantly in the hypothalamus of all benzene-treated groups (Figure IV-1). In the corpus striatum, the region richest in DA, the concentrations of this neurochemical increased significantly in animals receiving 8 and 40 mg/kg/day of benzene. Concomitantly, both DA metabolites, DOPAC and HVA, also increased significantly in this brain region (Figure IV-3). In other brain regions, the concentrations of DOPAC, the first acid product of the DA oxidative pathway, were significantly elevated only in midbrain (8 and 40 mg/kg/day) (Table IV-2). Amounts of DA, DOPAC and HVA in cerebellum, a region containing relatively low levels of these chemicals, were not determined.

Significant benzene-induced increases of 5-HT levels were seen in hypothalamus, midbrain, cerebral cortex, corpus striatum and medulla oblongata (Figure IV-1 and IV-4). No effect on 5-HT levels could be detected in the cerebellum, where the concentrations of this biogenic amine are relatively low. Accompanying increases of 5-HT, there were significant increases of its main metabolite 5-HIAA in the midbrain corpus striatum and cortex (Table IV-3). The hypothalamic 5-HIAA levels generally accumulated to coordinate its precursor chemical, 5-HT (Figure IV-1).

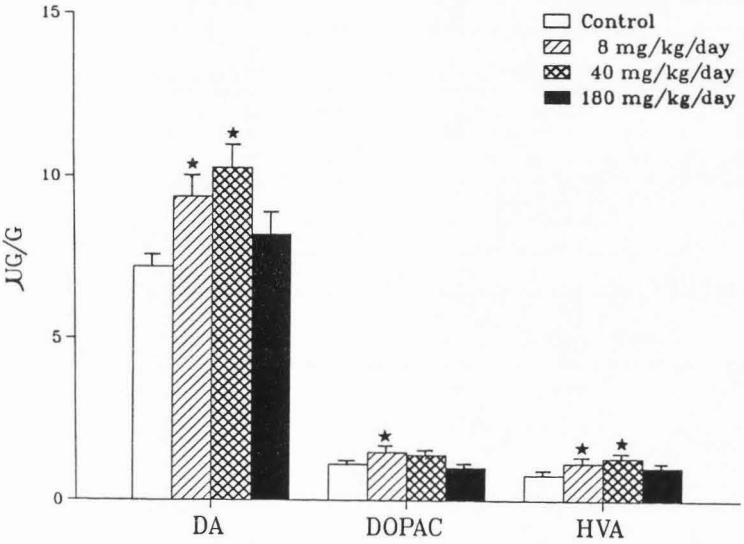

Fig. IV-3. Concentrations of dopamine and its major metabolites in the corpus striatum of mice exposed to benzene in drinking water for 4 weeks. Stars(*) denote significantly different from untreated control ($p<0.05$). Values are given as mean $\pm$ SE of five animals per gouup. DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; and HVA, homovanillic acid.

TABLE IV-2

EFFECTS OF BENZENE EXPOSURE ON REGIONAL CONCENTRATIONS OF BRAIN DOPAMINE (DA), 3,4-DIHYDROXYPHENYLACETIC ACID (DOPAC), AND HOMOVANILLIC ACID (HVA)

Chemicals	Doses[b] (mg/kg/day)	Concentrations (μg/g wet tissue)[a]		
		Medulla oblongata	Midbrain	Cortex
DA	Control	0.037 ± 0.005	0.265 ± 0.068	0.958 ± 0.142
	8	0.047 ± 0.006	0.494 ± 0.113	1.349 ± 0.178
	40	0.046 ± 0.003	0.496 ± 0.129	1.367 ± 0.086
	180	0.043 ± 0.006	0.330 ± 0.058	1.148 ± 0.105
DOPAC	Control	0.042 ± 0.006	0.118 ± 0.020	0.209 ± 0.018
	8	0.058 ± 0.008	0.217 ± 0.025*	0.249 ± 0.018
	40	0.054 ± 0.006	0.215 ± 0.038*	0.270 ± 0.033
	180	0.043 ± 0.006	0.120 ± 0.012	0.195 ± 0.021
HVA	Control	0.042 ± 0.003	0.138 ± 0.017	0.187 ± 0.023
	8	0.047 ± 0.006	0.179 ± 0.021	0.226 ± 0.008
	40	0.065 ± 0.004	0.218 ± 0.026	0.246 ± 0.019
	180	0.056 ± 0.007	0.156 ± 0.019	0.212 ± 0.008

[a] Values are given as mean ± SE (n=5)

[b] Benzene was administered continously via drinking water for 4 weeks

* Significantly ($p < 0.05$) different from control values

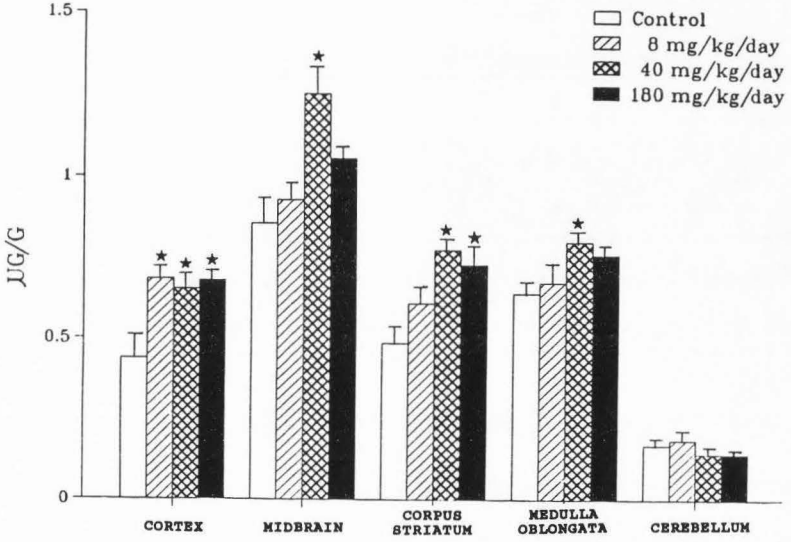

Fig. IV-4. Concentrations of serotonin in the various brain regions of mice exposed to benzene in drinking water for 4 weeks. Stars(*) denote significantly different from untreated control ($p<0.05$). Values are given as mean $\pm$ SE of five animals per gouup.

TABLE IV-3

EFFECTS OF BENZENE ON REGIONAL CONCENTRATIONS OF BRAIN 5-HYDROXYINDOLEACETIC ACID (5-HIAA)

Doses[b] (mg/kg/day)	Concentrations (μg/g wet tissue)[a]				
	Medulla oblongata	Cerebellum	Midbrain	Corpus striatum	Cortex
Control	0.400 ± 0.017	0.172 ± 0.010	0.552 ± 0.029	0.451 ± 0.057	0.225 ± 0.014
8	0.438 ± 0.035	0.192 ± 0.020	0.636 ± 0.013	0.598 ± 0.009*	0.317 ± 0.016*
40	0.494 ± 0.019	0.173 ± 0.007	0.741 ± 0.057*	0.683 ± 0.033*	0.311 ± 0.028*
180	0.452 ± 0.031	0.151 ± 0.007	0.638 ± 0.038	0.548 ± 0.024	0.285 ± 0.010*

[a] Values are given as mean ± SE (n=5)

[b] Benzene was administered continuously via drinking water for 4 weeks

* Significantly ($p < 0.05$) different from control values

DISCUSSION

This study monitored changes in selected regional brain monoamines that occurred after four weeks of benzene exposure in mice via drinking water. Since the concentration of any neurotransmitter does not by itself provide an appropriate indication of the turnover of that neurotransmitter to reflect monoaminergic neurophysiological activity (Commissiong, 1985), its metabolite(s) were also assayed. Assays in various regions of the brain is essential to detect potential perturbations of brain neurotransmitters because significant changes of certain neurochemicals may be concealed in certain minute brain regions and, therefore, remain undetected (Coulombe and Sharma, 1986). Due to the rapid changes in brain monoamine concentrations caused by the enzymes for both synthesis and catabolism (Faiman et al., 1973), the methods used to prepare brain tissues described earlier minimized the risk of postmorten alterations.

The regional concentrations of brain monoamine neurotransmitters and their metabolites obtained for the untreated control mice in this study were similar to those reported earlier for CD-1 mice (Sharma et al., 1986). Ingestion of benzene at the doses studied enhanced potential neurotoxic effects. Although selected neurotransmitters in the various discrete brain regions were significantly altered, no toxicological signs were observed during the period of treatment; the growth rate, and consumptions of food and water were not influenced by the benzene treatment. Similar treatment, however, caused macrocytic anemia at all dose levels of benzene (Chapter III).

Benzene had the greatest effect on NE in the hypothalamus and medulla oblongata; and on 5-HT in the hypothalamus, medulla oblongata

and midbrain. These increases were frequently associated with corresponding increases in VMA and 5-HIAA, respectively, suggesting an increased turnover of biogenic amines. Dopamine concentrations elevated significantly only in the corpus striatum, a brain region especially rich in this neurotransmitter, and in the hypothalamus. Elevated DOPAC and HVA levels were also observed in these brain regions. These findings are compatible with results reported by others (Paradowski et al., 1984; 1985; Juorio and Yu, 1985a,b). However, since their studies were performed on whole-brain homogenates or only in one or two selected brain regions; our studies provide confirmation of their observations. In rats, subcutaneous administrations of benzene in doses of 67.5-540 mg/kg/day for 4 weeks induced increases of brain NE and DA concentrations (Paradowski et al., 1985), and also produced accumulations of both 5-HT and 5-HIAA in the hypothalamus and striatum (Paradowski et al., 1984). The regional concentrations of the various monoamines increased enough to suggest that benzene-induced perturbations in CNS monoaminergic biosynthetic pathways, as is confirmed by the accompanying increases in the metabolites of these monoamines. Both synthesis and catabolism of these neurotransmitters increased following benzene intake. Synthesis of NE, DA and 5-HT more then compensated for their release and breakdown during the increased neuronal activity. In other words, their syntheses could cope with increased noradrenergic, dopaminergic and serotoninergic activities.

Despite rather nonspecific correlations, behavioral changes may also indicate the involvement of monoamines. Benzene-induced milk-licking, tremors, and hind-limb weakness indicated that this chemical possesses properties of neurobehavioral alterations in mice (Dempster et

al., 1984), which are often attributed to an imbalance in the concentrations of catecholamines and indoleamine (Leibowitz 1986; Willis and Smith, 1985). Furthermore, the subjective clinical indices of neurobehavior in humans exposed to benzene (250-500 ppm), e.g., vertigo, drowsiness, euphoria, giddiness, headache, and nervous irritability (Brief et al., 1980) may be mediated by alterations in regional brain monoamine levels.

The mechanism for the benzene-induced changes in levels of monoamines and their metabolites is not yet known. The effects of benzene on the major enzyme systems in the monoaminergic biosynthetic pathway have never been clearly shown. Benzene produced an induction of aromatic-L-amino acid decarboxylase (AADC) but no effect on monoamine oxidase (MAO) (Juorio and Yu, 1985a). However, due to its high enzymatic activity, the induction of AADC may not result in significant changes of endogenous monoamine levels (Cooper et al., 1986). Further investigations are needed to determine whether benzene alters the kinetics of the rate-limiting enzymes, tyrosine hydroxylase and tryptophan hydroxylase, in the catecholamines and indoleamine synthetic pathways, respectively. Benzene may interact with more than a single biological site, so data from nervous system molecular events, pharmacokinetics, as well as *in vivo* neurobehavioral changes are essential in the evaluation of the potential neurotoxic risk caused by oral ingestion of benzene.

Similar levels of benzene in drinking water caused alteration of various immune responses in CD-1 mice as described earlier (Chapter III). Although benzene is a hemopoietoxic chemical and can directly suppress immune functions, the increases in brain catecholamines will

have an additive adverse effect on the immune system via hypothalamus-pituitary-adrenal (HPA) axis. Increased levels of catecholamines can result in increased adrenal corticosteroid levels (Makara et al., 1980) which in turn are immunosuppressive (Claman, 1972). The pituitary-adrenal status after benzene treatment is currently being investigated in our laboratory.

In summary, administratering benzene to CD-1 mice for four weeks via drinking water significant elevated levels of NE, DA, and 5-HT, and their metabolites (VMA, DOPAC, HVA and 5-HIAA) in several brain regions studied. More detailed study of these phenomena will identify the mechanisms (membrane-mediated, metabolic, or both) that perturb the monoaminergic biosynthetic pathways. It is not known whether the neurotoxic effects of benzene are produced directly or indirectly (due to some metabolite(s) of this chemical). The data do not predict a no-effect level of benzene in drinking water.

CHAPTER V

EVALUATION OF TOLUENE EXPOSURE ON LEVELS OF REGIONAL BRAIN BIOGENIC MONOAMINES AND THEIR METABOLITES IN CD-1 MICE

INTRODUCTION

Chemical contamination of groundwater, which is a primary source of drinking water for approximately half the population of the United States, has become a great matter of public concern (U.S. Office of Technology Assessment, 1984). Of major concern is the probable subclinical, deleterious effects resulting from continuous long-term exposure to low levels of chemicals. Toluene, a major aromatic chemical, is produced in enormous quantities, and is extensively employed as a starting material for other chemicals, an important component in gasoline, and a "safe" replacement for benzene in solvent applications (Fishbein, 1985). It has been detected in concentrations up to 6.4 mg/L in finished water (Tardiff and Youngren, 1986). The neurotoxic properties of this chemical represent an important health hazard (Fishbein, 1985; Benignus, 1981a).

Toluene appears to have a pronounced effect on behavior; schedule-controlled behaviors have been reported to produce inverted U-shaped concentration-effect curves on response rates in experimental animals (Benignus, 1981b). Abnormal electroencephalographic (EEG) activity following toluene inhalation was found in humans (Grasso et al., 1984), as well as in animals (Naalsund, 1986; Dyer et al., 1984). Rigorous neurochemical studies are needed to better explain toluene's neurobehavioral effects. Several studies have indicated that

concentrations of various brain biogenic monoamines are affected by acute or subchronic administration of toluene via inhalation or intraperitoneal injection to rats. Most reports showed that brain norepinephrine (NE) increased while dopamine (DA) increased or remained unaltered after toluene treatment (Rea et al., 1984; Andersson et al., 1980; 1983; Arito et al., 1984; 1985). Significant alterations in brain concentrations of serotonin (5-HT) were also reported (Rea et al., 1984; Arito et al., 1984; 1985). None of those studies involved evaluation of regional neurotransmitters after prolonged oral exposure to toluene.

In this experiment we studied alterations in concentrations of regional brain biogenic amines (i.e., catecholamines, indoleamine) and their major metabolites in CD-1 mice continuously exposed to toluene via drinking water for 4 weeks. The neurochemicals selected for analysis are known to play an important role in physiology and behavior (Rogawski and Baker, 1985; Poirier and Bedard, 1984). The concentrations of metabolites were studied since steady-state transmitter concentrations alone are of limited value about how a toxicant perturbs the central nervous system. Changes in metabolite concentrations indicate the metabolic activity of neuronal pathways related to specific neurotransmitters (Commissiong, 1985). Discrete brain areas were chosen because examination of discrete brain regions demonstrate preferential effects on specific pathways. Recently, several studies in our laboratory have indicated that chemical-induced neurotoxicity might be related to the alterations of certain neurochemicals in certain minute brain regions (Coulombe and Sharma, 1985; 1986; Sharma et al., 1986).

MATERIALS AND METHODS

Animals

Adult male CD-1 mice (Charles River Breeding Laboratory Inc., Wilmington, MA) were used in all studies. Mice were kept in an AAALAC-accredited animal care facility with ambient temperature of 22-24^{o}C, 40-60% relative humidity, and a 12 h light dark cycle and acclimated one week prior to the beginning of the study. Commercial rodent chow (Wayne Products, Chicago, IL) and tap water were available *ad libitum*.

Chemical Treatment

Toluene (chromatography grade, 99.7% purity, Burdick and Jackson Labs. Inc., Muskegon, MI) was dissolved in drinking water to provide nominal concentrations of 20, 100, and 500 mg/L. Toluene has a solubility of 535 mg/L in fresh water at 25^{o}C (U.S. Environmental Protection Agency, 1980b). Exposures were conducted by continuously supplying the indicated concentrations of toluene in the drinking water for 28 days. Control animals received tap water without toluene. The treated water solutions were changed every three days to ensure freshness, and the volume of water ingested was recorded to estimate the dose of toluene intake. Food consumption was also monitored continuously, and animals were weighed weekly. Actual toluene concentrations in drinking water were determined by a gas chromatographic method (U.S. Environmental Protection Agency, 1986b).

Processing of Brain Tissues

Following 4 weeks of continuous exposure, mice were decapitated and their brains removed and immediately placed in an ice-cold glass petri dish. The brain was microdissected into six discrete regions:

hypothalamus, medulla oblongata, cerebellum, corpus striatum, cerebral cortex and midbrain according to the procedures of Glowinski and Iversen (1966). The midbrain includes the hippocampus, thalamus and subthalamus (Coulombe and Sharma, 1986). Each dissected brain region was promptly weighed in tared vials containing several volumes (in relation to tissue weight) of ice-cold 0.05 M perchloric acid with 0.1% cysteine then frozen on dry ice. To overcome possible diurnal alterations in regional brain neurochemicals, all animals were sampled between 10:00 and 12:00 AM on the same day. Tissue samples for chromatographic analysis were prepared as described in the methods section of Chapter IV.

Assay of Neurotransmitters and Their Metabolites

The concentrations of biogenic amines and their metabolites in various brain regions were assayed by high performance liquid chromatography (HPLC) with a multiple-electrode electrochemical detector (Mayer and Shoup, 1983). The HPLC system consisted of a Model LC-150 electrochemical analyzer, Model LC-3A amphoteric detector, Model LC-22A temperature controller, and a Biophase ODS-reversed phase column (Bio-Analytic System Inc.). The mobile phase, containing sodium octyl sulfate as ion-pair reagent, was prepared according to our modifications of the methods described previously (Mayer and Shoup, 1983). A constant flow rate of 1.4 ml/min was delivered, while the column temperature was maintained at 30^{o}C. The neurochemicals were oxidized by setting the potential at +0.7 V and the controller at 10 nA full scale. Data were recorded with a Hewlett-Packard (Avondale, PA) 3390A integrator from chromatograms derived from the responses of analytical standards (Sigma Chemical Co., St. Louis, MO). Standards were prepared by diluting

chemicals in 0.05 M perchloric acid containing 0.1% cysteine to produce concentrations of 10, 25, 100, 200, 500 ng/ml. The detector responses provided a linear correlation coefficient of > 0.98. Compounds assayed included the major catecholamines norepinephrine (NE), dopamine (DA), and their principal metabolites, 3-methoxy-4-hydroxymandelic acid (vanillymandelic acid, VMA), 3-4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA); indoleamine serotonin (5-hydroxytryptamine, 5-HT) and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA).

Statistical Analysis

The concentrations of monoamines and their metabolites determined from brain regions were expressed as μg/g wet tissue. One-way analysis of variance (ANOVA) and follow-up Fisher's least significant differences (LSD) test were used to analyze the data for differences between toluene-exposure and corresponding normal water control. A level of $p<0.05$ was considered statistically significant.

RESULTS

Gas chromatographic analyses revealed that concentrations of toluene in drinking water mixtures decreased approximately 25-35% over a 3-day period. The observed concentrations (overall average of the daily analysis) for the nominal concentrations of 0, 20, 100, and 500 mg/L were 0, 17 (±16%), 80 (±12%), and 405 (±14%) mg/L, respectively. Based on water consumption during the duration of exposure, the estimated toluene intake doses corresponded to 0, 5, 22, and 105 mg toluene/kg body weight/day.

At the doses used in this study, toluene did not adversely affect growth, mortality, body weights, nor did it produce any other overt

clinical symptoms of toxicity in mice. Food and water consumption throughout the study did not suggest any significant differences between treatment and control group.

The regional concentrations of brain biogenic amines (NE, DA, 5-HT) and their major metabolites (VMA, DOPAC, HVA, 5-HIAA) in CD-1 mice are shown in Figs. V-1 through V-3 and Tables V-1 through V-3. Toluene ingestion induced significant increases in concentration of these neurochemicals in several of the brain regions studied. In most cases where statistical differences are noticed, the increases exhibited a biphasic response which began at a dose of 5 mg/kg/day, peaked at 22 mg/kg/day, then decreased at 105 mg/kg/day. In several instances, the effects were dose-related. Figure V-1 indicates the effects of toluene on selected neurochemicals in the hypothalamus. The concentrations of NE increased significantly by 51, 63, and 34% in groups dosed with 5, 22, and 105 mg/kg/day, respectively, in this compartment. The accumulations of its metabolite, VMA, were not statistically significant. Significant increases of DA and DOPAC levels were detected in animals receiving toluene doses of 5 and 22 mg/kg/day, but their metabolite, HVA, was not influenced. Toluene (22 mg/kg/day) also significantly increased concentrations of both hypothalamic 5-HT and its metabolite 5-HIAA.

Figure V-2 illustrates the concentrations of the major amines and their metabolites in the corpus striatum following toluene treatment. Increases of DA concentrations in the corpus striatum, a brain region especially rich in this neurotransmitter, were dosed-related and exhibited significant differences in the two higher dosed animal groups as compared to those in the untreated animals. Toluene increased

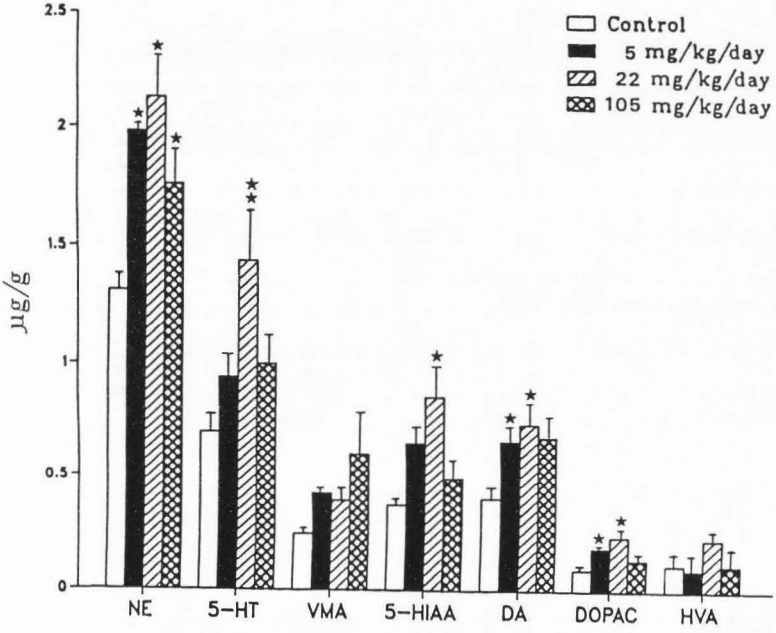

Fig. V-1. Concentrations of various biogenic monoamines and their metabolites in the hypothalamus of mice exposed to toluene in drinking water for 4 weeks. Stars (*) denote significant differences from control values at $p<0.05$. Values are given as mean $\pm$ SE of five animals per group. NE, norepinephrine; 5-HT, serotonin; VMA, vanillymandelic acid; 5-HIAA, 5-hydroxyindoleacetic acid; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; and HVA, homovanillic acid.

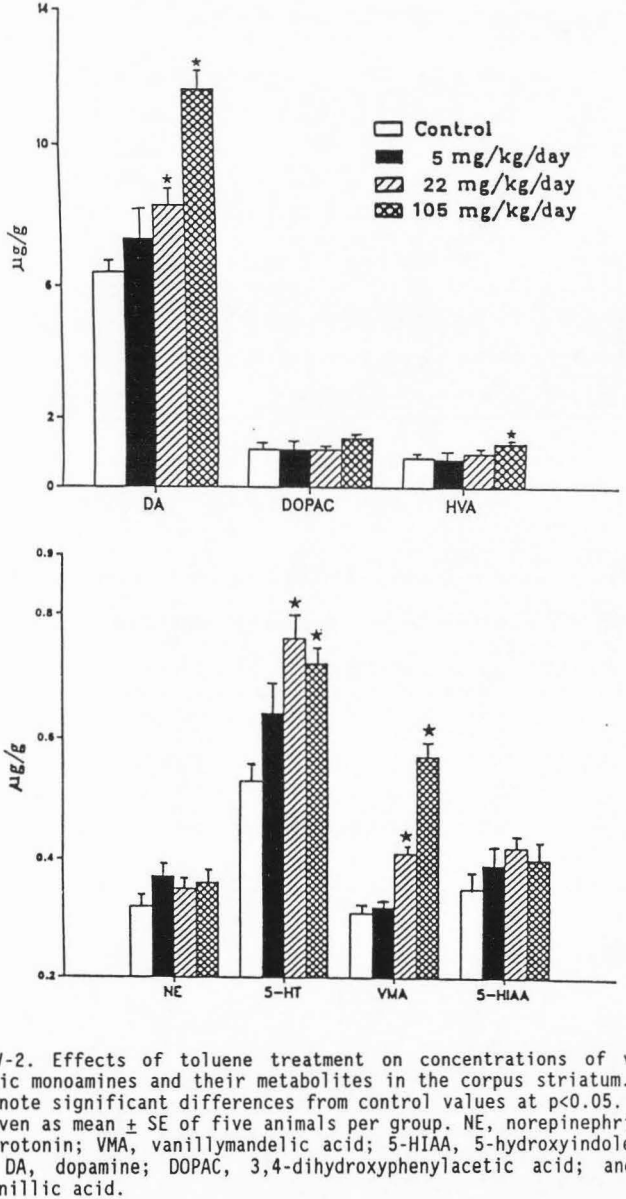

Fig. V-2. Effects of toluene treatment on concentrations of various biogenic monoamines and their metabolites in the corpus striatum. Stars (*) denote significant differences from control values at $p<0.05$. Values are given as mean $\pm$ SE of five animals per group. NE, norepinephrine; 5-HT, serotonin; VMA, vanillymandelic acid; 5-HIAA, 5-hydroxyindoleacetic acid; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; and HVA, homovanillic acid.

striatal HVA at the highest dose level only. No effects on NE were observed in this brain region, but VMA concentrations were significantly induced in animals dosed with 22 and 105 mg/kg/day. The two higher dose levels of toluene significantly elevated the hypothalamic 5-HT concentrations, while concentrations of 5-HIAA in this region were significantly higher only in the 105 mg/kg/day dosed animals.

Concentrations of biogenic amines and their major metabolite in the brain regions of cerebellum, midbrain and cerebral cortex are given in Table V-1 through V-3. The results shown in Table V-1 indicate that significant increases of NE were found in the midbrain in all toluene treatment groups. The concentrations of VMA were correspondingly increased in this brain region and showed statistical difference from untreated control values in the two higher dosed groups. Toluene also caused a significant increase of VMA in cerebral cortex at the highest dose. The amounts of DA and its metabolites DOPAC and HVA, were not obviously affected in these three brain areas (Table V-2). Although HVA increased occasionally in the cerebellum, the concentrations of this chemical are relatively low in this region. There were significant increases of 5-HT in midbrain in all three toluene dosed groups and in cerebral cortex in the 22 mg/kg/day dosed mice (Table V-3).

The effects of toluene ingestion on neurotransmitters and their metabolites in the medulla oblongata are presented in Figure V-3. Significant toluene-induced increases of NE and VMA were found at the 22 mg/kg/day dose only. Relatively high concentrations of 5-HT and 5-HIAA were noticed in this brain area. There were significant increases of 5-HT in the two higher toluene dosed animals and 5-HIAA in the 22 mg/kg/day dosed group. Dopamine and HVA in this brain region were not

TABLE V-1

EFFECTS OF TOLUENE EXPOSURE ON NE AND VMA CONCENTRATIONS IN CEREBELLUM, MIDBRAIN, AND CEREBRAL CORTEX

Chemicals	Dose[a] (mg/kg/day)	Concentration[b]		
		Cerebellum	Midbrain	Cerebral Cortex
NE	Control	0.330 ± 0.041	0.575 ± 0.011	0.359 ± 0.020
	5	0.335 ± 0.015	0.660 ± 0.031*	0.409 ± 0.029
	22	0.416 ± 0.023	0.671 ± 0.026*	0.460 ± 0.026
	105	0.377 ± 0.018	0.691 ± 0.019*	0.429 ± 0.021
VMA	Control	0.253 ± 0.015	0.305 ± 0.024	0.253 ± 0.017
	5	0.286 ± 0.009	0.320 ± 0.007	0.265 ± 0.006
	22	0.272 ± 0.020	0.356 ± 0.015*	0.250 ± 0.013
	105	0.244 ± 0.008	0.377 ± 0.011*	0.347 ± 0.006*

[a] Toluene was administered continuously via drinking water for 4 weeks

[b] μg neurochemical/g wet tissue, Mean ± SE (n=5)

* Significantly different from untreated control at $p<0.05$

TABLE V-2

EFFECTS OF TOLUENE EXPOSURE ON DA, DOPAC, AND HVA CONCENTRATIONS IN CEREBELLUM, MIDBRAIN, AND CEREBRAL CORTEX

Chemicals	Dose[a] (mg/kg/day)	Concentration[b]		
		Cerebellum	Midbrain	Cerebral Cortex
DA	Control	0.040 ± 0.004	0.270 ± 0.026	1.197 ± 0.093
	5	0.044 ± 0.006	0.488 ± 0.093	1.121 ± 0.130
	22	0.041 ± 0.007	0.310 ± 0.040	1.243 ± 0.159
	105	0.046 ± 0.005	0.393 ± 0.061	1.134 ± 0.084
DOPAC	Control	0.027 ± 0.003	0.141 ± 0.010	0.185 ± 0.008
	5	0.022 ± 0.002	0.192 ± 0.049	0.155 ± 0.017
	22	0.030 ± 0.005	0.126 ± 0.016	0.189 ± 0.013
	105	0.029 ± 0.003	0.124 ± 0.011	0.179 ± 0.013
HVA	Control	0.008 ± 0.008	0.126 ± 0.021	0.180 ± 0.019
	5	0.009 ± 0.009	0.161 ± 0.030	0.142 ± 0.012
	22	0.043 ± 0.005*	0.119 ± 0.009	0.180 ± 0.023
	105	0.020 ± 0.009	0.132 ± 0.008	0.170 ± 0.017

[a] Toluene was administered continuously via drinking water for 4 weeks

[b] μg neurochemical/g wet tissue, Mean ± SE (n=5)

* Significantly different from untreated control at $p<0.05$

TABLE V-3

EFFECTS OF TOLUENE EXPOSURE ON 5-HT AND 5-HIAA CONCENTRATIONS IN CEREBELLUM, MIDBRAIN, AND CEREBRAL CORTEX

Chemicals	Dose[a] (mg/kg/day)	Concentration[b]		
		Cerebellum	Midbrain	Cerebral cortex
	Control	0.222 ± 0.054	0.763 ± 0.017	0.527 ± 0.013
5-HT	5	0.191 ± 0.028	1.020 ± 0.046*	0.655 ± 0.007
	22	0.274 ± 0.027	1.102 ± 0.072*	0.788 ± 0.049*
	105	0.194 ± 0.027	0.982 ± 0.018*	0.654 ± 0.006
	Control	0.091 ± 0.022	0.370 ± 0.005	0.170 ± 0.007
5-HIAA	5	0.084 ± 0.012	0.417 ± 0.023	0.194 ± 0.013
	22	0.105 ± 0.016	0.417 ± 0.063	0.232 ± 0.026
	105	0.060 ± 0.005	0.322 ± 0.009	0.179 ± 0.007

[a] Toluene was administered continuously via drinking water for 4 weeks

[b] μg neurochemical/g wet tissue, Mean ± SE (n=5)

* Significantly different from untreated control at $p<0.05$

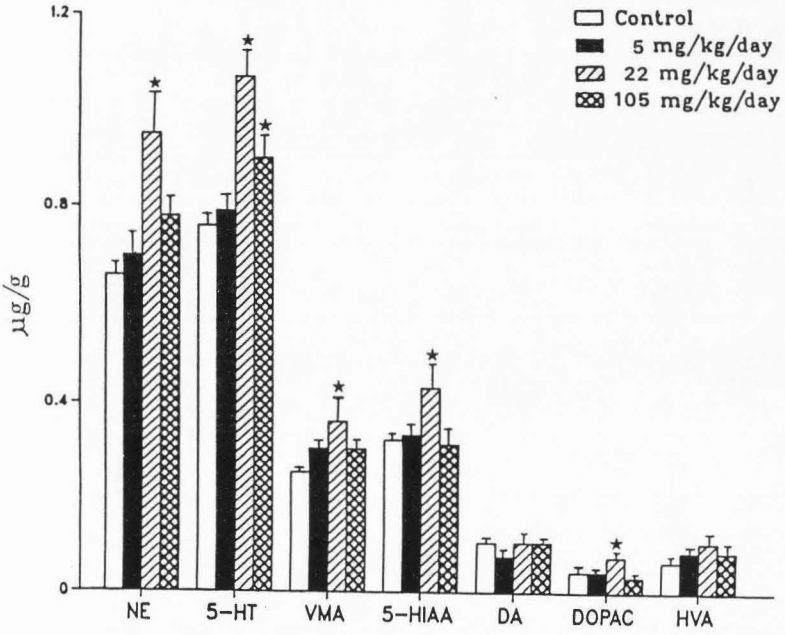

Fig. V-3. Effects of toluene treatment on concentrations of various biogenic monoamines and their metabolites in the medulla oblongata. Stars (*) denote significant differences from control values at $p<0.05$. Values are given as mean $\pm$ SE of five animals per group. NE, norepinephrine; 5-HT, serotonin; VMA, vanillymandelic acid; 5-HIAA, 5-hydroxyindoleacetic acid; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; and HVA, homovanillic acid.

affected by toluene treatment. A significant increase in DOPAC concentration was observed in the 22 mg/kg/day dosed group.

DISCUSSION

This study evaluated the potential of toluene to induce monoamine transmitter-related neurotoxicity in mice after 4 weeks of exposure via drinking water. The highest dose was lower than the solubility of toluene in fresh water at 25^{o}C, which is 535 mg/L (U.S. Environmental Protection Agency, 1980b). The lowest dose was slightly higher than the EPA maximum permissible concentration of 14.3 mg/L for ambient water (U.S. Environmental Protection Agency, 1986a), which was mainly based on a general toxicity evaluation of an oral dosing study in rats (Wolf et al., 1956).

The concentrations of neurochemicals gained in the control group in this study were similar to those reported earlier for CD-1 mice (Sharma et al., 1986). In addition, since diurnal variations and postmortem changes in neurochemical concentrations were avoided, values generally varied little within groups.

The findings indicate that ingestion of ambient water containing toluene at the EPA permissible concentration may have neurotoxic effects. Significant increases in regional concentrations of various neurochemicals in several brain regions caused by toluene ingestion would be sufficient to suggest toluene-induced perturbations in monoaminergic biosynthetic pathways. The corresponding increases in their metabolites support the validity of these findings. Noradrenergic neurons and serotonergic neurons appeared to be highly vulnerable to toluene while dopaminergic neurons were rather resistant. Toluene

dramatically affected NE concentrations in the hypothalamus, medulla oblongata, midbrain and corpus striatum, and 5-HT concentrations in the hypothalamus, medulla oblongata, midbrain, cerebral cortex and corpus striatum. Increased concentrations of NE and 5-HT along with their respective metabolites (VMA and 5-HIAA) in these regions suggest that syntheses of NE and 5-HT more than compensate for their releases and breakdown during increased neuronal activities. The significant increase in concentrations of DA and its metabolites in the hypothalamus may be a secondary effect since they are not major neurochemicals in this brain compartment. Although toluene treatment significantly increased DA concentrations in the corpus striatum, only slight increases of its metabolites DOPAC and HVA were observed. In addition to these two brain areas, no apparent effects on DA or its metabolites were found in any of the other brain regions.

Increases in steady-state levels of NE, DA and 5-HT have been reported in whole-brain homogenates or discrete brain areas of rats who continuously inhaled 100, 300, or 1000 ppm toluene for 8 hr (Rea et al., 1984). Subchronic repeated exposure of rats to toluene vapor also increased concentrations of catecholamines and their turnover rates in hypothalamus or median eminence (Andersson et al., 1980; 1983). After a single ip administration of 200 mg toluene/kg body weight to rats, Arito et al. (1984) reported a significant increase in the regional concentrations of NE, 5-HT and their respective metabolites, 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG) and 5-HIAA, however, alterations in DA and its metabolites DOPAC and HVA were not detected. In their follow-up study, however, 14 consecutive days of ip injection of toluene in rats significantly increased NE and MHPG only (Arito et al., 1985).

Pronounced increases in the concentrations of serum corticosterone, growth hormone (GH), follicle-stimulating hormone (FSH) and prolactin (PRL) were found in rats under the influence of toluene exposure via inhalation (Andersson et al., 1980; 1983). Hypothalamic monoaminergic mechanisms are known to have an important role in the secretion of these types of adenohypophyseal hormones.

Results in the present study also support the possible involvement of monoamine metabolism in the available behavioral and neurophysiological reports of toluene-induced bell-shaped concentration-effect curves, i.e., initially increasing rates and at higher levels decreasing rates of response (Dyer et al., 1984; Benignus, 1981b). Alterations in the brain concentrations of neurotransmitters and their metabolites have been correlated with abnormal behavioral and physiological functions (Rogawski and Baker, 1985; Willis and Smith, 1985; Poirier and Bedard, 1984).

The mechanisms of these changes in concentrations of biogenic amines and their metabolites are not yet known. Changes in the amounts of neurotransmitters in neurons may result from the destruction of cellular elements in which transmitters reside, or from perturbations that profoundly alter neuronal activity or metabolism. Uptake of toluene in the central nervous system is highest in the cerebrum, and is rapidly eliminated from brain tissue in rats (Savolainen, 1978). Subacute treatment of rats with toluene via inhalation was reported to reduce affinities in striatal DA receptors (D2 type) and cortical 5-HT receptors at a high concentration (3,000 ppm) (Celani et al., 1983), and the number of 5-HT binding sites in hippocampus and pons plus medulla oblongata at a concentration of 7,000 ppm toluene (Yamawaki et al.

1982). A down regulation activity at the DA and 5-HT synapses, resulting in a major metabolic effort of the presynaptic monoamine nerve cell to maintain normal monoaminergic transmission, was suggested by these workers. In contrast, one study demonstrated that no neurostructural changes in the rat brain were found following subchronic inhalation of less than 1,500 ppm toluene (Lewis and Holdsworth, 1982). The absence of neurostructural changes together with the reported increases in response rates of neurobehavioural and electrophysiological effects following exposure to low toluene concentrations mentioned above suggest that toluene-induced neurotoxicity may result from elevated concentrations of significant neurotransmitters.

The toluene-induced increase in brain biogenic monoamine metabolism may have an additive adverse effect via the hypothalamo-pituitary-adrenal (HPA) axis. The role of HPA status after toluene treatment is currently being investigated. It is important to note that significant changes in concentrations of neurochemicals in several specific brain regions occurred at the dose near the current U.S. Environmental Protection Agency criterion level (i.e., 14.3 mg/L) for toluene in water. Further investigations to re-evaluate the safety limits of low-level toluene exposure via drinking water seem warranted.

CHAPTER VI

IMMUNOTOXICOLOGICAL EVALUATION OF SUBCHRONIC TOLUENE EXPOSURE IN CD-1 MICE

INTRODUCTION

The initial studies of this series of subclinical effects of groundwater contaminants focused on benzene (Chapter III, VI). Toluene, another major aromatic hydrocarbon of environmental significance, is produced in enormous quantities and is widely used in a broad spectrum of applications as a solvent, an important component in gasoline, and an intermediate in the synthesis of a multitude of organic chemicals (Fishbein, 1985). It has been detected in finished water supplies in concentrations up to 6.4 mg/L (Tardiff and Youngren, 1986). Potential for exposure to toluene exists for both industrial workers and the general public.

Toluene exposure is reported to produce a variety of pathologic states in humans beings or experimental animals, including cardiac sensitization, kidney and liver damage, and various neurological disorders (Benignus, 1981a,b; Hayden et al., 1977). We demonstrated that toluene ingestion causes perturbation of regional brain monoamine metabolisms in the central nervous system of mice (Chapter V). In addition, toluene was found to be embryotoxic in rats, mice, and rabbits (Courtney et al., 1986; Ungvary and Tatrai, 1985; Nawrot and Staples, 1979). Alterations in lymphocyte functions, protein kinase C activity and clastogenic effects also have been noted (Mohtashamipur et al., 1987; Roghani et al., 1987; Suleiman, 1987; Dean, 1985; Fishbein, 1985; Schmid et al., 1985). Both *in vivo* and *in vitro* studies have indicated

that toluene, via oxidation of the aromatic ring, is activated to give reactive intermediates that bind covalently to biomolecules (Pathiratne et al., 1986b).

Despite the many toxicologic studies of toluene in experimental animals, only few published studies have examined its immunotoxic potential (Fishbein, 1985; U.S. Environmental Protection Agency, 1980b). Recently, Aranyi et al. (1985) reported a significant suppression in host defense mechanisms against microorganism infections in mice that inhaled toluene at concentrations ranging from 2.5 to 500 ppm. Because of its benzene-like structure and lipophilic nature, and its common occurrence in groundwater, a primary source of drinking water for approximately half the population of the United States, it is important to fully understand the immunotoxicity of toluene. Toxicity on the immune system may be relevant to determine significance to human health following chronic low-level exposure to toluene.

This study was designed to evaluate the general toxicity, hematological response, interleukin-2, and both humoral and cell-mediated immunity in mice exposed to various concentrations of toluene via oral ingestion for 28 days. The lowest concentration of toluene employed approached the EPA maximum permissible level (14.3 mg/L) for drinking water (U.S. Environmental Protection Agency, 1986a).

MATERIALS AND METHODS

Animals

Six-week-old male CD-1 mice were obtained from Charles River Breeding Laboratory Inc. (Wilmington, MA) and quarantined 1 week prior to being used. Mice were housed in groups of five per cage in a room

maintained at a constant temperature (22-24^{o}C) and humidity (40-60%) with a 12-h light/dark cycle before and during the experiment. Commercial rodent chow (Wayne Products, Chicago, IL) and water were available *ad libitum*.

Exposure Regimens

Toluene (chromatoquality grade, 99.7 % purity Burdick and Jackson Labs. Inc., Muskegon, MI) was dissolved in normal tap water in concentrations of 20, 100, and 500 mg/L. Toluene has a solubility of 535 mg/L in fresh water at 25^{o}C (U.S. Environmental Protection Agency, 1980b). Exposures were conducted by administering the toluene solutions to mice continuously for 28 days via drinking water; control animals received tap water without toluene. Drinking water was prepared and changed at least every 3 days. Food and water consumption were monitored continuously, and animals were weighed once a week. The water was sampled daily to confirm toluene concentrations by gas chromatography (U.S. Environmental Protection Agency, 1986b).

Gross Observations and Hematology

Body, spleen, thymus, liver and kidney weights of animals were determined immediately upon sacrifice at the end of 28 days continuous exposure. Gross pathological examinations were performed on all mice. Total erythrocytes and leukocytes of blood samples were counted with a Coulter counter. Differential leukocyte counts were evaluated by Wright's stained smears. The single splenocyte suspensions were prepared aseptically as described by Sharma and Gehring (1979). Unless stated otherwise, all splenocyte manipulations were performed in complete RPMI tissue culture medium containing RPMI 1640 medium

supplemented with 5% heat-inactivated fetal calf serum, 100 units/mL penicillin and 100 μg/mL streptomycin.

Mitogen-Stimulated Lymphocyte Proliferation

Splenocyte lymphoproliferative responses to mitogens were determined with optimal concentrations of the T-cell mitogens phytohemagglutinin (PHA, 6.0 μg/mL; Wellcome Reagents Ltd., Beckenham, England), and concanavalin A (Con A, 2.5 μg/mL; Sigma Chemical Co., St. Louis, MO), the B-cell mitogen *E. coli* lipopolysaccharide (LPS, 20 μg/mL; Sigma), and the T- and B-cell mitogen pokeweed mitogen (PWM, 30 μg/mL; Gibco). Cells were plated in 96-well sterile flat-bottom microtiter plates. Triplicate cultures containing $5x10^5$ viable (i.e., trypan blue-excluding) cells per well in 0.15 mL of medium with or without mitogen were prepared for splenic cells from individual treated or control mice. Cultures were incubated at 37°C for 48 hr in a humidified atmosphere with 4.0% CO_2 and harvested after a 6-hr pulse with tritiated thymidine (^{3}H-TdR, 0.5 μCi/well; New England Nuclear, Boston, MA) on glass fiber filters using an automatic cell harvester. Cell-incorporated radioactivity (dpm) was quantitated with a liquid scintillation counter (Model 2660, Packard Instrument Co., Downers Grove, IL), and compared against background incorporation with media alone.

Mixed Lymphocyte Culture (MLC) Responses

The responses of MLC were assessed by measurement of the lymphoproliferation of splenocytes (responders) from toluene-treated or control CD-1 mice after co-culture with mitomycin C-treated YAC-1 mouse lymphoma cells (stimulators), a mouse lymphoma cell line (H-2^a) of A/Sn

origin, at responder-stimulator ratios of 1:1, 2:1, and 4:1. The complete RPMI culture medium was supplemented with $5x10^{-5}$ M 2-mercaptoethanol (2-ME). After a 72-hr incubation, cells were pulsed with ^{3}H-TdR for 6 hr and harvested and counted for the cell-incorporated radiolabel as described for the mitogenesis assays above. Spontaneous ^{3}H-TdR incorporation was quantitated in cultures incubated without YAC-1 cells.

Antibody Plaque-Forming Cell (PFC) Responses

In a separate experiment, each mouse from control and exposed groups was sensitized by ip injection with 0.25 mL of a 20% washed SRBC suspension 4 days before the end of toluene exposure. Numbers of IgM plaque-forming cells (PFC) were quantitated by the methods of Cunningham and Szenberg (1968). Results are expressed as PFC/10^{6} splenocytes and as PFC/total splenic cells. The titer of anti-SRBC antibody in the serum collected from mice used in the PFC assay was analyzed by a double sandwich enzyme-linked immunosorbent assay (ELISA) as previously reported (Chapter III).

Interleukin-2 (IL-2) Assays

Interleukin-2 production was assayed by the methods described elsewhere (Gillis et al., 1978; Exon et al., 1983) with minor modifications. Briefly, aliquots of splenic lymphocytes (5×10^{5}) obtained from chemical-treated or control animals were cultured in RPMI media with 10% FCS, $5x10^{-5}$ M 2-ME, and antibiotics with or without Con A at the concentration of 2.5 ug/mL, to stimulate IL-2 production. At 48 hr, the culture supernatants were collected by removing the cells with centrifugation. Supernatants were assayed for IL-2 content by the

ability to enhance proliferation of an IL-2-dependent murine T-helper cell line (HT-2) of BALB/c origin, originally established by J. Watson (1979). The growth of HT-2 cells was maintained in the supernatants of Con A-stimulated rat splenic lymphocyte cultures. Serial two-fold dilution of IL-2-containing supernatant samples (100 µL) and $4X10^3$ HT-2 cells (50 µL) were placed into triplicate wells of a 96-well microtiter plate. Cultures were incubated at 37°C in a humidified atmosphere with 4.0% CO_2 for 24 hr and harvested after a 4-hr pulse with 0.4 µci of ^{3}H-TdR per well. The cells were harvested onto glass fiber filters and the amount of ^{3}H-TdR incorporation (dpm) was determined by scintillation counting. A supernatant of Con A-stimulated rat splenic lymphocyte culture was used as positive control. Non-Con A-stimulated supernatants from CD-1 mouse splenic lymphocyte cultures were tested as negative control to assess background induction of IL-2.

Statistical Analysis

All data were expressed as the means and their standard errors. Statistical evaluation was carried out by a one-way analysis of variance (ANOVA) and Fisher's least mean significant differences (LSD) test. A level of $p<0.05$ was considered statistically significant.

RESULTS

Concentrations of toluene in the drinking water mixtures as determined by gas chromatography decreased approximately 25-35% over a 3-day period, when water was changed. The actual concentrations (overall mean of the daily analysis) for the nominal concentrations of 0, 20, 100, and 500 mg/L were 0, 17 (± 16%), 80 (±12%), and 405 (±14%) mg/L, respectively. Based on toluene concentration in water during the

exposure and the estimated water intake, doses of toluene were 0, 5, 22, 105 mg/kg/body weight/day.

Animals exposed to toluene at the doses used did not produce mortality or exhibit overt clinical symptoms of toxicity over a 4-week period. Overall, no significant change in food and water consumption between treatment and control groups was observed during the study. No gross lesions were found at the time of sacrifice, on any of the organs of mice in all treatment groups.

The selected organ and body weight data for toluene-dosed mice are presented in Table VI-1. No differences were observed between group and those of the control group. Liver weights were significantly increased, while the weight of a thymus was decreased, at the highest dose of toluene (i.e., 105 mg/kg/day). No changes were found in spleen or kidney weights.

Table VI-2 shows the effects of toluene on spleen cellularity, erythrocyte and leukocyte counts and absolute differentials. Total splenocytes did not show a significant decrease in any treatment groups. No significant differences were observed in any of selected hematological parameters, although a nonsignificant decrease in total circulating leukocyte counts and lymphocyte and neutrophil populations was noted in mice receiving toluene at the 105 mg/kg/day dose.

The splenocyte lymphoproliferative response data for optimal stimulating concentrations of the T- and B-cell mitogen PWM, T-cell mitogens Con A and PHA, the B-cell mitogen LPS are shown in Fig. VI-1. Significant reductions in ^{3}H-TdR uptake were found for spleen cells cultured in the absence of mitogen or in the presence of PWM from the 5, 22, and 105 mg/kg/day dosage groups. Lymphocyte responsiveness to Con A

TABLE VI-1

ORGAN AND BODY WEIGHT OF MICE FOLLOWING 4 WEEKS OF TOLUENE EXPOSURE

Dose[a] (mg/kg/day)	Body weight (g)[b]		Organ weight (g/100 g body weight)[b]			
	Day 0	Day 28	Spleen	Liver	Kidney	Thymus
0	20.62 ± 0.13	34.86 ± 1.18	0.34 ± 0.03	5.67 ± 0.07	1.62 ± 0.05	0.19 ± 0.02
5	20.56 ± 0.02	34.26 ± 0.64	0.31 ± 0.01	6.09 ± 0.17	1.72 ± 0.06	0.18 ± 0.01
22	20.16 ± 0.31	33.18 ± 0.21	0.33 ± 0.01	6.32 ± 0.17	1.68 ± 0.03	0.18 ± 0.02
105	20.00 ± 0.23	35.12 ± 0.72	0.28 ± 0.01	6.73 ± 0.14*	1.68 ± 0.05	0.13 ± 0.02*

[a] Toluene was administered continuously via drinking water for 4 weeks

[b] Values are given as mean ± SE (n=5)

* Significantly different from control values at $p<0.05$

TABLE VI-2

EFFECTS OF FOUR WEEKS OF TOLUENE EXPOSURE ON SPLEEN CELLULARITY AND SELECTED BLOOD PARAMETERS

Dose[a] (mg/kg/day)	Total splenocyte[c] ($\times 10^{-7}$)	Erythrocyte[c] ($10^6/mm^3$)	Leukocyte[c] ($10^3/mm^3$)	Leukocyte absolute differentials[c]		
				Lymphocyte ($10^3/mm^3$)	Neutrophil ($10^3/mm^3$)	Other[b] ($10^3/mm^3$)
0	7.71 ± 0.27[c]	9.30 ± 0.26	5.96 ± 1.02	4.40 ± 0.62	1.13 ± 0.38	0.41 ± 0.08
5	6.18 ± 0.52	9.87 ± 0.49	6.34 ± 0.57	4.46 ± 0.38	1.46 ± 0.39	0.42 ± 0.09
22	6.57 ± 0.91	8.79 ± 0.85	6.43 ± 0.57	4.50 ± 0.78	1.42 ± 0.48	0.50 ± 0.07
105	6.58 ± 0.47	9.38 ± 0.38	3.90 ± 0.36	2.81 ± 0.82	0.82 ± 0.16	0.36 ± 0.05

[a] Toluene was administered continuously via drinking water for four weeks

[b] Including monocyte, eosinophil and basophil

[c] Values are given as mean ± SE (n=5)

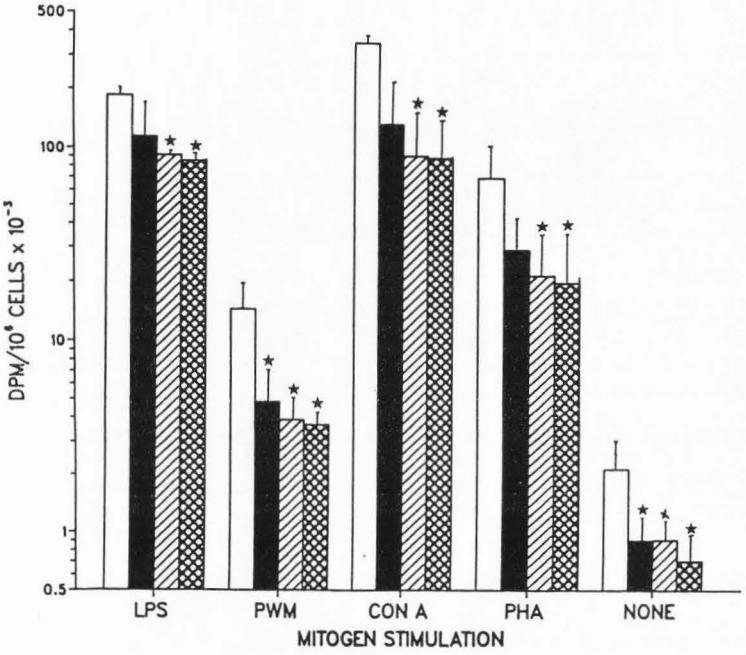

Fig. VI-1. Effect of toluene exposure on splenocyte lymphoproliferative responses to LPS, PWM, Con A, and PHA mitogens. Toluene was administered continuously to mice via drinking water for 4 weeks, control mice received tap water without toluene. The bars (left to right), ☐, ■, ▨, and ▩, indicate toluene at 0 (control), 5, 22, and 105 mg/kg/day concentrations, respectively. Stars (*) denote significantly different from control ($p<0.05$). Values represent mean ± SE of 5 animals per group.

and PHA was suppressed significantly for mice exposed to toluene at the two highest doses. Splenocytes from toluene-treated mice also exhibited a decreased response to LPS at the 105 mg/kg/day dose.

Figure VI-2 illustrates the effect of toluene exposure on the mixed lymphocyte cultures. The ability of splenic lymphocytes to proliferate in response to alloantigens (YAC-1 lymphocytic cells) was unchanged by exposure to toluene at the two low dosages, but a significant difference was seen in the highest dosed group.

The results of primary antibody response (T-cell dependent) to SRBC are presented in Table VI-3. A significant reduction in PFC/10^6 splenocytes or PFC/total spleen was seen in animals that received toluene at 105 mg/kg/day. However, changes in the total number of recovered splenocytes or α-SRBC antibody as determined by ELISA were not significant in any toluene-treated group.

Splenocytes taken from mice treated with 105 mg/kg/day toluene produced significantly less IL-2 after induction by Con A in culture (Table VI-4). The IL-2 activity was reduced to less than half in the highest treatment group, compared with controls. There was no change in IL-2 synthesis from spleen cells of mice exposed in the two low dosage groups.

DISCUSSION

Results of evaluations of the hematotoxic potential of toluene ingestion via drinking water for 4 weeks revealed that the chemical was nontoxic. There is general agreement that toluene does not have the hematotoxic properties of benzene (Fishbein, 1985; U.S. Environmental Protection Agency, 1980b). Wolf et al. (1956) conducted a long-term

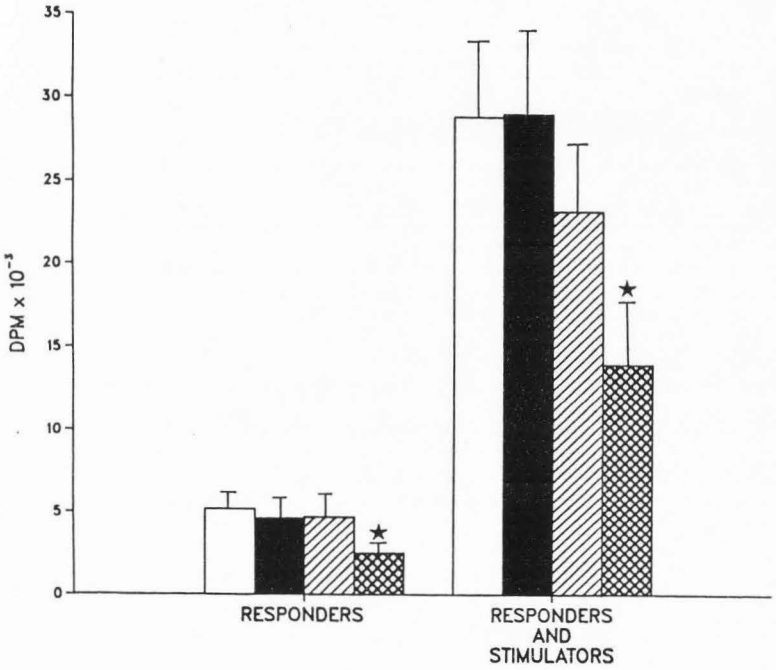

Fig. VI-2. Effect of toluene exposure via drinking water on mixed lymphocyte culture (MLC) responses. See legend of Fig. VI-1 for details. Responders indicate ^{3}H-thymidine incorporation in splenic cells alone, whereas responders and stimulates indicate the ^{3}H-thymidine incorporation when the splenocytes was cocultured in the presence of mitomycin C-treated YAC-1 cells. Several stimulator: responder ratios were used, but data are presented only from the 1:2 ratio, which gave optimal stimulation. Mean $\pm$ SE of 5 animals per group, each cultured in triplicate. Stars (*) denote significantly different from control ($p<0.05$).

TABLE VI-3

EFFECTS OF 4 WEEKS OF TOLUENE EXPOSURE ON ANTIBODY PLAQUE FORMING CELLS (PFC) AND ANTIBODY TITER (α-SRBC)[a]

Dose[b] (mg/kg/day)	Total splenocytes ($\times 10^{-7}$)	PFC/10^6 splenocytes	PFC/spleen	α-SRBC
0	20.16 ± 2.75	1,184 ± 90	238,545 ± 37,429	0.44 ± 0.04
5	16.68 ± 1.19	985 ± 105	166,015 ± 30,231	0.55 ± 0.06
22	21.78 ± 3.05	973 ± 116	217,891 ± 47,845	0.43 ± 0.05
105	13.66 ± 1.79	631 ± 27*	89,936 ± 13,269*	0.32 ± 0.03

[a] Mice were sensitized with thymic-dependent antigen sheep erythrocyte (SRBC) four days before the end of toluene exposure. Splenocytes were analyzed for antibody plaque forming cell and sera were detected for antibody titer (α-SRBC). Values are given as mean ± S.D. (n=5)

[b] Toluene was administered continuously via drinking water for four weeks

* Significantly different from control values at $p<0.05$

TABLE VI-4

EFFECTS OF TOLUENE EXPOSURE ON INTERLEUKIN-2 SYNTHESIS BY CON A-STIMULATED MOUSE T-LYMPHOCYTE[a]

Dose (mg/kg/day)	^{3}H-Thymidine uptake (DPM x 10^{-3})	Stimulation index[b]
0	12.95 ± 1.98	15.70 ± 2.39
5	11.13 ± 2.57	13.49 ± 3.11
22	10.21 ± 1.38	12.38 ± 1.68
105	5.93 ± 1.26*	7.18 ± 1.53*
Positive control[c]	14.58 ± 0.23	17.66 ± 0.27
Negative control[d]	1.34 ± 0.76	1.62 ± 0.68

[a] Several serial dilutions of IL-2 sources (supernatants of splenocyte culture) which showed a decreasing incorporation of ^{3}H-thymidine to IL-2 dependent murine T-helper cell clone, HT-2 cell, were used. Only data obtained from a 1:2 diluted ratio were presented. Values are given as mean ± SE (n=5)

[b] Stimulation index (S.I.) = dpm (culture with dilution of supernatant)/dpm (culture with media alone)

[c] Rat spleen conditioned medium (supernatant of Con A-stimulated rat splenocyte culture)

[d] Supernatant of non-Con A-stimulated splenocyte cultures of untreated mice

* Significantly different from the untreated control at $p<0.05$

toxicity study in which toluene was given orally to rats, and indicated no adverse effects on the cell counts of circulating blood and bone marrow. In a study of toluene-benzene interaction in mice, Andrews et al. (1977) noted that toluene had no effect on incorporation of ^{59}Fe into developing erythrocytes.

Consistent alterations in the immune parameters examined were generally found only in mice receiving the highest dose of toluene (105 mg/kg/day). The observed effects of suppressed humoral and cellular immunity, and decreased IL-2 synthesis at this dosage were accompanied by a decrease in thymus weight. Thymus involution is consistent with the impairment of T-cell function. The suppressed proliferation responses of lymphocytes from exposed mice to specific mitogen signal was found at the lower toluene doses, but proliferation of lymphocytes were not suppressed in response to allogeneic stimulation, and *in vivo* T-dependent antibody production response was not suppressed. Other studies have demonstrated only marginal correlation between lymphocyte response to mitogens and alterations in host resistance or any other immunologic function assay (Dean, et al., 1985b). The ability of lymphocytes to recognize and respond to foreign histocompatibility antigens (versus mitogens) represents a critical juncture in the generation of an appropriate immune response. After exposure to toluene vapor at concentrations as low as 2.5 ppm, mice exhibited increased susceptibility to respiratory infection with *Streptococcus zooepidemicus* and decreased pulmonary bactericidal activity to *Klebisella pneumoniae* (Aranyi et al., 1985). Several reports have appeared in the literature which link exposure of toluene to altered immunocompetence in animals and humans (U.S. National Toxicology Program, 1987; U.S. Environmental

Protection Agency, 1980b).

Treatment of mice with toluene at the highest dose level significantly reduced IL-2 synthesis. Inhibition of this basic immunoregulatory lymphokine may be a basic mechanism by which toluene mediates immunotoxic effects via oral ingestion. IL-2, produced by helper T-cell following stimulation with either T-cell mitogens or allogeneic cells, is known to augment numerous immune responses including proliferation and generation of cytotoxic T-cells and residual helper-T cells, and to enhance the antibody-forming cell responses (Farrar et al., 1982; 1986; Gillis et al., 1978).

Toluene caused immunotoxicity at the 105 mg/kg/day dose and may also be linked to the induction of corticosteroid release as a result of stimulating effects on the hypothalamic-pituitary-adrenocortical (HPA) axis. This exposure regimen has been demonstrated to activate mouse HPA status, resulting in increased concentrations of the hypothalamic norepinephrine and its major metabolite, vanillymandelic acid, which in turn leads to increased secretions of adrenocorticotropic hormone (ACTH) and corticosterone from pituitary gland and adrenal gland, respectively (Chapter VII). Corticosteroids, the end products of HPA axis, are known to impair various aspects of immunocompetence and to inhibit IL-2 as well as IL-1 synthesis (Goodwin et al., 1986; Cupps et al., 1985; Snyder and Unanue, 1982; Gillis et al., 1979a,b). Increased corticosteroid concentrations can control the clonal expansion (driven by IL-2) of the committed cells of the immune system (Besedovsky et al., 1985). The helper T-cell is believed to be the most susceptible lymphocyte subset (Esterling and Rabin, 1987; Verbruggen et al., 1987). Consequently, decrease of helper T-/suppressor T-cell ratio could result in

immunosuppression.

Benzene-induced toxicity has been attributed to various reactive intermediates (Irons, 1985). Similarly, it has been indicated that formation of cresol from aromatic oxidation of toluene occurs through an arene oxide intermediate which, in turn, is a reactive metabolite and is responsible for the binding of toluene to biomacromolecules (Pathiratne et al., 1986a; Bakke and Scheline, 1970). Superoxide anions are also formed during the formation of secondary metabolites, such as semiquinones and quinones, from the oxidation of cresol (Pathiratne et al., 1986a), suggesting that covalent binding of toluene to biological macromolecules involves more than one reactive metabolite. However, the covalent binding effects of toluene to biomacromolecules were considerably less than those of benzene, which could account for the differences in degree of immunotoxicity of toluene and benzene (Chapter III). Further studies are needed to determine the precise biological site of action of toluene. Toluene treatment was found to induce the activities of microsomal monoxygenenase, increasing the concentration of cytochrome p-450 in the liver and some non-hepatic tissues (Pathiratne et al., 1986b; Pyykko, 1980; 1983). Increase of liver weights in the highest dosed group of our study may support the validity of these findings.

For the protection of human health from the toxic properties of toluene ingested through water, the current U.S. Environmental Protection Agency (1986a) criterion level for toluene in water is 14.3 mg/L. This permissible concentration is extrapolated from a general toxicity evaluation of an oral dose study in rats (Wolf et al., 1956), which at the time was thought to be well below any toxicity-based

criterion. Although at the doses used in this study, toluene did not elicit any general toxicity and hematotoxicity, the effects of toluene on immune function should be examined in more detail and in different animal species to more accurately assess the potential hazard to human health of chronic low-level exposure to toluene in drinking water.

CHAPTER VII

CHANGES IN HYPOTHALAMIC-PITUITARY-ADRENOCORTICAL AXIS ACTIVITY AND IMMUNE FUNCTION DURING CONTINUOUS ORAL INGESTION OF BENZENE AND TOLUENE IN MICE

INTRODUCTION

The hypothalamic-pituitary adrenocortical (HPA) axis is a highly sensitive index of enviromental changes and may be augmented by a broad range of stimuli such as chemical pollultants, infections, and many other various "stressors". It is critically involved in the regulation of behavioral and physiological adaptations by releasing biogenic amines from the hypothalamus, adrenocorticotropic hormone (ACTH) from anterior pituitary, and corticosteroids from adrenal cortex (Axelrod and Reisine, 1984; Dunn and Kramarcy, 1984). Changes in HPA activity are known to result in marked modulation of immune functions (Lumpkin, 1987; Besedovsky et al., 1985; Ader, 1981).

Many organic solvents have been associated with a number of neurophysiological and psychological disturbances (Baker et al., 1985; Grasso et al., 1984; Savolainen, 1977). Benzene and toluene, universal solvents are considered as important health hazards (Fishbein, 1984; 1985). Previous studies have indicated that both chemicals have a pronounced effect on metabolisms of brain neurotransmitters in rats via inhalation exposure or the injection route (Arito et al., 1985; Paradowski et al., 1985; Rea et al., 1984; Andersson et al., 1980; 1983). Recently we found that prolonged oral ingestion of either benzene or toluene significantly altered both biosyntheses and catabolisms of regional brain biogenic amines in mice (Chapter IV, V).

It was therefore desirable to ascertain whether exposure to these chemicals affected the secretion of pituitary-adrenocortical (PA) hormones. The specific roles of the brain monoaminergic neural system in mediating PA hormone releases have received much attention over the years but still remain ill-defined (Axelrod and Reisine, 1984; Dunn and Kramarcy, 1984; Ganong, 1980).

The purpose of this study was to investigate the changes in hypothalamic catecholamine metabolisms and correlate them with plasma ACTH and serum corticosterone levels in CD-1 mice during continuous oral administration of benzene or toluene via drinking water. Since it has been recognized, corticosteroids, the end products of HPA axis, have powerful effects on immune function (Besedovsky et al., 1985; Cupps et al., 1985), we also examined if benzene or toluene altered the synthetic abilities of lymphocyte-derived interleukin-2 (IL-2) to evaluate the hypothesis that chemicals may have an additional adverse effect on immune system via HPA status. IL-2 is a soluble glycoprotein that causes lymphocyte proliferation when it binds to cells via a specific membrane receptor, various immunoenhancing activities have been ascribed to this cytokine (Farrar et al., 1982; 1986). Corticosteroids has been reported to inhibit IL-2 production (Goodwin et al., 1986; Gillis et al., 1979a,b).

MATERIALS AND METHODS

Animals

Adult male CD-1 mice were obtained from Charles River Breeding Laboratory Inc. (Wilmington, MA) and quarantined upon arrival for one week prior to use. Animals were housed in groups of 5 per cage in a

room at a constant temperature (22-24^{o}C) and with a 12 hour light cycle before and during the experiment. Commercial rodent chow (Wayne Products, Chicago, IL) and water were available *ad libitum*.

Treatments

Analytical reagent grade benzene (99.9% purity, J.T. Baker Chemical Co., Phillipsburg, NJ) and a chromatoquality grade of toluene(99.7% purity, Burdick and Jackson Labs. Inc., Muskegon, MI) were dissolved in normal tap water to make the desired concentrations of 40, 200 and 1000 mg/L for benzene and 20, 100 and 500 mg/L for toluene, respectively. The solubility of benzene in fresh water at 25^{o}C is 1780 mg/L (U.S. Environmental Protection Agency, 1980a), while toluene has a solubility 535 mg/L (U.S. Environmental Protection Agency, 1980b). Exposures were conducted by free-choice administration of chemical-treated solutions to mice for 28 days via drinking water; control animals received tap water. Food and water consumption was monitored continuously, and animals were weighed once each week. Actual benzene and toluene concentrations in the drinking water of mice were confirmed by gas chromatography (U.S. Environmental Protection Agency, 1986b).

Twenty-eight days after exposure to benzene or toluene, mice were killed by decapitation. The brains were removed quickly, and the hypothalamic regions were dissected out by the procedures of Glowinski and Iversen (1966). The brain tissue was promptly weighed in a tared vial containing 0.3 mL of ice-cold 0.05 M perchloric acid with 0.1% cysteine and frozen at -80^{o}C until analyzed. Trunk blood was collected into chilled EDTA tubes, and plasma samples for ACTH assay were separated by centrifugation (within one hour) at 1500 x g for 20 min at 4^{o}C. Serum samples for measuring corticosterone levels were collected

once before and at 2, 7, 14, and 28 days after exposure via venepuncture. Spleens were removed aseptically and the single-splenocyte suspensions were prepared for interleukin-2 assay (described later).

Neurochemical Assays

Concentrations of hypothalamic NE and its metabolite, VMA, were assayed by high performance liquid chromatography (HPLC) with electrochemical detection using the methods described elsewhere (Mayer and Shoup, 1983; Sharma et al., 1986). The homogenized brain tissues were centrifuged (10,000 x g, 30 min) and the supernatants were filtered through regenerated cellulose filter of 0.2 μm pore size (Bio-Analytical System, Inc., West Lafayette, IN) prior to chromatography. The amounts of NE and VMA were determined by measuring the height of the individual peaks and comparing them with the peak of various standards (Sigma Chemical Co., St. Louis, MO). The detector response provided a linear correlation coefficient of >0.98 for the standard curve. The HPLC system consisted of a model LC-150 electrochemical analyzer, Model LC-3A amphoteric detector, Model LC-22A temperature controller, and a Biophase ODS-reversed phase column (Bio-Analytic System, Inc.). A 0.15 M monochloroacetate buffer (pH 3.0) containing sodium octyl sulfate as ion-pair reagent served as mobile phase (Mayer and Shoup, 1983). The instrument settings were: flow rate 1.4 mL/min, column temperature 30^{o}C, detector potential +0.7V, and controller 10nA full scale.

Radioimmunoassays

Materials from commercial radioimmunoassay kits were used for determination of plasma ACTH and serum corticosterone concentrations

(Radioassay Systems Laboratories, Inc., Carson, CA). The kit offers a highly sensitive method of measuring ACTH and corticosterone by monitoring the competitive binding of radioisotope-labeled hormones (^{125}I-ACTH and ^{3}H-corticosterone) and non-labeled hormones (standards) with their corresponding specific antibodies. Separation of antibody-bound labeled antigen (hormone) from the unbound labeled antigen was achieved by precipitation. The radioactivities of precipitates were than counted and amounts of ACTH (pg/mL) and corticosterone (ng/mL) were measured from the respective standard curves.

Interleukin-2 (IL-2) Assays

Splenic lymphocytes for IL-2 assays were prepared from chemical-treated or control animals according to the procedures of Sharma and Gehring (1979) and suspended in RPMI1640 media (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Hylone Sterile Systems, Inc, Logan, UT), 5 X 10^{-5} M 2-mercaptoethanol, and 100 units penicillin and 100 µg streptomycin per mL. Interleukin-2 production was assayed by the methods described elsewhere (Gillis et al., 1978; Exon et al., 1983) with our minor modifications as described in the methods section of Chapter VI.

Statistical Analyses

All data are expressed as means±SE. Statistical determinations of the neurochemical, ACTH, and IL-2 data were performed by one-way analysis of variance (ANOVA) and follow-up Fisher's least significant differences (LSD) test were used to analyze the data for differences between chemical-treated and control animals. A fixed-effect, two way ANOVA was used for analyzing corticosterone data to evaluate the effects

of benzene or toluene concentrations and exposure time. Differences were considered significant at $p<0.05$.

RESULTS

The average concentrations of benzene or toluene in the drinking water mixtures as determined by gas chromatography are indicated in Table VII-1. There was an approximate 30% loss of both solvents over a 3-day period, when water was changed. Based on daily analysis the overall means of observed concentrations were 78 (±12)%, 83 (±9)%, and 79 (±6)% of the 40, 200, and 1000 mg/L benzene nominal concentrations, respectively. Likewise, actual toluene concentrations in the drinking water were 83 (±14)%, 80 (±10)%, and 81 (±11)% of the 20, 100, and 500 mg/L intended levels. The observed concentration values are used to designate low, medium and high group dosing.

General Toxicity Evaluation

At the doses used both benzene and toluene did not elicit mortality or any other overt clinical symptoms of toxicity in mice. Comparison of the body weight gains of each treated group with those of the control groups did not suggest any consistent differences through the treatment period. There were also no significant changes between treatment and control groups in food and water consumptions.

Neurochemical

The effects of benzene or toluene exposure on the concentrations of hypothalamic NE and its major metabolite, VMA, are presented in Table VII-1. Both solvents produced marked increases in hypothalamic NE concentrations. There was a dose-related response for increased NE and

TABLE VII-1

CONCENTRATIONS OF NOREPINEPHRINE (NE) AND ITS METABOLITE VANILLYMANDELIC ACID (VMA) IN THE HYPOTHALAMUS OF MICE EXPOSED TO BENZENE OR TOLUENE[a]

Concentration in water, mg/L			Neurochemical concentration[b]	
Chemical	Nominal	Observed	NE	VMA
	0	0	1.632 ± 0.422	0.260 ± 0.016
Benzene	40	31	2.284 ± 0.247	0.304 ± 0.037
	200	166	2.571 ± 0.281*	0.367 ± 0.054
	1000	790	2.637 ± 0.123*	0.395 ± 0.033
	0	0	1.307 ± 0.067	0.238 ± 0.018
Toluene	20	17	1.981 ± 0.034*	0.422 ± 0.015
	100	80	2.125 ± 0.167*	0.393 ± 0.046
	500	405	1.762 ± 0.136*	0.596 ± 0.210

[a] Chemical was administered continuously to mice via drinking water for 4 weeks

[b] In μg neurochemical/g wet tissue, Mean ± SE (n=5)

* Significantly different from untreated control at $p<0.05$

VMA with increasing levels of benzene. Significant benzene-induced increases of NE were found in the 166 and 790 mg/L dose groups. The mean concentrations of VMA were correspondingly increased by 42 and 52% in these two treatment groups, although the findings were not statistically significant. Toluene produced significant elevations of NE at all dose levels but increases were maximal at the medium dose (80 mg/L). The three toluene doses also caused apparent accumulations of VMA with 66-150% increases.

Plasma ACTH

Figure VII-1 shows the effects of benzene or toluene exposure on plasma concentrations of ACTH. The results suggest that increase in ACTH secretion was correlated with activation in hypothalamic NE metabolisms. Mice exposed to benzene rose ACTH by 1.6, 1.8, and 2.5-fold in the 31, 166, and 790 mg/L dose groups, respectively. Toluene also caused dose-related elevation of this hormone by 2.1, 2.6, and 3.8-fold at the three dose levels. Significant differences from untreated control values were found in the high dose groups of both chemical treatments.

Serum Corticosterone

The corticosterone response to benzene or toluene was assessed once before and at 2, 7, 14, and 28 days after exposure. Comparisons of solvent-exposure mice with same-time controls revealed the corticosterone levels were significantly higher in mice exposed to the 790 mg/L dose at 7 and 28 days and the 166 mg/L dose at the 7th day (Fig. VII-2). The marked increases (1.8 fold) of corticosterone at day 7 in the 31 mg/L dose exposure were not significant due to the large

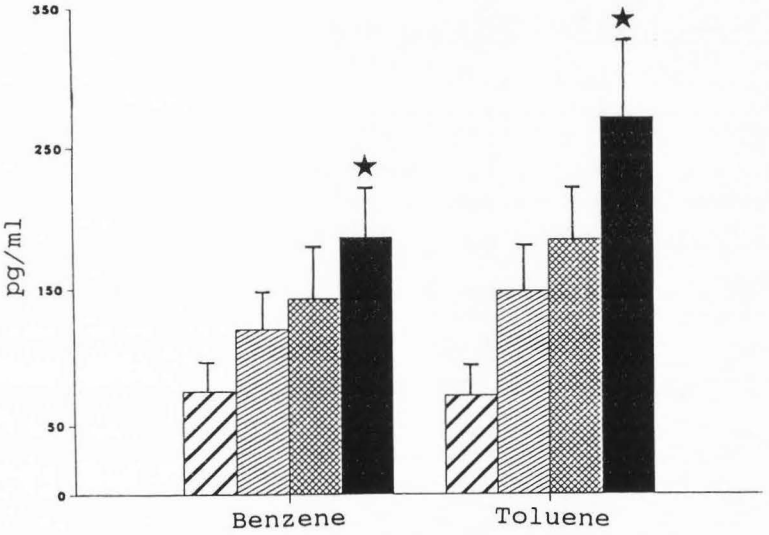

Fig. VII-1. Effect of 4 — weeks benzene and toluene exposures via drinking water on concentrations of plasma ACTH in mice (▨ , control; ▨ , 31 mg/L for benzene and 17 mg/L for toluene; ▩ , 166 mg/L for benzene and 80 mg/L for toluene; ■ , 790 mg/L for benzene and 405 mg/L for toluene). Stars (*) denote a significant effect ($p<0.05$) relative to control. Values represent mean $\pm$ SE (n=5).

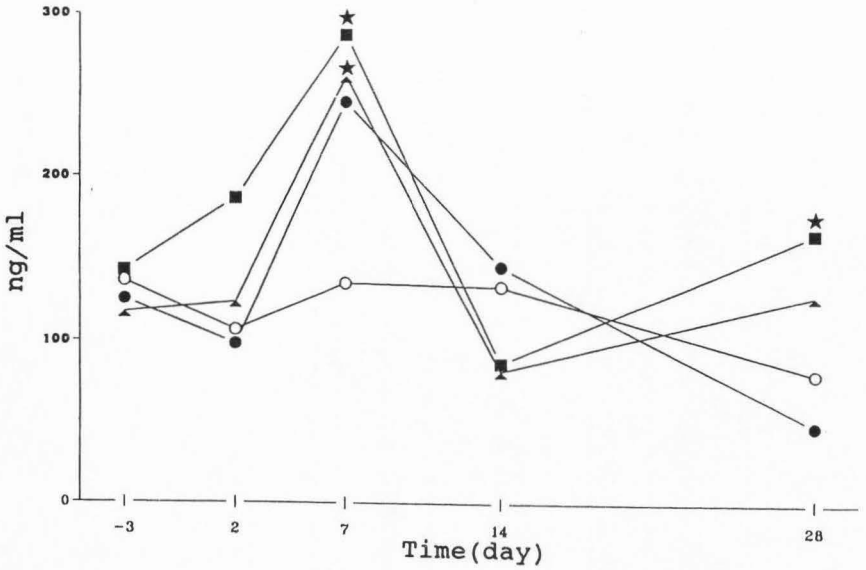

Fig. VII-2. Serum corticosterone concentrations in control and benzene-exposed mice (○ , control; ● , 31 mg/L; ▲ , 166 mg/L; ■ , 790 mg/L). Values represent mean ± SE (n=5). Stars (*) denote values significantly different from same-time control values at $p<0.05$.

variation. Toluene elevated corticosterone levels at 14 and 28 days at the highest dose (405 mg/L) only (Fig. VII-3). Two-way analysis of variance showed a significant interactive effect of benzene or toluene on plasma corticosterone over the exposure time (Table VII-2). During a period of 28-day benzene exposure, corticosterone increased and reached maximal response at the 7th day, then declined to or below control level at the 14th day. However, re-elevated corticosterone concentrations were detected in the 790 mg/L dose group at the termination of exposure. The elevated corticosterone caused by the 405 mg/L dose toluene did not return to normal levels during the 28-day exposure.

IL-2 Synthesis

The effects of benzene or toluene ingestion on IL-2 synthesis are illustrated in Table VII-3. Splenocytes obtained from mice exposed to benzene at various concentrations for four weeks and stimulated with Con A *in vitro* elicited a biphasic dose-related response in IL-2 productions as determined by the proliferation of HT-2 cells. Mice receiving 166 or 790 mg/L benzene manifested a significant suppression of ^{3}H-TdR incorporation into DNA of HT-2 cells. Splenic cells taken from mice treated with toluene produced a lesser change in IL-2 synthesis; a significant depression was detected in the highest toluene-dosed group (405 mg/L) only.

DISCUSSION

Oral administration of various dosages of benzene or toluene via drinking water to CD-1 mice for four weeks markedly stimulated the HPA activity. Hypothalamic NE activity was highly elevated by both organic solvents, indicated by the increased concentrations of NE, and a

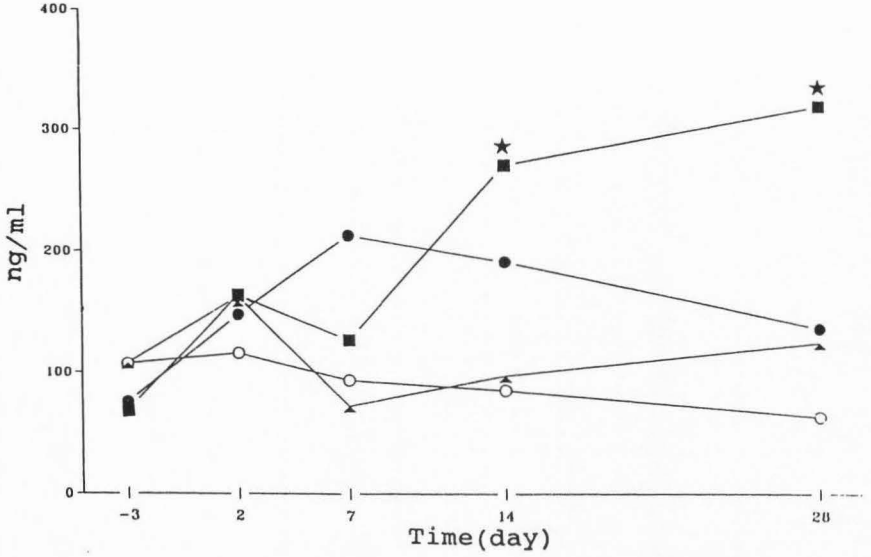

Fig. VII-3. Serum corticosterone concentrations in control and toluene-exposed mice (○ , control; ● , 17 mg/L; ▲ , 80 mg/L; ■ , 405 mg/L). Values represent mean ± SE (n=5). Stars (*) denote values significantly different from same time control values at $p<0.05$.

TABLE VII-2

RESULTS OF A FIXED-EFFECT, TWO-WAY ANALYSIS OF VARIANCE FOR SERUM CORTICOSTERONE DATA

Chemical	Source of variance	DF	MS	F ratio
Benzene	Main effects	7	35637.83	7.83*
	dose	3	13957.62	3.09*
	time	4	51897.99	11.40*
	Interaction	12	8643.25	1.90*
	Residual	80	4551.74	
	Total	99	7245.69	
Toluene	Main effects	7	27177.28	3.78*
	dose	3	43529.58	6.06*
	time	4	14913.07	2.08
	Interaction	12	19386.72	2.70*
	Residual	80	7179.18	
	Total	99	10072.89	

DF: degrees of freedom; MS: mean squares (variability)

* $p<0.05$

TABLE VII-3

EFFECTS OF BENZENE AND TOLUENE ON IL-2 PRODUCTION BY CON A-STIMULATED MOUSE T-LYMPHOCYTE[a]

Chemical	Solvent Concentration (mg/L)	^{3}H-Thymidine uptake (DPM x 10^{-3})
	0	16.63 ± 0.62
Benzene	31	20.78 ± 1.36
	166	9.40 ± 1.25*
	790	4.92 ± 0.79*
	0	17.07 ± 1.38
Toluene	17	14.07 ± 1.73
	80	13.75 ± 0.86
	405	10.30 ± 1.32*
	Positive control[b]	18.92 ± 0.59
	Negative control[c]	1.80 ± 0.47

[a] Several dilutions including 1:1, 1:2, 1:4, and 1:8 of IL-2 sources (Con A-stimulated supernatants) which showed a decreasing incorporation of ^{3}H-thymidine to IL-2 dependent murine T-helper cell clone, HT-2 cell, were used. Only data obtained from a 1:1 diluted ratio were presented. Values are given as mean ± SE (n=5)

[b] Rat spleen conditioned medium (Con A-stimulated supernatant)

[c] Non-Con A-stimulated CD-1 mouse lymphocyte cultural supernatant

* Significantly different from untreated control at $p<0.05$

parallel trend in its major metabolite, VMA, in this brain region. At the same time these were accompanied by increased ACTH/corticosterone releases into blood. Increases in NE concentration, metabolism or turnover have been reported in the hypothalamus of rats exposed to toluene (Arito et al., 1985; Andersson et al., 1980; 1983) or in whole-brain homogenates of rats exposed to benzene (Paradowski et al., 1985). Significant elevations of serum corticosterone were also found in rats following toluene exposure via inhalation (Andersson et al., 1980).

The present study provides evidence that organic solvent-induced ACTH/corticosterone releases from PA axis is stimulated by the hypothalamic noradrenergic neuronal pathway. The specific role of NE in the release of PA hormones has remained controversial, but several reports involving direct measurement of NE turnover or metabolism suggest a stimulatory effect of NE neuronal activity on ACTH/corticosterone productions (Smythe et al., 1983; 1987; Johnston et al., 1985; Hary et al., 1984; Hedge et al., 1976). These workers also reported a highly significant correlation between NE neuronal activity and plasma ACTH/corticosterone levels after chemical exposures. It was assumed, therefore, that AP hormone effects were mediated via hypothalamic noradrenergic facilitation of corticotropin releasing factor (CRF), which then acts on the pituitary gland to release ACTH (Smythe et al., 1987; Vale et al., 1981). Lesions of the hypothalamus were shown to block both changes in CRF content in median eminence and plasma ACTH after a variety of stimuli (Dallman et al., 1987). Corticotrophin releasing factor was thought to be the major but not the sole means of releasing ACTH/corticosterone from PA axis (Axelrod and Reisine, 1984). It has been previously shown that AP hormone responses

to chemical-induced stress were inhibited significantly by clonidine (α_2-adrenoreceptor agonist) and increased by yohimbine (α_2-adrenoreceptor blocker) (Smythe et al., 1983; 1987), both chemicals have greater presynaptic than postsynaptic activity. The α_1 and β_2-adrenergic agonists have also been reported to stimulate ACTH releases *in vivo* by acting on the hypothalamic-pituital axis (Axelrod and Reisine, 1984). The blockade of postsynaptic adrenergic receptors was able to significantly blunt the ACTH release induced by chemical stress (Hary et al., 1984). In reviewing the roles of monoamine neurotransmitters in pituitary ACTH release Ganong (1980) interpreted, however, that NE concentrations were inhibitory to ACTH secretion. It is noteworthy that his postulation was mainly based on either neuropharmacological manipulation of brain NE levels or the uses of NE agonist or antagonist drugs. The measurement of steady-state transmitter concentrations alone is of limited value regarding the NE neuronal activity and the relationship between NE and ACTH secretion (Commissiong, 1985; Hedge et al., 1976).

Corticosterone secreted by adrenal cortex is dependent on stimulation of the hypothalamic-pituitary axis. The possibility that the increased NE activity is due to a positive feedback of corticosterone has been excluded by the demonstrations that 1) exogenously administered corticosterone to unstressed rats had no effect on hypothalamic NE status nor on ACTH release, and 2) corticosterone-deficient (adrenalectomized) rats exhibited enhanced hypothalamic noradrenergic neuronal activity and ACTH release (Smythe et al., 1983). The latter finding suggests that decreased corticosterone may exert a negative feedback on central NE neuronal activity which, in turn,

increase ACTH release. The negative feedback system on the HPA status, however, can be overridden by a second control system with a higher priority. When the animal or man is subjected to a variety of environmental challenges, which are collectively referred to as "stress" (e.g. chemical intoxication, infection, psychologic stress, and many other environmental stressors), noradrenergic neuronal activity and ACTH secretion is vigorously stimulated in spite of the fact that circulating corticosterones are much higher than those required to inhibit ACTH production in the "unstressed" condition (Ader and Cohen, 1985).

Treatment of mice with benzene significantly reduced IL-2 production at the two higher doses, while toluene caused a significant inhibition of IL-2 synthesis at the highest dose level. Benzene has been associated with hematotoxicity and associated immunotoxic effects (Fishbein, 1984; Chapter III). Exposure of mouse T-lymphocytes to *p*-benzoquinone, one of the major metabolites of benzene, was reported to inhibit the formation of IL-2 (Post et al., 1985). Although there is general agreement that toluene does not elicit the hematotoxic properties of benzene, adverse effects on lymphocyte and macrophage functions, and host resistance to infectious microorganisms have been noted (Suleiman, 1987; Fishbein, 1985; Aranyi et al., 1985). We recently found that a 4-week toluene ingestion resulted in suppression of T-lymphocyte-related immune functions at the concentration of 405 mg/L (Chapter VI). IL-2 has been shown to augment numerous immune responses (Farrar et al., 1982). Inhibition of this immunoregulatory lymphokine synthesis via HPA axis stimulation may be a possible mechanism by which toluene mediated its immunotoxic effects via oral ingestion. Additional studies need to be performed to determine the

precise biological site of action of this chemical. The reactive metabolite(s)-mediated covalent binding of toluene to biological macromolecules has been reported (Pathiratne et al., 1986b).

The time-course of the corticosterone response of mice during benzene exposure revealed that serum corticosterone was elevated during the initial period of exposure but returned to control, or lower, values despite the continued exposure to the solvent. The cycling effect of benzene on corticosterone with time may be brought about by a feedback inhibition of ACTH release, despite the presence of a stressor. From the perspective of the general adaptation syndrome (GAS) response to a variety of stressors including many pollutants (Selye, 1950), benzene may activate the HPA axis, resulting in the process of adaptations to an altered environment following a period of alarm. However, re-elevated corticosterone levels detected in the high dose group at 28 day suggest that mice were unable to completely maintain adaptation at this dose level of benzene. Likewise, mice generally adapted to toluene exposure at the low and medium doses. Corticosterone remained elevated throughout the duration of the high dose toluene exposure and suggested that adaptation did not occur. If adaptation of GAS does not occur, the organism may progress into producing deleterious effects including immunological dysfunction (Makara et al., 1980).

It is obvious from this study that organic solvents such as benzene and toluene have, at least partially, an additive adverse effect on immune function via activated HPA status. Corticosteroids, the end products of HPA axis, are well known to impair various aspects of immunocompetence (Besedovsky et al., 1985; Cupps et al., 1985) and have been shown to inhibit IL-2 as well as IL-1 productions (Snyder and

Unanue, 1982; Gillis et al., 1979a,b). Exogenous IL-2 also reverses the inhibition of mitogen-induced T cell proliferation by corticosteroids (Gillis et al., 1979a,b). Increased corticosteroid concentration can control the clonal expansion (deriven by IL-1 and IL-2) of committed cells of the immune system that have high affinities for antigen (Besedovsky et al., 1985). Recently, Goodwin et al., (1986) found leukotriene B_4, the 5-lipoxygenase product in arachidonic acid metabolism, reversed the corticosteroid-induced suppression of T cell proliferation and IL-2 secretion, and concluded that corticosteroids, by inhibiting leukotriene B_4 production, also inhibited IL-2 synthesis, resulting in a decreased T-cell proliferation. Lymphocyte from ACTH-treated calves which have increased plasma cortisol concentrations have an impaired capacity to produce IL-2 (Blecha and Baker, 1986).

CHAPTER VIII

EFFECTS OF REPEATED ORAL EXPOSURE TO COMBINATIONS OF BENZENE AND TOLUENE ON IMMUNOLOGIC RESPONSES IN MICE

INTRODUCTION

Toxicity of enviromental pollutants may not necessarily involve exposure to a single chemical exposure, but may reflect the combined effects of several chemicals (Lewtas, 1985). Exposures to mixtures of chemically related substances are frequently encountered. In such cases the balance between different pathways of metabolism for chemicals could be radically changed (Sipes and Gandolfi, 1986). Alteration in metabolism of a second compound may be beneficial or detrimental, depending upon the toxicity of the parent compound versus its metabolite(s).

Exposure to benzene has been associated with hematotoxicity and immunotoxicity (Brief et al., 1980; Goldstein, 1983; Chapter III). Benzene is a carcinogen and mutagen (Aksoy, 1985; Dean, 1985; Ereson et al., 1985). It has been associated with an increased incidences of leukemia in humans (Rinsky et al., 1987), and several solid tumors (Maltoni et al., 1985) and possibly leukemia/lymphoma in rodents (Snyder et al., 1980; Cronkite et al., 1984). The embryotoxic/teratogenic potential of this chemical in experimental animals has been noted (Green et al., 1978; Kuna and Kupp, 1981; Ungvary and Tatrai, 1985). Although the mechanism of these toxicities is not well elucidated, studies have shown that benzene itself is not the primary toxicant but is converted by liver microsomal enzymes or bone marrow peroxidases to a reactively oxidative metabolite(s) to exert its toxic effects (Irons, 1985;

Sawahata et al., 1985; Eastmond et al., 1987b).

Benzene is frequently employed in industry with toluene. Pretreatment of rats with toluene has been reported to increase the *in vitro* liver microsomal metabolism of benzene (Pathiratne et al., 1986b). However, *in vivo* coadministration of benzene with toluene, which competitively inhibited benzene metabolism (Ikeda et al., 1972; Andrews et al., 1977; Sato and Nakajima, 1979), prevented benzene toxicity such as myelotoxic (Andrews et al., 1977; Tunek et al., 1981; 1982) and genotoxic (Tice et al., 1982; Tunek et al., 1982; Gad-El-Karim et al., 1984; 1985a; 1986) effects.

We have recently reported that oral ingestion of benzene or toluene possesses immunotoxic potentials in mice (Chapter III and VI), and that alteration of immune function in toluene-exposed animals was generally evident at a relatively high dose. To our knowledge, no studies have been published on evaluation of interaction-mediated immunotoxicity after exposure to combinations of benzene and toluene. The present study was therefore undertaken to assess the effects of repeated exposure to various concentrations of benzene and toluene, alone or combined, on hematological and immunological responses in CD-1 mice. Since both these chemicals are known groundwater contaminants (U.S. Office of Technology Assessment, 1984; U.S. Environmental Protection Agency, 1986a), oral administration via drinking water was employed.

MATERIALS AND METHODS

Animals

Male CD-1 mice were obtained from Charles River Breeding Laboratories (Wilmington, MA). Animals were acclimated in an AAALAC-

accredited animal care facility with an ambient temperature of 22-24°C, 40-60% relative humidity, and a 12-hr light/dark cycle for one week prior to treatment. Commercial rodent chow (Wayne Products, Chicago, IL) and tap water were available *ad libitum*. Animals were randomly distributed into control and treatment groups of 5 mice each.

Chemical Treatments

Analytical reagent grade benzene (99.9% purity, J. T. Baker Chemical Co., Phillipsburg, NJ) and a chromatoquality grade of toluene (99.7% purity, Burdick and Jackson Labs. Inc., Muskegon, MI) were dissolved in normal tap water to make the desirable concentrations. Two experiments were conducted. In the first experiment, the nominal concentrations employed were 200 mg/L benzene, 400 mg/L toluene, and 200 mg benzene + 400 mg toluene/L for the mixture. The concentration of toluene was reduced to 100 mg/L in the second experiment. The solubility of benzene in fresh water at 25°C is 1,780 mg/L (U.S. Environmental Protection Agency, 1980a), while toluene has a solubility of 535 mg/L (U.S. Environmental Protection Agency, 1980b). Treatments were achieved by continuously administering the test material(s) in drinking water to mice at the intended concentrations for 28 days. Control animals received normal tap water without test materials. The water was provided in glass water bottles with stainless-steel sipper tubes equipped with ball bearings. The treated solutions were changed every three days to ensure freshness, and bottles were shaken frequently during the experiment. Fluid and food intake was monitored continuously, and animals were weighed weekly. Actual benzene or toluene concentrations were determined daily by gas chromatography (U.S. Environmental Protection Agency. 1986b).

Gross Observations and Hematology

At the end of 28 days of continuous chemical exposures, mice were killed by decapitation, and gross pathological examinations were performed. Selected organs, i.e., liver, spleen, thymus, and kidney were removed, trimmed and weighed immediately. Total blood erythrocyte and leukocyte counts were determined on a Coulter Model ZB-I blood cell counter (Coulter, Electronics, Inc., Hialeah, IL). Differential counts were determined manually by Wright's stained blood smears. Single splenocyte suspensions (> 95% viability by trypan blue exclusion) were prepared aseptically as described by Sharma and Gehring (1979). Cell concentration was measured for assay of total spleen cellularity. The splenocyte number was adjusted to the desired concentration for each immune function assay. Unless otherwise indicated, all splenocyte manipulations were performed in RPMI 1640 tissue culture medium that contained 5% heat-inactivated fetal calf serum (FCS), 100 units/mL penicillin and 100 μg/mL streptomycin (referred to as a complete medium).

Mitogen-Stimulated Lymphoproliferation

The proliferative responses of splenocytes to mitogens were determined by using a microculture assay. Splenocytes from individual animals were added in 0.1 mL ($5x10^5$ cell) to triplicate wells of a 96-well flat-bottomed microtiter plate. The cells were incubated for 48 hr at 37^oC in a humidified 4.0% CO_2 and air mixture with or without optimal concentrations of the purified T-cell mitogens phytohemagglutinin (PHA, 12.5 μg/mL; Wellcome Reagents LTD., Beckenham, England) or concanavalin A (Con A, 2.5 μg/mL; Sigma Chemical Co., St. Louis, MO), B-cell mitogen *E. coli* lipopolysaccharide (LPS, 20

μg/mL; Sigma), and the T- and B-cell mitogen pokeweed mitogen (PWM, 30 μg/mL) and were harvested after a 6-hr pulse with tritiated thymidine (^{3}H-TdR, 0.5 μCi/well, New England Nuclear, Boston, MA) onto fiber glass strips with a multiple sample harvester (Model M12, Biomedical Research and Development Laboratory, Rockville, MD). Amounts of ^{3}H-TdR incorporation into DNA of dividing lymphocytes was quantitated with a liquid scintillation counter (Model 2660, Packard Instrument Co., Downers Grove, IL).

Mixed Lymphocyte Culture (MLC) Reaction

MLC responses were performed under similar conditions as mitogen assays by using ratios of 2:1 and 4:1 responders (splenocytes) to stimulators (mitomycin C-blocked YAC-1 lymphoma cells of A/Sn origin, H-2^a). The complete RPMI culture medium was supplemented with $5x10^{-5}$ M 2-mercaptoethanol (2-ME). After 72 hr in 37°C/4% CO_2 humidified incubation, the cells were pulsed with ^{3}H-TdR for 6 hr and harvested for counting the cell-incorporated radioactivity as described above. Spontaneous ^{3}H-TdR incorporation was determined in cultures incubated without YAC-1 cells.

Cell-Mediated Cytolysis (CMC) Response

Cell-mediated cytotoxicity of splenic cytotoxic T-lymphocytes (CTL) was generated *in vitro* against YAC-1 tumor cells as described by Grabstein (1980) with minor modifications (Chapter III). The splenocytes (responders) were co-cultured with mitomycin C-blocked YAC-1 cells (stimulators) in RPMI complete medium supplemented with $5x10^{-5}$ M 2-ME for 5 days. These cultured splenic cells (effectors) were washed, counted, and incubated with YAC-1 cells (targets) previously

radiolabeled with ^{51}Cr-sodium chromate (New England Nuclear) at effector: target (E:T) ratios of 100:1, 50:1; 25:1, 12:1, and 6:1. A constant number of ^{51}Cr-labeled target cells (2×10^4) were added to each well of a plastic 96-well microtiter plate followed by the addition of effector cells. After 4 hr incubation at 37°C, cells were pelleted by centrifugation, and radioactivity in the supernatant of cultures was measured with a gamma counter (Model 3320, Packard). The ^{51}Cr labeled target cells in the presence of culture medium or 0.5% sterilized saponin alone served as the spontaneous release and total release of radioactivity, respectively. Percentage of specific cytotoxicity was calculated as described earlier (Grabstein, 1980).

Interleukin-2 (IL-2) Assay

Supernatants containing IL-2 were prepared by incubating splenocytes (5×10^5) obtained from chemical-treated or control mice with or without Con A at the concentration of 2.5 μg/mL in RPMI 1640 medium supplemented with 10% FCS, 5×10^{-5} M 2-ME, and antibiotics for 48 hr at 37°C/4% CO_2. After incubation, the cell-free supernatant aliquots (100 μL) were collected by removing the cells with centrifugation at 400 x g for 10 minutes and stored at -70°C until analysis for IL-2 activity.

A single-blind microassay, using IL-2-dependent murine T-helper cell line (HT-2) of BALB/C origin (Watson, 1979), was used to quantitate IL-2 as described (Gillis et al., 1978; Exon et al., 1983) with minor modifications as described in the methods section of Chapter VI.

Antibody Production Response

In a separate experiment, each animal from exposed and control groups were immunized ip with 0.25 mL of a 20% suspension of saline-

washed sheep red blood cells (SRBC) 4 days before the end of chemical treatments. Numbers of antibody plaque-forming cells (PFC) were quantitated by the methods of Cunningham and Szenberg (1968). Results were expressed as antibody PFC/10^6 splenocytes and as antibody PFC per spleen.

The level of anti-SRBC antibody (α-SRBC) in the serum collected from mice utilized in PFC assay was analyzed by a double sandwich enzyme-linked immunosorbent assay (ELISA) as previously reported (Chapter III).

Statistical Analysis

All data were expressed as the means and their standard errors. A one way analysis of variance and Fisher's least significant difference (LSD) test were used to determine the statistical significance ($p<0.05$) of experimental data.

RESULTS

Based on gas chromatographic analyses, the overall average of daily observed concentrations of test materials in the drinking water of mice was 166 ($\pm$ 9.5%) mg/L for the 200 mg/L nominal concentration of benzene, 80 ($\pm$ 12%) mg/L for the toluene 100 mg/L group, and 325 ($\pm$ 13.5%) mg/L for the toluene 400 mg/L group. The benzene or toluene concentrations in the drinking water of the mixture groups were the same as in the groups of chemical alone.

Gross Observations

Animals exposed to various concentrations of treated water over a 4-week period did not exhibit overt clinical symptoms of toxicity. Food

and water consumption were not affected by the test chemical(s). There were no apparent differences in body, spleen, kidney and liver weights between control and treatment groups (Table VIII-1). No gross lesions were detected at autospy in any organ from mice treated with test chemical(s). The animals treated with benzene (166 mg/L), showed significant reduction in thymus mass. Toluene (325 mg/L) reduced the effect of benzene on the thymus weight. Both toluene concentrations (325 mg/L and 80 mg/L)) had no significant adverse effect on weight of thymus.

Hematology

Table VIII-2 shows the effects of benzene and toluene, alone or combined, on selected blood parameters. Total blood erythrocyte, leukocyte, and lymphocyte counts were depressed in mice treated with benzene alone. Concurrent exposure of either dose of toluene (80 and 325 mg/L) with benzene (166 mg/L) resulted in increases of erythrocyte counts when compared to the benzene treatment alone. Toluene at 80 mg/L did not alleviate benzene-induced leukocytopenia and lymphocytopenia. There were significant differences in leukocyte and lymphocyte counts between toluene (80 mg/L) and benzene plus toluene (166 plus 80 mg/L) treatments. No changes in hematological responses were observed in groups treated with toluene alone when compared to the control values.

Mitogen-Stimulated Lymphoproliferation

The lymphoproliferative response data for optimal concentrations of the B-cell mitogen LPS, T-cell mitogens Con A and PHA,and the B- and T-cell mitogen PWM are summarized in Table VIII-3. A significant decrease in ^{3}H-TdR uptake was observed for splenocytes from the 166 mg/L benzene

TABLE VIII-1

ORGAN AND BODY WEIGHTS OF MICE FOLLOWING 4-WEEKS OF EXPOSURE TO BENZENE AND TOLUENE, ALONE OR COMBINED[a]

Concentration in water, mg/L	Body weight (g)		Organ weight (g/100 g body weight)			
	Day 0	Day 28	Spleen	Kidney	Liver	Thymus
Experiment I						
Control	27.02 ± 0.73	34.36 ± 0.78	0.31 ± 0.01	1.46 ± 0.05	5.77 ± 0.16	0.14 ± 0.01
Benzene, 166	27.02 ± 0.56	35.70 ± 1.01	0.28 ± 0.02	1.59 ± 0.08	6.28 ± 0.17	0.09 ± 0.02[b]
Toluene, 325	27.46 ± 0.48	37.32 ± 1.34	0.29 ± 0.02	1.54 ± 0.04	5.92 ± 0.17	0.11 ± 0.01
Benzene, 166 +Toluene, 325	26.78 ± 0.37	34.22 ± 0.39	0.28 ± 0.01	1.56 ± 0.05	5.77 ± 0.16	0.11 ± 0.01
Experiment II						
Control	23.38 ± 0.79	31.12 ± 1.02	0.29 ± 0.01	1.79 ± 0.10	6.19 ± 0.13	0.17 ± 0.01
Benzene, 166	22.50 ± 0.72	30.44 ± 0.75	0.24 ± 0.92	1.73 ± 0.11	6.29 ± 0.17	0.14 ± 0.01[b]
Toluene, 80	25.38 ± 0.75	34.72 ± 1.20	0.29 ± 0.03	1.74 ± 0.05	6.02 ± 0.17	0.15 ± 0.01
Benzene, 166 +Toluene, 80	25.42 ± 0.75	34.14 ± 1.25	0.26 ± 0.01	1.73 ± 0.08	6.38 ± 0.23	0.14 ± 0.01[b]

[a] Values are given as mean ± SE (n=5)
[b] Significantly different from untreated controls ($p<0.05$)

TABLE VIII-2

EFFECTS OF EXPOSURE TO BENZENE AND TOLUENE, ALONE OR COMBINED, ON SELECTED BLOOD PARAMETERS

Concentration (mg/L)	Erythrocyte[a] (10^6/mm^3)	Leukocyte[a] (10^3/mm^3)	Leukocyte absolute differentials (10^3/mm^3)[a]		
			Lymphocyte	Neutrophil	Other[b]
Experiment I					
Control	9.73 ± 0.18	6.90 ± 1.08	5.19 ± 1.03	1.17 ± 0.11	0.54 ± 0.09
Benzene, 166	7.09 ± 0.25[c]	4.44 ± 0.16[c]	2.43 ± 0.16[c]	1.61 ± 0.11	0.31 ± 0.04
Toluene, 325	9.78 ± 0.23	6.46 ± 0.61	4.67 ± 0.54	1.24 ± 0.05	0.55 ± 0.08
Benzene, 166 +Toluene,325	9.45 ± 0.38[d]	5.62 ± 0.35	3.60 ± 0.31	1.57 ± 0.16	0.44 ± 0.07
Experiment II					
Control	9.36 ± 0.18	7.54 ± 0.62	5.66 ± 0.58	1.36 ± 0.22	0.53 ± 0.08
Benzene, 166	6.77 ± 0.35[c]	4.80 ± 0.59[c]	2.78 ± 0.52[c]	1.34 ± 0.26	0.57 ± 0.19
Toluene, 80	10.42 ± 0.34	7.55 ± 0.81	5.52 ± 0.76	1.58 ± 0.17	0.54 ± 0.07
Benzene, 166 +Toluene, 80	9.28 ± 0.25[d]	5.06 ± 0.60[c,e]	2.93 ± 0.54[c,e]	1.72 ± 0.11	0.40 ± 0.14

[a] Values are given as mean ± SE (n=5)
[b] Including monocytes, eosinophils and basophils
[c] Significantly different from untreated controls ($p<0.05$)
[d] Significantly different from benzene treatment alone ($p<0.05$)
[e] Significantly different from toluene treatment alone ($p<0.05$)

TABLE VIII-3

EFFECTS OF EXPOSURE TO BENZENE AND TOLUENE, ALONE OR COMBINED, ON SPLENIC LYMPHOCYTE PROLIFERATIVE RESPONSE TO MITOGENS

Concentration (mg/L)	Splenocyte[a] ($x10^{-7}$)	Mitogenic responses[a,b]				
		None	LPS	PWM	Con A	PHA
Experiment I						
Control	8.94 ± 1.90	2.02 ± 0.48	79.36 ± 20.14	8.99 ± 0.43	85.45 ± 39.75	103.40 ± 34.64
Benzene, 166	7.90 ± 1.67	1.07 ± 0.29	15.01 ± 3.22	2.53 ± 0.83[c]	13.79 ± 3.48[c]	14.18 ± 2.15[c]
Toluene, 325	8.61 ± 1.16	1.59 ± 0.52	32.14 ± 5.24	7.72 ± 2.11	34.87 ± 7.37	43.53 ± 13.55
Benzene, 166 +Toluene,325	9.26 ± 0.75	3.17 ± 0.59[d,e]	125.41 ± 23.93[d,e]	10.66 ± 1.31[d]	174.51 ± 83.59[d]	119.57 ± 2.50[d]
Experiment II						
Control	10.80 ± 1.14	2.10 ± 0.71	55.52 ± 10.24	6.72 ± 1.83	72.03 ± 13.33	ND[f]
Benzene, 166	7.80 ± 0.05	0.52 ± 0.23	4.07 ± 1.10[c]	3.31 ± 1.32	10.55 ± 2.82[c]	ND
Toluene, 80	11.38 ± 1.87	1.49 ± 0.28	27.43 ± 9.92	4.49 ± 0.53	35.72 ± 8.77	ND
Benzene, 166 +Toluene, 80	10.74 ± 0.70	2.05 ± 0.72	6.02 ± 1.68[c]	3.79 ± 1.06	23.55 ± 2.82[c]	ND

[a] Values are given as mean ± SE (n=5)
[b] dpm x $10^{-3}/10^{6}$ splenic cells: response evaluated by incorporation of [methyl-^{3}H]-thymidine into day 2 splenocyte cultures for 6 hours pulsing
[c] Significantly different from untreated controls ($p<0.05$)
[d] Significantly different from benzene treatment alone ($p<0.05$)
[e] Significantly different from toluene treatment alone ($p<0.05$)
[f] ND, not determined

dosage group in the presence of all mitogens tested. Toluene (80 or 325 mg/L) did not significantly suppress the proliferation of splenocytes stimulated with mitogen, although apparent reductions (50-60%) were observed for the mitogenesis by LPS, Con A, and PHA. Toluene at a concentration of 325 mg/L not only completely prevented the benzene-produced depression in mitogenesis but enhanced the ^{3}H-TdR uptake when compared to the control values. No antagonistic effects on mitogen-stimulated lymphoproliferation were found when benzene-exposed mice were simultaneously treated with toluene at the 80 mg/L dose. The proliferation of spleen cells cultured in the absence of mitogen exhibited a similar mitogen-stimulated response as described above.

Mixed Lymphocyte Culture (MLC) Response

The results of MLC are presented in Fig. VIII-1. They showed that splenocytes from control and chemical(s)-treated mice exhibit different abilities to recognize histocompatible murine YAC-1 lymphoma cells (H-2^{a}), and to proliferate after recognition. A significant decrease in ^{3}H-TdR incorporation into the DNA of dividing splenic lymphocytes was observed for mice exposed to benzene (166 mg/L), toluene (325 mg/L), and benzene (166 mg/L) + toluene (80 mg/L). A high toluene concentration (325 mg/L) protected against benzene-induced suppression of MLC responses.

Cell-Mediated Cytolysis (CMC) Response

Figure VIII-2 illustrates the effects of chemical exposures on CTL generation to YAC-1 lymphoma cells. The results of ^{51}Cr-release assay revealed that benzene (166 mg/L) significantly impaired the capacity of spleen cell populations to lyse tumor cells (target cells) after four

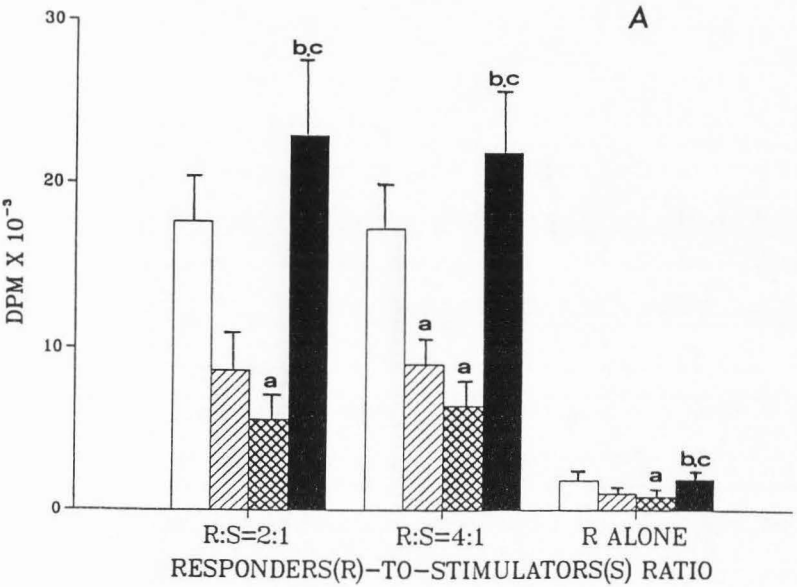

Fig. VIII-1. Effects of benzene and toluene, alone or combined, exposure on mixed lymphocyte culture (MLC) responses. Chemical(s) were administered continuously to mice via drinking water for 4 weeks, while control mice received tap water. A: the bars (left to right), ▭, ▨, ▩, and ■, indicate control, benzene (166 mg/L) alone, toluene (325 mg/L) alone, and benzene (166 mg/L) + toluene (325 mg/L), respectively. B: the bars (left to right), ▭, ▨, ▩, and ■, indicate control, benzene (166 mg/L) alone, toluene (80 mg/L), and benzene (166 mg/L) + toluene (80 mg/L), respectively. (a) Denotes significantly different from control ($p<0.05$); (b) denotes significantly different from benzene treatment alone ($p<0.05$); (c) denotes significantly different from toluene treatment alone ($p<0.05$). Responder cells were the splenocytes of CD-1 mice and stimulator cells were the allogenic YAC-1 lymphoma cells ($H\text{-}2^a$) of A/Sn origin. The stimulator cells were treated with mytomycin-C before addition to the culture. MLR was evaluated by incorporation (dpm x 10^{-3}) of [methyl-^{3}H]-thymidine into day 3 MLR cultures for 6 hours pulsing. Values are given as mean ± SE (n=5).

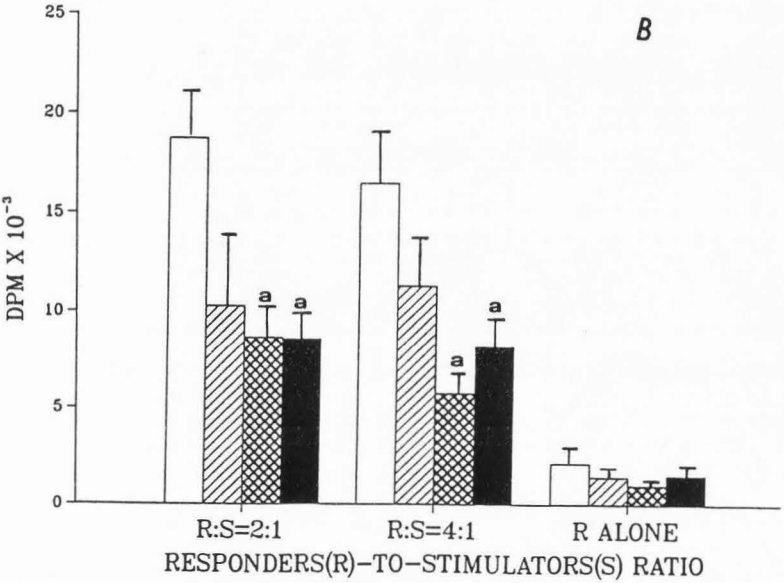

Fig. VIII-1 (Continued)

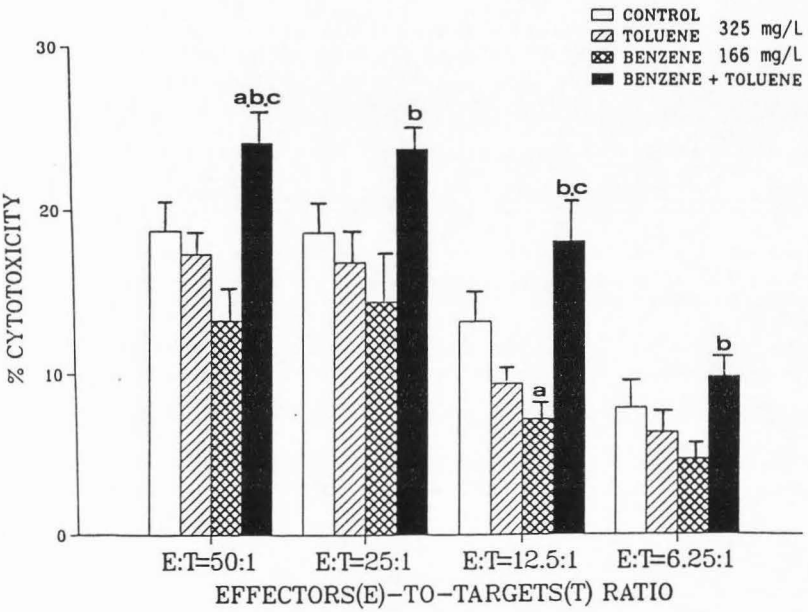

Fig. VIII-2. Effects of benzene and toluene alone or combined, exposure on cytotoxic lymphocyte functions. See legend of Fig. VIII-1 for detail. Splenocyte population (effector cells) was generated from mixed lymphocyte culture (day 5) and ^{51}Cr-labeled YAC-1 lymphoma cells were used as target cells. All determinations were done in triplicate for each E:T ratio. Percent specific cytotoxicity was calculated as percent of total releasable counts, corrected for spontaneous release of radiolabel. Spontaneous release was less than 8% of total releasable counts for all assays.

weeks of oral ingestion. Toluene (325 mg/L) itself had no noticeable effect on the CMC response. Concomitant administration of benzene and toluene resulted in a significant elevation of CMC activity when compared to the control, benzene, or toluene treatment alone.

Antibody Production

The effects of chemical treatments on the capacity of T-cells, B-cells and macrophages to cooperate in the production of antibody to a T-cell-dependent antigen, SRBC, are presented in Table VIII-4. Mice exposed to benzene (166 mg/L) had significantly reduced numbers of IgM antibody PFC per 10^6 splenocytes and per spleen, and reduced levels of α-SRBC serum antibodies. Toluene (325 mg/L) itself had no effect on α-SRBC antibody level and PFC/spleen, although a reduction of PFC as expressed on specific activity (PFC/10^6 spleen cells) was noted. Toluene alleviated the adverse effects of benzene on both PFC numbers and -SRBC antibody levels. No significance was detected in the total of recovered splenocyte among any of the chemical treatment groups (Table VIII-4).

IL-2 Synthesis

Ability of the supernatants from Con A-stimulated splenocyte cultures to promote the growth of an IL-2-dependent T-cell line, HT-2, are presented in Table VIII-5. The results clearly indicate that benzene (166 mg/L) exposure suppressed IL-2 secretions. Splenocytes taken from mice treated with 325 mg/L toluene produced less but statistically equivalent IL-2. Spleen cells obtained from animals exposed to a mixture of benzene (166 mg/L) and toluene (325 mg/L) and stimulated with Con A in culture produced significantly higher levels of IL-2 than benzene or toluene treatment alone.

TABLE VIII-4

EFFECTS OF EXPOSURE TO BENZENE AND TOLUENE, ALONE OR COMBINED, ON THE ANTIBODY RESPONSES TO T-DEPENDENT ANTIGEN SHEEP ERYTHROCYTE (SRBC)[a]

Concentration (mg/L)	Total splenocytes ($\times 10^{-7}$)	PFC/10^6 splenocytes	PFC/spleen ($\times 10^{-3}$)	α-SRBC antibody titer
Control, 0	22.01 ± 2.29[b]	1485 ± 66	337.36 ± 44.82	0.78 ± 0.07
Benzene, 166	17.81 ± 1.18	833 ± 89[b]	151.41 ± 21.53[b]	0.47 ± 0.05[b]
Toluene, 325	19.02 ± 1.03	1190 ± 109[b]	229.51 ± 32.30	0.67 ± 0.03
Benzene, 166 +Toluene,325	22.96 ± 1.61	1972 ± 159[b,c,d]	454.90 ± 42.48[c,d]	1.24 ± 0.10[b,c,d]

[a] Mice were sensitized with SRBC 4 days before the end of exposure. Splenic cells were analyzed for antibody forming cells (plaque-forming cell, PFC) and sera were detected for antibody titer (α-SRBC). Values are given as mean ± SE (n=5)

[b] Significantly different from untreated controls ($p<0.05$)

[c] Significantly different from benzene treatment alone ($p<0.05$)

[d] Significantly different from toluene treatment alone ($p<0.05$)

TABLE VIII-5

EFFECTS OF SIMULTANEOUS EXPOSURE TO BENZENE AND TOLUENE ON INTERLEUKIN-2 SYNTHESIS BY CON A-STIMULATED MOUSE T-LYMPHOCYTE[a]

Concentration in Water, mg/L	^{3}H-Thymidine uptake (DPMx10^{-3})	Stimulation[b] index
Experiment I		
Control	11.07 ± 1.09	13.42 ± 1.32
Benzene, 166	6.31 ± 0.36[c]	7.65 ± 0.43[c]
Toluene, 325	8.09 ± 1.26	9.81 ± 1.53
Benzene, 166 +Toluene,325	12.65 ± 1.40[d,e]	15.21 ± 1.77[d,e]
Experiment II		
Control	12.20 ± 2.41	14.79 ± 2.93
Benzene, 166	4.22 ± 0.59[c]	5.13 ± 0.70[c]
Toluene, 80	10.07 ± 0.63	11.71 ± 0.77
Benzene, 166 +Toluene, 80	6.19 ± 0.50[c,e]	7.51 ± 0.61[c,e]

[a] Several doubling dilutions of IL-2 sources (Con A-stimulated supernatants) which showed a decreasing incorporation of ^{3}H-thymidine to IL-2 dependent murine T-helper cell clone, HT-2 cell, were used. Only data obtained from 1:1 diluted ratio are presented. Uptakes of ^{3}H-thymidine by HT-2 cells in presence of positive control IL-2 source (supernatants of Con A-stimulated rat splenocyte cultures) and negative control IL-2 source (supernatants of non-Con A-stimulated splenocyte cultures of untreated CD-1 mice) were 18.92 ± 0.59 and 1.62 ± 0.60, respectively. Values are given as mean ± SE (n=5)

[b] Stimulation index (S.I.) = dpm (culture with dilution of supernatant/dpm (culture with media alone)

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

[d] Significantly different from the benzene treatment alone at $p<0.05$

[e] Significantly different from the toluene treatment alone at $p<0.05$

DISCUSSION

The present study reports on the development of an animal model for simultaneous subchronic exposure of benzene and toluene via oral ingestion in which the immunotoxic interactions between the agents were evaluated. The results demonstrate that the combination of ingested benzene and sufficient amounts of toluene is less hematotoxic and immunotoxic than ingested benzene alone. Compared to treatment with benzene alone, the combined exposure (166 mg benzene + 325 mg toluene/L) produced a markedly protective effect on benzene-induced depressions of total blood erythrocyte, leukocyte, and lymphocyte counts, and abnormalities of immune-associated parameters, including humoral and cellular immunity.

Alterations in immunocompetence following a four-week oral ingestion of benzene alone was evidenced by a significant involution of thymus and a loss of circulating lymphocytes, both as useful indicators of immune function. The sensitivity of lymphocytes to benzene revealed the immunotoxic potential of this agent. Evidence from several recent studies implicated the immune system as a target organ for benzene. Administration of benzene by inhalation or injection to experimental animals has been shown to suppress the mitogenic response of B- and T-lymphocyte proliferations (Rozen and Snyder, 1985; Rozen et al., 1984; Wierda et al., 1981), humoral antibody response as measured by plaque-forming cells to sheep erythrocytes (Aoyama, 1986; Wierda et al., 1981), and host resistance to the pathogenic microorganisms, such as *Listeria monocytogenes* (Rosenthal and Snyder, 1985) and *Klebsiella pneumoniae* (Aranyi et al., 1986). Recently, it has also been reported that exposure to 100 ppm benzene (5 days/week x 4 weeks) via inhalation in

mice depressed the mixed lymphocyte culture (MLC) response and tumor lytic ability of cytotoxic T lymphocytes, and ruled out the possibilities that depressions in immune function are due to an induction of suppressor cell activity (Rosenthal and Snyder, 1987). As MLC responsiveness is generally thought to represent the initial phases in the induction of cytotoxic T cells (CTL), the benzene-induced loss of CTL lytic ability to tumor cells might be explained by an impaired ability of T cells to recognize foreign tissue or tumor-associated antigens during T-helper-cell-mediated induction of CTL cells. In the present study benzene ingestion significantly reduced the production of IL-2, a known important immunoregulatory lymphokine and a necessary component in MLC response and CTL generation (Farrar et al., 1982).

Toluene, a widely used organic solvent, is an agent of environmental and occupational significance (U.S. National Academy of Sciences, 1982). This agent is known to possess the potential to significantly alter the metabolism and resulting bioactivity of certain other chemicals, thereby influencing their toxicities (Hayden et al., 1977; Fishbein, 1985). In animal models toluene exposure is associated with the induction of microsomal cytochrome P-450 dependent enzymes and conjugation enzymes (Pyykko, 1980; Toftgard et al., 1982; Pathiratne et al., 1986a), and depletion of liver glutathione (van Doorn et al., 1980). Time-course of effects of toluene on several microsomal monoxygenases in liver or some non-hepatic tissues of rats exposed to toluene has been reported (Pyykko, 1983).

The reasonable explanation for reduced benzene immunotoxicity is that toluene decreases the levels of toxic benzene metabolites. Metabolism of benzene is required for its toxicity (Irons, 1985; Snyder

et al., 1983). Benzene metabolites have been shown to produce a variety of alterations in immune function. The ability of phenol, hydroquinone, and catechol to suppress lymphocyte growth and function *in vitro* correlates with their capacity to undergo oxidation and with their concentration in the bone marrow or lymphoid organs (Irons et al., 1981; Pfeifer and Irons, 1981; 1982; 1983). Hydroquinone and its oxidation product, *p*-benzoquinone, inhibit proliferation and differentiation in lectin-stimulated lymphocytes in culture at concentrations that are not cytotoxic, while phenol or catechol suppress lymphocyte growth or function and only at concentrations that result in cell death. Suppression of lymphocyte blastogenesis by hydroquinone is probably mediated by the interaction of *p*-benzoquinone with sulfhydryl (SH) groups on tubulin (Irons et al., 1981; 1984; Pfeifer and Irons, 1983). Post et al. (1985) demonstrated that exposure to *p*-benzoquinone in mice completely inhibits the proliferation and production of the T-cell lymphokine, interleukin-2, by concanavalin A (Con A)-stimulated T-lymphocytes. Hydroquinone and catechol have been shown to reduce the frequency of spleen and bone marrow progenitor B-lymphocytes and to inhibit polyclonal plaque-forming cells (Wierda and Irons, 1982).

Toluene is a substrate for cytochrome P-450 and has been shown to competitively inhibit benzene biotransformation *in vitro* or *in vivo*. The conversion of benzene to phenols in rats, mice or *in vitro* has been reported to be suppressed when benzene was given in combination with toluene (Ikeda et al., 1972; Andrews et al., 1977; Sato and Nakajima, 1979; Gilmour et al., 1986). Andrews et al. (1977) coadministered benzene and toluene to mice or rabbits and observed a marked reduction in levels of benzene metabolites in bone marrow, coupled with a

compensatory increase in pulmonary excretion of unmetabolized benzene. It was also demonstrated using liver microsomes *in vitro* that toluene is a competitive inhibitor of benzene metabolism. When benzene and toluene were administered in combination to rats intraperitoneally, their disappearance rates from blood and the rates of urinary excretion of their metabolites were delayed compared with those observed when they were given separately. Their metabolic interaction was found to be dose-dependent (Sato and Nakajima, 1979). Several studies have indicated that toluene can significantly influence the myelotoxic and genotoxic effects of benzene. It was demonstrated that toluene protects against benzene-induced depression of red cell ^{59}Fe uptake in bone marrow of mice (Andrews et al., 1977). Toluene not only alleviates the benzene-induced leukopenia but the benzene-induced increase in leukocyte alkaline phosphatase activity (Li et al., 1986). Toluene significantly decreased the adverse effects of benzene on granulopoietic stem cells (CFU per tibia) and on tibial bone marrow cellularity in mice (Tunek et al., 1981; 1982). When benzene-exposed mice were simultaneously given an ip injection of toluene, the sister chromatid exchange (SCE) incidence was reduced by up to 90% compared with animals exposed to benzene alone (Tice et al., 1982). Gad-El-Karim et al. (1984; 1985a; 1986) found that toluene antagonized benzene's myeloclastogenicity and metabolism when mixtures of both chemicals were administered orally to CD-1 mice. On the other hand, toluene-induced protection against benzene toxicities might be related to the enhancement of detoxication reactions, i.e., conjugation reactions. Pretreatment of rats with toluene has been found to increase the *in vitro* liver microsomal metabolism of benzene (Pathiratne et al., 1986b).

Toluene treatment alone (at doses used) did not show the dramatic immunosuppresive effects of benzene. A previous study has indicated that alteration in immune functions of mice ingesting toluene is generally evident at relatively high doses (Chapter VI). Alterations in host resistance to infectious microorganisms, lymphocyte and macrophage functions has also been noted (Aranyi et al., 1985; Fishbein, 1985; Suleiman, 1987). Similar to the metabolite(s)-mediated mechanism of benzene toxicity, it has been reported that formation of cresol from aromatic oxidation of toluene occurs through an arene oxide intermediate which, in turn, is a reactive metabolite and is responsible for the binding of toluene to biomacromolecules (Pathiratne et al., 1986a; Bakke and Scheline, 1970). Superoxide anions are also formed during the formation of secondary metabolites, such as semiquinones and quinones, from the oxidation of cresol (Pathiratne et al., 1986a). This suggests that covalent binding of toluene to biological macromolecules involves more than one reactive metabolite. However, the covalent binding effect of toluene to biomacromolecules is considerably less than that of benzene. This could account for the differences in the degree of immunotoxicity of toluene and benzene in this study.

Enhancements of immune responses in the combined exposure (166 mg benzene + 325 mg toluene/L) group, when compared to the untreated control, might involve the fact that concurrent administration of toluene decreases benzene metabolism and results in low amounts of reactive oxidative metabolites of benzene. Several studies have indicated that low concentrations of benzene or its metabolite(s) possess promotive effects on the immune and hemopoietic systems (Chapter III; Gaido and Wierda, 1985; Garnett et al., 1983; Harigaya et al.,

1981).

In conclusion, the present study demonstrates that oral ingestion of toluene in combination with benzene over a four-week period causes antagonistic effects on benzene immunotoxicity, while toluene itself shows no strong immunosuppressions. Since time of exposure to toluene relative to the time of exposure to a second chemical is quite important, further studies involved in the time-course of effects of toluene on benzene metabolism or toxicity seem to be warranted in order to better assess their interaction mechanisms.

CHAPTER IX

EFFECTS OF REPEATED ORAL EXPOSURE TO COMBINATIONS OF BENZENE AND TOLUENE ON REGIONAL BRAIN MONOAMINE METABOLISM IN MICE

INTRODUCTION

Exposure to chemicals in combinations has received much attention. The possible modes by which chemicals modify the toxicity of other compounds include an interaction at the target site, an interaction with drug-metabolizing enzymes, a modification of the absorption site changing the body burden, or alteration of the chemical or physical characteristics of the compounds leading to an alteration in biological action (Mitchell, 1976; Sipes and Gandolfi, 1986). Such interactions may be very critical in environmental situations and the investigations of synergism and antagonism are important areas.

Benzene and toluene, the major aromatic hydrocarbons, are known to produce numerous toxicological effects on a variety of organ systems (Fishbein, 1984; 1985). Both compounds are extensively coemployed in industry, chemistry, and used as important components in gasoline. The potential for human exposure to one or both of these agents is great (U.S. National Academy of Sciences, 1982). Several *in vitro* or *in vivo* studies have indicated that toluene is a competitive inhibitor of the biotransformations of benzene (Ikeda et al., 1972; Andrews et al., 1977; Sato and Nakajima, 1979; Gilmour et al., 1986), and can significantly alter the hematotoxic and genotoxic effects of benzene (Andrews et al., 1977; Tunek et al., 1981; 1982; Tice et al., 1982; Gad-El-Karim et al., 1984; 1985a; 1986). We have recently reported that toluene has an

antagonistic effect on benzene immunotoxicity (Chapter VIII). However, none of these studies involved evaluation of interaction-induced neurotoxicity after simultaneous exposure to the mixtures of these two compounds. Neurological effects produced by either benzene or toluene alone have been characterized (Brief et al., 1980; Evans et al., 1981; Benignus, 1981a,b; Dempster et al., 1984). Changes in regional brain catecholamine and indoleamine metabolisms in mice following benzene or toluene ingestion have also been noticed (Chapters IV and V).

The present study was designed to assess the effects of continuous exposure to various levels of benzene and toluene, alone or combined, on concentration of regional brain monoamines and their major metabolites in CD-1 mice. The results of this investigation should help our understanding of the possible mechanisms of solvent action on the central nervous system.

MATERIALS AND METHODS

Animals

Male CD-1 mice obtained from Charles River Lab. Inc. (Wilmington, MA) and 6 weeks old on arrival were used. After 1-week acclimatization in a AAALAC-accredited animal care facility with an ambient temperature of 21 $\pm$ 1^{o}C, a relative humidity of 50 $\pm$ 10%, and a 12-h light-dark cycle (7 a.m. - 7 p.m.), animals were randomized into control and test groups of 5 mice each. A standard commercial rodent chow (Wayne Products, Chicago, IL) and water were available *ad libitum* throughout the entire experimental period.

Chemical Exposures

Benzene (analytical reagent grade, 99.9% purity; J. T. Baker Chemical Co., Philipsburg, NJ) and toluene (chromatoquality grade, 99.7% purity; Burdick and Jackson Labs. Inc., Muskegon, MI) were dissolved in normal tap water to make the desirable concentrations. Two experiments were conducted. The first experiment was designed primarily to study a combination exposure of 200 mg benzene plus 400 mg toluene/L. The results of this experiment were compared to single exposures of benzene (200 mg/L) and toluene (400 mg/L) that were conducted simultaneously. The concentration of toluene was reduced to 100 mg/L in the second experiment. Benzene and toluene have a solubility of 1,780 and 535 mg/L, respectively, in fresh water at 25°C (U.S. Environmental Protection Agency, 1980 a,b). Exposures were achieved by continuously administering chemical(s)-treated solutions to mice for 28 days. Water and food consumption was monitored continuously, and animals were weighed weekly. Actual concentrations of benzene and toluene were confirmed daily by gas chromatography (U.S. Environmental Protection Agency, 1986b).

Processing of Brain Tissues

Twenty-eight days after continuous exposure to test material(s), mice were decapitated and brains were microdissected immediately, on an ice-cold plate, into six discrete regions: hypothalamus, medulla oblongata, cerebellum, corpus striatum, cerebral cortex and the remainder of the brain, midbrain, according to the procedures of Glowinski and Iversen (1966). The midbrain includes the hippocampus, thalamus and subthalamus (Coulombe and Sharma, 1986). Each dissected brain region was promptly weighed in tared vials containing several volumes (in relation to tissue weight) of ice-cold 0.05 M perchloric

acid with 0.1% cysteine. To overcome possible diurnal alternations in regional brain neurochemicals (Matsumoto et al., 1981). All animals of each experiment were sampled between 9:30 and 11:30 a.m. on the same day. Tissue samples for chromatographic analysis were prepared as described in the methods section of Chapter IV.

Assay of Neurochemicals

Brain regional concentrations of the major catecholamines norepinephrine (NE), dopamine (DA), and their principal metabolites, 3-methoxy-4-hydroxymandelic acid (vanillymandelic acid, VMA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA); the indoleamine serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in perchloric acid extracts were simultaneously determined by high performance liquid chromatography with eletrochemical detection (HPLC-ECD) using the methods described elsewhere (Mayer and Soup, 1983). The HPLC system consisted of a Model LC-150 electrochemical analyzer, Model LC-3A amphoteric detector, Model LC-22A temperature controller, and a 250 x 4.6 mm ODS--5 μm--reversed phase column (Bio-Analytic Systems Inc.). A 0.15 M monochloroacetate buffer (PH 3.0) containing sodium octyl sulfate as ion-pair reagent served as mobile phase. The instrument settings were: flow rate 1.4 mL/min, column temperature 30^{o}C, detector potential +0.7 V, controller 10 nA full scale, and sample volume 20 μL. The amounts of neurochemicals were determined by measuring the height of the individual peaks and comparing them with the peak of various reference compounds (Sigma Chemical Co., St. Louis, MO). The detector response provided a linear correlation coefficient of >0.98 for the standard curves. Data were collected with a Hewlett-Parkard (Avondale, PA) 3390A integrator.

Statistical Analysis

The concentrations of monoamines and their metabolites obtained from brain regions were expressed as μg/g wet tissue. All data were given as mean ± SE. Analysis of variance (ANOVA) and followed-up Fisher's least significant differences (LSD) test were used to determine the statistical differences ($p<0.05$) of experimental data.

RESULTS

Concentrations of test material(s) in the drinking water mixtures of mice as determined by gas chromatography decreased approximately 30% over a three-day period, when the water was changed. Overall means of the daily analyses for the nominal concentrations of 200 mg/L benzene, 100 mg/L toluene, and 400 mg/L toluene were 166 (± 9.5%), 80 (± 12%), and 325 (± 13.5%) mg/L, respectively. The benzene or toluene concentrations in the drinking water of the mixture groups were the same as the group treated with benzene or toluene alone.

Repeated exposure to benzene and toluene, alone or combined, over a four-week period did not adversely affect growth, body weights, water and food consumption, nor did it elicit mortality and any other overt clinical symptoms of toxicity and gross treatment-related behavioral alterations in test animals.

At the doses used, both benzene and toluene treatments (alone or combined) induced significant increases in concentrations of biogenic monoamines (NE, DA, 5-HT) and their major metabolites (VMA, DOPAC, HVA, 5-HIAA) in several of the brain regions studied. Furthermore, in several instances, the increased metabolite concentrations were greater in the combined treatment than when either chemical was used alone. Fig. IX-1

and Table IX-1 show the effects of chemical(s) exposures on concentrations of selected neurotransmitters and their metabolites in the hypothalamus. When compared to the untreated control, significant increases of NE in the hypothalamus, the region containing the highest level of this amine, were found in the benzene (166 mg/L), toluene (80 and 325 mg/L), and benzene plus toluene (166 plus 325 mg/L) mixture groups (Fig. IX-1A). Dopamine concentrations increased significantly in the hypothalamus of animals treated with 166 mg/L benzene, 80 mg/L toluene, or 166 mg benzene + 325 mg toluene/L (Fig. IX-1B). Elevated levels of 5-HT also appeared in this region of mice exposed to benzene (166 mg/L) and toluene (325 mg/L), alone or in combination (Fig. IX-1C). Increases of various monoamine metabolites in the hypothalamus were noticed and statistical differences were detected in the 166 mg/L benzene (HVA, 5-HIAA), 325 mg/L toluene (5-HIAA), and their combination groups (VMA, HVA, 5-HIAA) (Table IX-1).

Concentrations of monoamines and their major metabolites in the corpus striatum are illustrated in Fig. IX-2 and Table IX-2, respectively. Benzene (166 mg/L) had no obvious effects on the striatal NE, while toluene increased NE at a concentration of 80 mg/L. Coadministration of benzene with toluene (80 mg/L) resulted in an increase of this neurochemical when compared to control (Fig. IX-2A). Increases of DA in the corpus striatum, a brain area especially rich in DA, were seen in animals exposed to benzene alone or its combination with either dose of toluene (80 and 325 mg/L) (Fig. IX-2B). Concentrations of striatal 5-HT in animals receiving benzene and toluene (325 mg/L), alone or together, were significantly higher than those in the untreated control (Fig. IX-2C). Increases of amine metabolites in

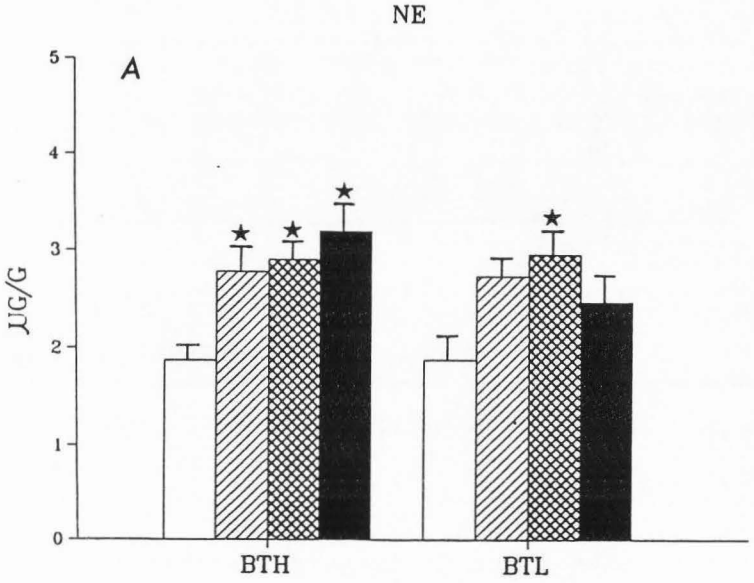

Fig. IX-1. Effects of benzene and toluene, alone or combined, exposure on monoamine concentrations in the hypothalamus of CD-1 mice. A: effects of chemical treatments on norepinephrine; B: effects of chemical treatments on dopamine; C: effects of chemical treatments on serotonin; BTH: exposure to 166 mg/L benzene, 325 mg/L toluene, and combination of benzene (166 mg/L) and toluene (325 mg/L); BTL: exposure to 166 mg/L benzene, 80 mg/L toluene, and combination of benzene (166 mg/L) and toluene (80 mg/L). The bars (left to right), ▭, ▨, ▩, and ■, indicate treatment groups of control, benzene, toluene, and benzene + toluene mixture, respectively. Values are given as mean ± SE (n=5). Stars (*) denote significant differences from control values at $p<0.05$.

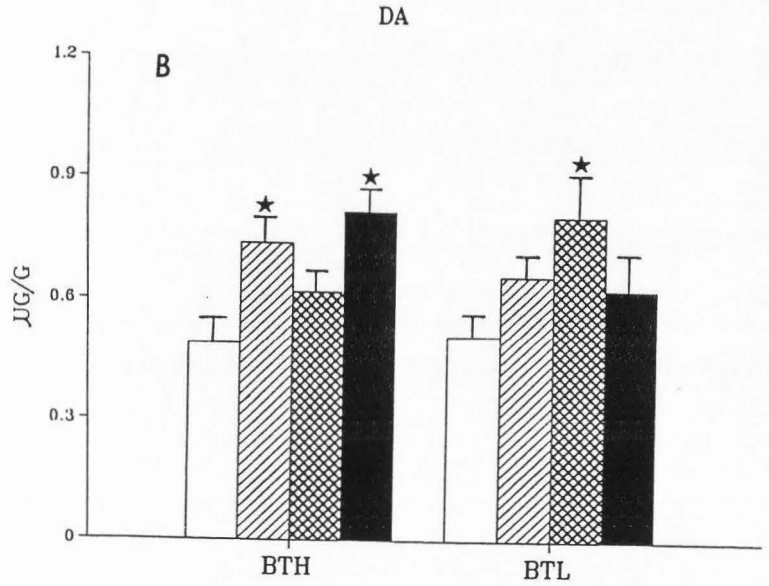

Fig. IX-1 (Continued)

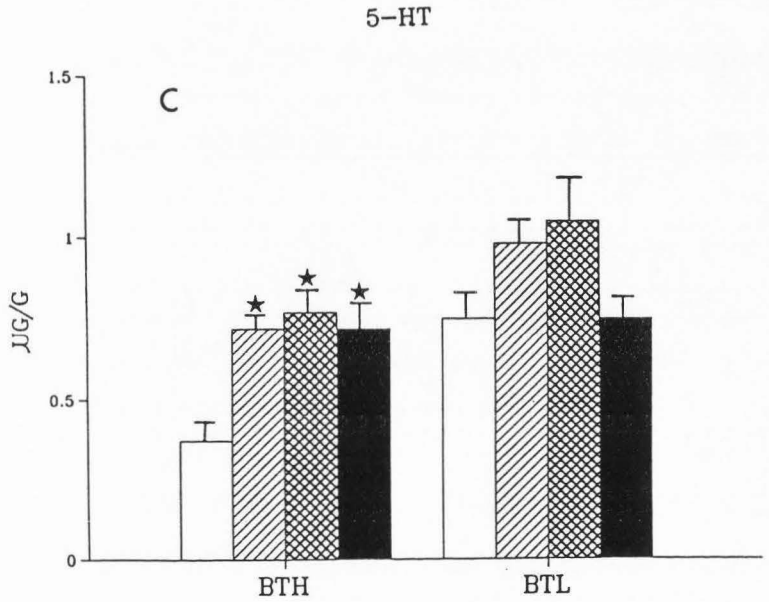

Fig. IX-1 (Continued)

TABLE IX-1

CONCENTRATIONS OF MONOAMINE METABOLITES IN HYPOTHALAMUS OF MICE TREATED WITH BENZENE AND TOLUENE, ALONE OR COMBINED[a]

Concentration in water, mg/L	Neurochemical concentration[b]			
	VMA	DOPAC	HVA	5-HIAA
Experiment I				
Control	0.202 ± 0.031	0.224 ± 0.038	0.156 ± 0.016	0.316 ± 0.066
Benzene, 166	0.275 ± 0.054	0.368 ± 0.074	0.260 ± 0.026[c]	0.686 ± 0.037[c]
Toluene, 325	0.266 ± 0.037	0.351 ± 0.054	0.243 ± 0.025	0.677 ± 0.046[c]
Benzene, 166 +Toluene,325	0.383 ± 0.045[c]	0.424 ± 0.048	0.288 ± 0.031[c]	0.756 ± 0.057[c]
Experiment II				
Control	0.219 ± 0.042	0.206 ± 0.030	0.156 ± 0.020	0.601 ± 0.058
Benzene, 166	0.270 ± 0.030	0.338 ± 0.050	0.435 ± 0.140	1.180 ± 0.183[c]
Toluene, 80	0.266 ± 0.038	0.273 ± 0.014	0.259 ± 0.040	0.836 ± 0.175
Benzene, 166 +Toluene, 80	0.261 ± 0.032	0.263 ± 0.022	0.259 ± 0.032	0.708 ± 0.076[d]

[a] Chemical(s) was (were) administered continuously to mice via drinking water for 4 weeks. Abbreviations for various chemicals have been described in text

[b] In μg neurochemical/g wet brain tissue, mean ± SE (n=5)

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

[d] Significantly different from benzene treatment alone at $p<0.05$

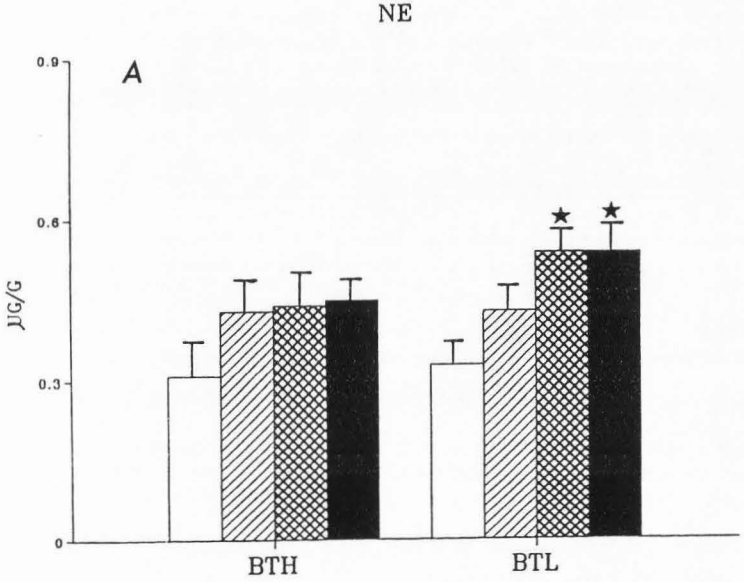

Fig. IX-2. Effects of benzene and toluene, alone or combined, exposure on monoamine concentrations in the corpus striatum. A: effects of chemical treatments on norepinephrine; B: effects of chemical treatments on dopamine; C: effects of chemical treatments on serotonin. The bars (left to right) indicate treatment groups of control, benzene, toluene, and benzene + toluene mixture, respectively. Values are given as mean ± SE (n=5). Stars (*) denote significant differences from control values at $p<0.05$. BTH: exposure to 166 mg/L benzene, 325 mg/L toluene, and combination of benzene (166 mg/L) and toluene (325 mg/L); BTL:exposure to 166 mg/L benzene, 80 mh/l toluene, and combination of benzene (166 mg/L) and toluene (80 mg/L).

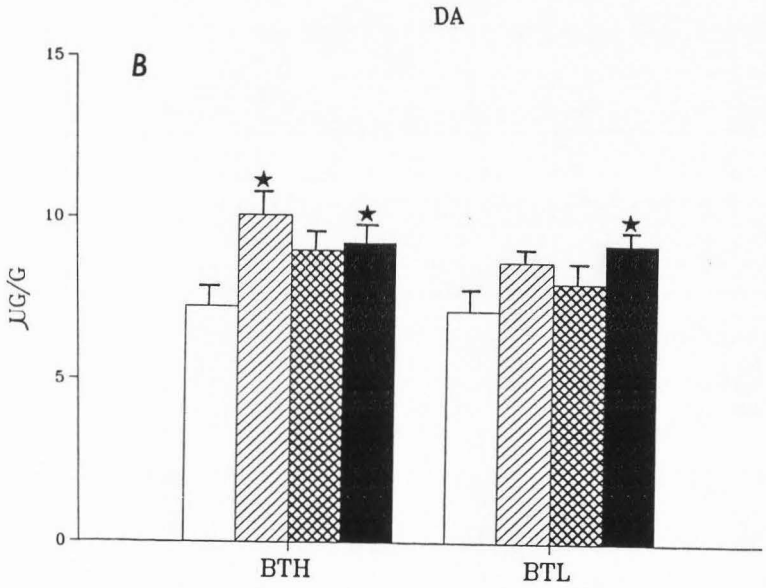

Fig IX-2. (Continued)

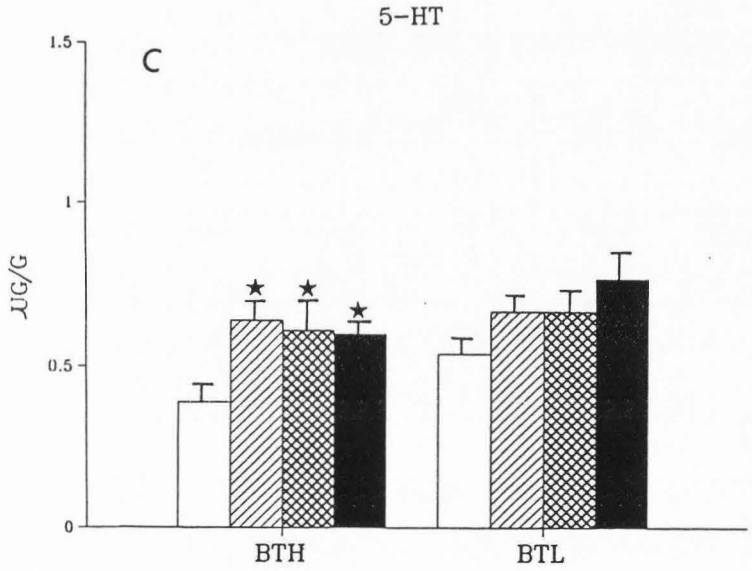

Fig. IX-2. (Continued)

this brain region were detected in the 166 mg/L benzene (VMA, HVA), 80 mg/L toluene (VMA, HVA), and both combination groups (DOPAC, HVA). There were significantly higher concentrations of striatal DOPAC and HVA in the benzene (166 mg/L) plus toluene (325 mg/L) and benzene (166 mg/L) plus toluene (80 mg/L) groups, respectively, than in the single chemical treatment groups (Table IX-2).

Figure IX-3 showed the effects of benzene and toluene, alone or combined, on major amines in the cerebral cortex, midbrain, medulla oblongata, and cerebellum. Benzene caused increases of NE in the cortex, midbrain, and medulla oblongata. Toluene significantly increased NE in the medulla oblongata. Concurrent exposures of toluene (325 mg/L) with benzene (166 mg/L) reduced the benzene-induced effects on this neurotransmitter in the cortex and midbrain (Fig. IX-3A). Significant higher concentrations of DA were also observed in the cerebral cortex of animals receiving both toluene doses, i.e., 80 and 325 mg/L (Fig. IX-3B). Amounts of DA in the cerebellum, a region containing relatively low levels of this catecholamine, were not determined. Benzene (166 mg/L) increased 5-HT concentrations in the cortex, midbrain and medulla oblongata, while toluene-induced increases of this indoleamine were detected in the cortex and midbrain of mice treated with 325 mg/L toluene (Fig. IX-3C).

Concentrations of the major metabolites of biogenic amines in the brain regions of cortex, midbrain, medulla oblongata, and cerebellum are given in Table IX-3 - IX-6. The results shown in Table IX-3 indicate that significant increases of the NE metabolite, VMA, were observed in the midbrain and medulla oblongata of mice coadministered with benzene (166 mg/L) and toluene (325 mg/L) as compared to the untreated control

TABLE IX-2

CONCENTRATIONS OF MONOAMINE METABOLITES IN CORPUS STRIATUM OF MICE TREATED WITH BENZENE AND TOLUENE, ALONE OR COMBINED[a]

Concentration in water, mg/L	Neurochemical concentration[b]			
	VMA	DOPAC	HVA	5-HIAA
Experiment I				
Control	0.122 ± 0.017	1.206 ± 0.038	0.930 ± 0.068	0.547 ± 0.069
Benzene, 166	0.162 ± 0.010	1.425 ± 0.074	1.281 ± 0.088[c]	0.671 ± 0.059
Toluene, 325	0.138 ± 0.010	1.458 ± 0.054	1.088 ± 0.053	0.582 ± 0.047
Benzene, 166 +Toluene,325	0.109 ± 0.012	1.920 ± 0.158[c,d,e]	1.272 ± 0.054[c]	0.722 ± 0.036
Experiment II				
Control	0.156 ± 0.014	0.822 ± 0.076	0.886 ± 0.107	0.408 ± 0.036
Benzene, 166	0.249 ± 0.022[c]	1.068 ± 0.054	1.209 ± 0.045[c]	0.491 ± 0.056
Toluene, 80	0.199 ± 0.041[c]	1.161 ± 0.058	1.280 ± 0.044[c]	0.537 ± 0.053
Benzene, 166 +Toluene, 80	0.223 ± 0.037	1.249 ± 0.098[c]	1.510 ± 0.066[c,d,e]	0.564 ± 0.078

[a] Chemical(s) was (were) administered continuously to mice via drinking water for 4 weeks

[b] In μg neurochemical/g wet brain tissue, mean ± SE (n=5)

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

[d] Significantly different from benzene treatment alone at $p<0.05$

[e] Significantly different from toluene treatment alone at $p<0.05$

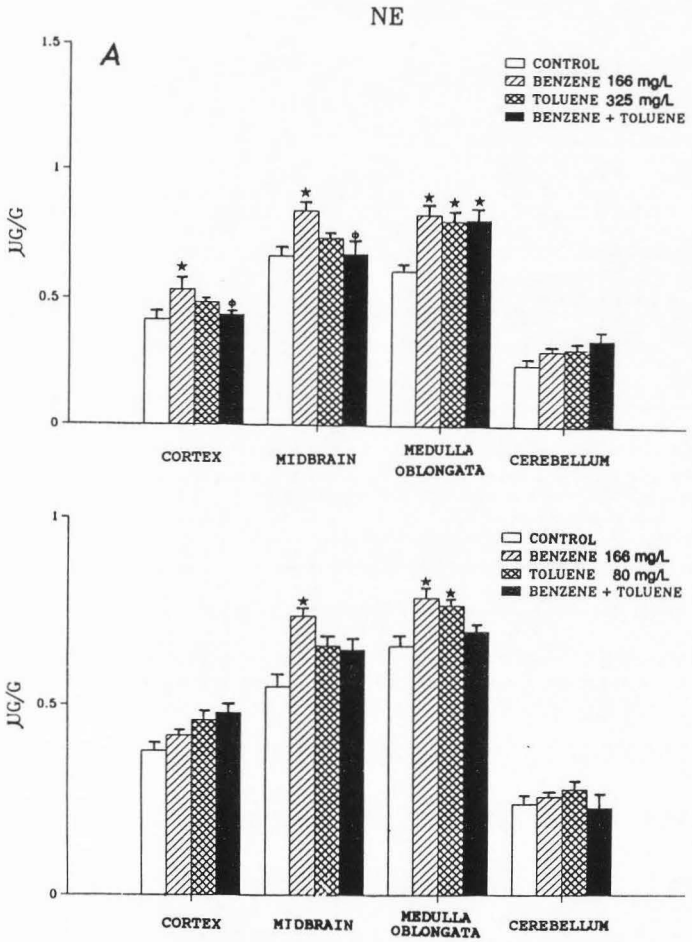

Fig. IX-3. Effects of benzene and toluene, alone or combined, exposure on monoamine concentrations in cerebral cortex, midbrain, medulla oblongata, and cerebellum. A: effects of chemical treatments on norepinephrine; B: effects of chemical treatments on dopamine; C: effects of chemical treatments on serotonin. Stars (*) and (Φ) denote significant ($p<0.05$) differences from untreated control and benzene treatment alone, respectively. Values are given as mean $\pm$ SE (n=5).

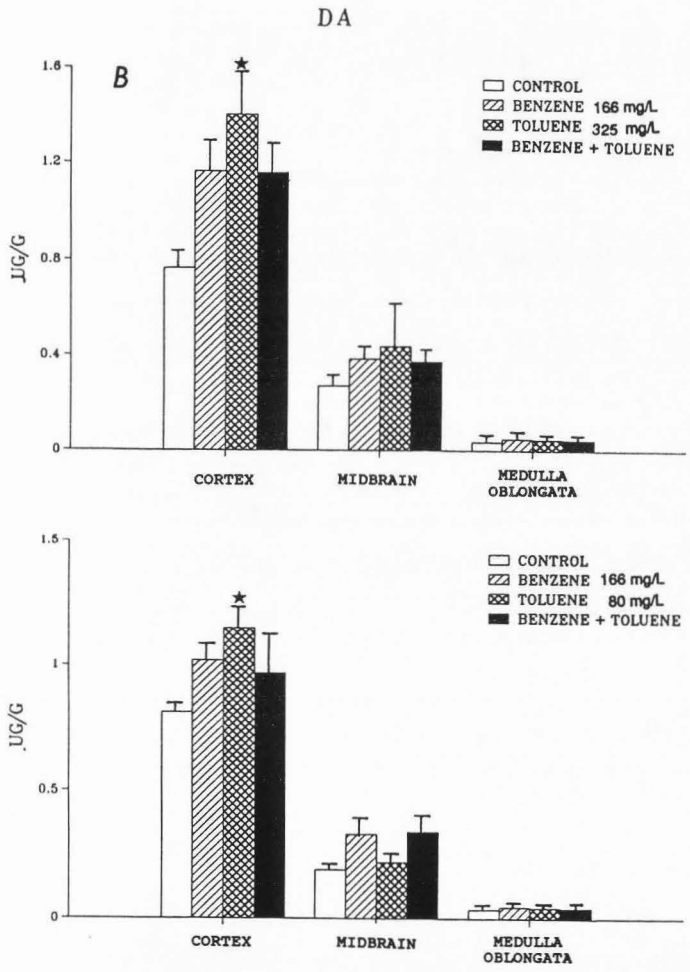

Fig. IX-3. (Continued)

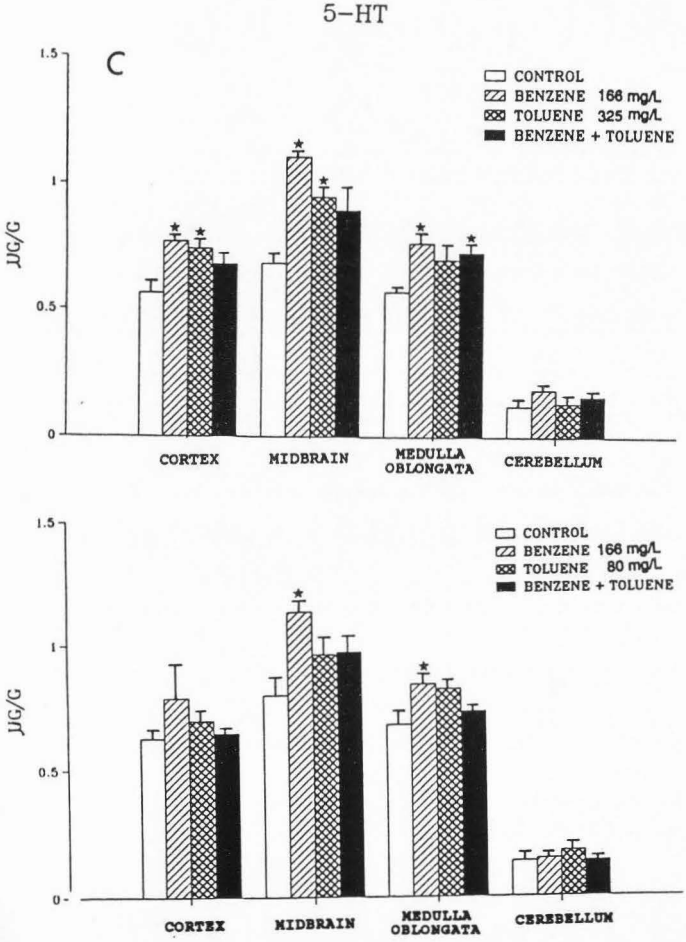

Fig. IX-3. (Continued)

TABLE IX-3

CONCENTRATIONS OF VANILLYMANDELIC ACID (VMA) IN CEREBRAL CORTEX, MIDBRAIN, MEDULLA OBLONGATA AND CEREBELLUM OF MICE TREATED WITH BENZENE AND TOLUENE, ALONE OR COMBINED[a]

Concentration in water, mg/L	Neurochemical concentration[b]			
	Cortex	Midbrain	Medulla oblongata	Cerebellum
Experiment I				
Control	0.106 ± 0.020	0.085 ± 0.020	0.107 ± 0.007	0.117 ± 0.015
Benzene, 166	0.147 ± 0.018	0.078 ± 0.004	0.120 ± 0.003	0.099 ± 0.017
Toluene, 325	0.131 ± 0.006	0.101 ± 0.005	0.168 ± 0.025[c]	0.143 ± 0.045
Benzene, 166 +Toluene,325	0.132 ± 0.009	0.133 ± 0.012[c,d]	0.208 ± 0.012[c,d]	0.152 ± 0.030
Experiment II				
Control	0.220 ± 0.033	0.166 ± 0.033	0.150 ± 0.011	0.121 ± 0.033
Benzene, 166	0.251 ± 0.015	0.198 ± 0.041	0.178 ± 0.024	0.132 ± 0.015
Toluene, 80	0.218 ± 0.029	0.164 ± 0.032	0.212 ± 0.020	0.164 ± 0.041
Benzene, 166 +Toluene, 80	0.224 ± 0.030	0.167 ± 0.025	0.235 ± 0.036	0.137 ± 0.018

[a] Chemical(s) was (were) administered continuously to mice via drinking water for 4 weeks

[b] In μg neurochemical/g wet brain tissue, mean ± SE (n=5)

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

[d] Significantly different from benzene treatment alone at $p<0.05$

or benzene treatment alone. Toluene (325 mg/L)-treated animals also showed higher concentrations of VMA in the medulla oblongata than control group. Occasional increases of DOPAC and HVA, the main acid metabolites of DA, were detected in the cortex and medulla oblongata (Table IX-4 and IX-5). The effects of solvent exposures, alone or combined, on the concentration of the 5-HT metabolite, 5-HIAA, in these brain areas are illustrated in Table IX-6. Benzene (166 mg/L) caused elevations of 5-HIAA in midbrain. Mice exposed to toluene (80 mg/L) had higher 5-HIAA concentrations in the cortex, midbrain and medulla oblongata than control group. Simultaneous exposure of benzene and toluene raised this neurochemical in the midbrain and cortex when compared to the untreated control. Concentrations of 5-HIAA in the cortex of mice treated with benzene plus toluene (166 plus 80 mg/L) were significantly different from benzene treatment alone.

DISCUSSION

Benzene and toluene are known neurotoxicants. Alteration of neurotoxicity has been previously shown to occur between different neurotoxicants (Savolainen, 1977; Abou-Donia et al., 1985a,b). This study was undertaken to investigate the possible interaction effects between benzene and toluene, on the catecholamine and indoleamine metabolism. The neurochemicals selected for evaluation are known to play an important role in physiology and behavior (Rogawski and Baker, 1985; Poirier and Bedard, 1984). Changes in concentrations of their metabolites provide an appropriate indication of neural activity (Commissiong, 1985). Assays in various brain regions are essential to detect potential perturbations of a specific neurotransmitter(s).

TABLE IX-4

CONCENTRATIONS OF 3,4-DIHYDROXYPHENYLACETIC ACID (DOPAC) IN CEREBRAL CORTEX, MIDBRAIN, AND MEDULLA OBLONGATA OF MICE TREATED WITH BENZENE AND TOLUENE, ALONE OR COMBINED[a]

Concentration in water, mg/L	Neurochemical concentration[b]		
	Cortex	Midbrain	Medulla oblongata
Experiment I			
Control	0.205 ± 0.009	0.212 ± 0.031	0.062 ± 0.018
Benzene, 166	0.232 + 0.028	0.175 ± 0.006	0.066 ± 0.013
Toluene, 325	0.273 + 0.034	0.216 ± 0.040	0.040 ± 0.005
Benzene, 166 +Toluene,325	0.283 ± 0.033	0.183 ± 0.011	0.066 ± 0.011
Experiment II			
Control	0.137 ± 0.005	0.127 ± 0.014	0.030 ± 0.002
Benzene, 166	0.175 ± 0.008	0.134 ± 0.007	0.047 ± 0.005
Toluene, 80	0.200 ± 0.020[c]	0.117 ± 0.016	0.040 ± 0.004
Benzene, 166 +Toluene, 80	0.193 ± 0.019[c]	0.169 ± 0.031	0.056 ± 0.007[c]

[a] Chemical(s) was (were) administered continuously to mice via drinking water for 4 weeks

[b] In μg neurochemical/g wet brain tissue, mean ± SE (n=5)

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

TABLE IX-5

CONCENTRATIONS OF HOMOVANILLIC ACID (HVA) IN CEREBRAL CORTEX, MIDBRAIN, AND MEDULLA OBLONGATA OF MICE TREATED WITH BENZENE AND TOLUENE, ALONE OR COMBINED[a]

Concentration in water, mg/L	Neurochemical concentration[b]		
	Cortex	Midbrain	Medulla oblongata
Experiment I			
Control	0.155 ± 0.014	0.129 ± 0.010	0.050 ± 0.007
Benzene, 166	0.218 ± 0.047	0.193 ± 0.068[c]	0.064 ± 0.007
Toluene, 325	0.234 ± 0.044	0.178 ± 0.024	0.054 ± 0.008
Benzene, 166 +Toluene,325	0.211 ± 0.018	0.157 ± 0.012	0.057 ± 0.006
Experiment II			
Control	0.165 ± 0.010	0.163 ± 0.019	0.030 ± 0.005
Benzene, 166	0.220 ± 0.011[c]	0.194 ± 0.014	0.047 ± 0.007[c]
Toluene, 80	0.260 ± 0.021[c]	0.154 ± 0.009	0.040 ± 0.004[c]
Benzene, 166 +Toluene, 80	0.256 ± 0.019[c]	0.220 ± 0.026	0.056 ± 0.004[c]

[a] Chemical(s) was (were) administered continuously to mice via drinking water for 4 weeks

[b] In μg neurochemical/g wet brain tissue, mean ± SE (n=5)

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

TABLE IX-6

CONCENTRATIONS OF 5-HYDROXYINDOLEACETIC ACID (5-HIAA) IN CEREBRAL CORTEX, MIDBRAIN, MEDULLA OBLONGATA, AND CEREBELLUM OF MICE TREATED WITH BENZENE AND TOLUENE, ALONE OR COMBINED[a]

Concentration in water, mg/L	Neurochemical concentration[b]			
	Cortex	Midbrain	Medulla oblongata	Cerebellum
Experiment I				
Control	0.283 ± 0.023	0.550 ± 0.011	0.371 ± 0.036	0.167 ± 0.009
Benzene, 166	0.361 ± 0.019	0.705 ± 0.036[c]	0.456 ± 0.033	0.165 ± 0.003
Toluene, 325	0.336 ± 0.017	0.628 ± 0.030	0.432 ± 0.031	0.162 ± 0.017
Benzene, 166 +Toluene,325	0.344 ± 0.021	0.708 ± 0.025[c]	0.489 ± 0.030	0.178 ± 0.008
Experiment II				
Control	0.245 ± 0.010	0.491 ± 0.020	0.390 ± 0.034	0.135 ± 0.016
Benzene, 166	0.263 ± 0.010	0.657 ± 0.044[c]	0.480 ± 0.020	0.142 ± 0.011
Toluene, 80	0.318 ± 0.020[c]	0.670 ± 0.075[c]	0.513 ± 0.035[c]	0.163 ± 0.010
Benzene, 166 +Toluene, 80	0.305 ± 0.009[c,d]	0.669 ± 0.043[c]	0.449 ± 0.010	0.151 ± 0.003

[a] Chemical(s) was (were) administered continuously to mice via drinking water for 4 weeks

[b] In µg neurochemical/g wet brain tissue, mean ± SE (n=5)

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

[d] Significantly different from benzene treatment alone at $p<0.05$

Recently, several studies have indicated that chemical-induced neurotoxicity might be related to the alterations of certain neurochemical(s) in certain minute brain region(s) of mice or rats (Coulombe and Sharma, 1985; 1986; Sharma et al., 1986).

Significant increases in concentrations of various biogenic amines and their respective metabolites in several brain regions caused by benzene treatment alone would be sufficient to suggest benzene-induced alterations in brain monoamine metabolisms. In rats a four-week prolonged sc administration of benzene has been shown to increase the contents of NE and DA in brain and other internal organs such as spleen, liver, and kidney (Paradowski et al., 1985), and also produced accumulations of both 5-HT and its metabolite, 5-HIAA, in the hypothalamus and corpus striatum (Paradowski et al, 1984). Intraperitoneal administration of benzene to mice caused increased striatal levels of 5-HT and 5-HIAA (Juorio and Yu, 1985a). The mechanism for benzene-induced changes in levels of monoamines and their metabolites is not yet known. Benzene produced an induction of aromatic-L-amino acid decarboxylase (AADC) but showed no effect on monoamine oxidase (Juorio and Yu, 1985a). However, due to its high enzymatic activity, the induction of AADC may not result in significant changes of endogenous monoamine levels (Cooper et al., 1986). Further investigations are needed to determine whether benzene alters the kinetics of the rate-limiting enzyme, tyrosine hydroxylase and tryptophan hydroxylase, in the catecholamine and indoleamine metabolic pathways, respectively.

Toluene ingestion also caused dramatic effects on brain monoamine metabolisms. Several studies have indicated that concentrations of

various brain biogenic amines are affected by acute or subchronic administration of toluene via inhalation or ip injection to rats. Most reports showed that brain NE increased while DA increased or remained unaltered after toluene treatment (Rea et al., 1984; Andersson et al., 1980; 1983; Arito et al., 1984; 1985). Significant alterations in brain concentrations of 5-HT were also reported (Rea et al., 1984; Arito et al., 1984; 1985). We have recently demonstrated that toluene-induced increases in CNS neurotransmitter metabolisms exhibited a biphasic dose-effect in mice (Chapter V), and that toluene, by significantly increasing concentrations of hypothalamic NE and a parallel trend in its major metabolite VMA, markedly stimulated the hypothalamic-pituitary-adrenocorticol (HPA) axis, resulting in an elevated serum corticosterone and a decreased immune function (Chapter VII).

Increases in the regional concentrations of brain neurochemicals produced by the combined exposures, compared to the untreated control, indicate the absence of protective effect on monoamine-related neurotoxicity between benzene and toluene, at least at the doses administered in this study. Toluene has been shown to alleviate benzene-induced hematotoxicity (Andrews et al., 1977; Tunek et al., 1981; 1982; Li et al., 1986), genotoxicity (Tice et al., 1982, Gad-El-Karim et al., 1984; 1985a; 1986), and immunotoxicity (Chapter VIII). Contrarily, the fact that concurrent administration of two compounds raised the concentrations of biogenic amine metabolites in few instances when compared to either chemical treatment alone suggests that there are marginal additive effects of the combined treatment.

One possible explanation for these phenomena is that toluene competitively inhibits the biotransformation of benzene (Ikeda et al.,

1972; Andrews et al., 1977; Sato and Nakajima, 1979; Gilmour et al., 1986). When benzene and toluene were administered in combination to rats intraperitoneally, their disappearance rates from blood and the rates of urinary excretion of their metabolites were delayed compared with those observed when they were given separately (Sato and Nakajima, 1979). The prolonged half-life of both benzene and toluene, which have been reported to be readily taken up by the CNS and possess high ability to modify membrane environments (Baker et al., 1985), may render CNS cell more susceptible to the toxic effects of these organic solvents. The effects of two or more solvents in combination may be potentiating rather than additive (Savolainen, 1977).

However, the possible role of oxidative metabolism and tissue binding in the CNS has been suggested as a factor for benzene and toluene in particular (Savolainen, 1977; Baker et al., 1985). The decreased concentrations of regional brain monoamines and their metabolites produced by phenol, a major metabolite of benzene, following a four-week repeated exposure in mice (Hsieh et al., 1988) together with the reported delayed excretion of this benzene metabolite caused by a sufficient amount of toluene (Sato and Nakajima, 1979) support the findings in the present study. Additional research on *in vivo* neurobehavioral assays, pharmacokinetics, and nervous system molecular events may provide relevant information to realistically evaluate neurotoxic mechanisms of benzene-toluene interaction.

SUMMARY AND CONCLUSION

Benzene and toluene are major aromatic hydrocarbon compounds with enviromental and occupational significance. The basic immunotoxic and neurotoxic mechanisms of these chemicals and their interactions were evaluated in this study by utilizing various immunological and neuro-endocrinobiochemical parameters in CD-1 mice continuously exposed to 0, 31, 166, and 790 mg/L benzene and 0, 17, 80, and 405 mg/L toluene, respectively, in drinking water for four weeks.

Benzene exposure caused a dose-dependent reduction in numbers of peripheral blood leukocytes, lymphocytes, erythrocytes and resulted in a severe macrocytic anemia. Splenic lymphocyte proliferation to both B cell and T cell mitogens (lipopolysaccharide, pokeweed mitogen, concanavalin A, and phytohemagglutinin) was followed by a dose-related biphasic responsiveness, enhanced at the lowest dose (31 mg/L) and depressed in the higher dosage groups (166 and 790 mg/L). Cell-mediated immunity as measured by mixed-lymphocyte culture response to allogeneic cells and cytotoxic lymphocyte activity to YAC-1 tumor cells exhibited a similar biphasic phenomenon. Antibody production as assessed by enumeration of sheep red blood cell (SRBC)-specific plaque-forming cells (PFC) indicated a significant suppression of PFC in animals exposed to 166 and 790 mg/L benzene. A decrease in anti-SRBC antibody titer corresponded to the numbers of PFC. The findings indicate that oral ingestion of benzene produced a biologically significant alteration in both humoral and cellular immune responses.

Benzene also induced both synthesis and catabolism of regional brain monoamine neurotransmitters. In the hypothalamus, the brain region

richest in NE, concentrations of NE increased by 40, 58 and 61% when mice received doses of 31, 166 and 790 mg/L benzene, respectively. Significant increases of NE were also observed in the medulla oblongata and cerebellum. Dopamine concentrations increased significantly in the hypothalamus and corpus striatum. Increases of catecholamine metabolites were seen in a number of brain regions: midbrain (DOPAC), corpus striatum (VMA, DOPAC, HVA), cerebral cortex (VMA) and cerebellum (VMA). Benzene treatment significantly increased 5-HT concentrations in the hypothalamus, corpus striatum, midbrain, cerebral cortex and medulla oblongata. Concomitant with increases of 5-HT, 5-HIAA increased in hypothalamus, corpus striatum, midbrain, cerebral cortex and medulla oblongata.

Toluene is not as strong an immunotoxicant as its analogue benzene. No effects on hematological parameters, including erythrocytes, leukocytes and their differentials were noticed. Splenocyte lymphoproliferation to alloantigens decreased at the 405 mg/L toluene dose only. Numbers of SRBC-specific PFC decreased in the 405 mg/L dosed animals, however, no significant change was observed in anti-SRBC antibody level. Toluene (405 mg/L) also adversely affected IL-2 synthesis. It appeared that suppresion of immune functions of mice ingesting toluene was generally evident at relatively high doses, except for splenic lymphocyte responses to selected mitogens.

The maximum toluene-induced increases in brain biogenic amines and their metabolites occurred at a toluene concentration of 80 mg/L. In the hypothalamus, the concentrations of NE significantly increased by 51, 63, and 34% in groups dosed with 17, 80, and 405 mg/L toluene, respectively. Increases of NE were also observed in the medulla

oblongata and midbrain. Correspondingly, concentrations of VMA increased in various brain regions. Concentrations of DA were higher in the corpus striatum and hypothalamus. Alterations in levels of the DA metabolites, DOPAC and HVA, were marginal. Toluene increased 5-HT in all dissected brain regions except cerebellum, and 5-HIAA levels in the hypothalamus, corpus striatum and cerebral cortex. Noradrenergic and serotonergic neurons appeared to be highly vulnerable to toluene while dopaminergic neurons were rather resistant.

The concentrarions of hypothalamic NE and VMA, plasma ACTH and serum corticosterone were increased following both chemical exposures. Comparisons of benzene-treated mice with appropriate-time-controls revealed that corticosterone levels were significantly higher in mice after 7 days (166 and 790 mg/L benzene) and at 28 days (790 mg/L of benzene). Toluene elevated corticosterone levels at 14 and 28 days at the 405 mg/L exposure. IL-2 production was suppressed in the two higher benzene-treated groups, while toluene decreased IL-2 synthesis at the 405 mg/L concentration only. Results indicated that both benzene and toluene ingestion stimulated the hypothalamic-pituitary-adrenocortical (HPA) axis, resulting in elevation of corticosterone which has been reported to inhibit IL-2 production and impair immunocompetence.

Toxicity of environmental pollutants may be expressed as combined effects of the chemicals. Benzene frequently occurs in a cocontaminated enviroment with toluene, a competitive inhibitor of the biotransformation of benzene. When coadministered with benzene (166 mg/L), toluene (325 mg/L) completely inhibited benzene-induced immunotoxicity, i.e., involution of thymic mass and suppressions of both B- and T-cell mitogeneses, mixed lymphocyte culture response to

alloantigens, the ability of cytotoxic lymphocytes to lyse tumor cells, antibody production response to SRBC, and IL-2 secretion by Con A-stimulated mouse T-cells. However, the low dose of toluene (80 mg/L) did not protect against benzene-induced depressions of immune function. The results demonstrate that toluene, in sufficient amounts, has an antagonistic effect on benzene immunotoxicity. When compared to the untreated controls, mice given the combined treatments of benzene and toluene exhibited elevated regional concentrations of brain amines and their metabolites in several brain regions. Increased concentrations of biogenic amine metabolites in a few brain regions were greater in the combined exposures of benzene and toluene than when either chemical was used alone; there was a marginal additive effect between these two types of neurotoxicants.

The present study demonstrates that both benzene and toluene possess the potential for inducing immunotoxic and neurotoxic effects in CD-1 mice following four weeks of continuous exposure via drinking water. Of particular importance was the fact that the neurotoxic effects were evident at the lowest dose of benzene or toluene treatment, and a no-effect level could not be established. Further studies involving lower levels of exposure are necessary to fully assertain the safety of these compounds in drinking water. The results of combination studies suggest that benzene immunotoxicity is mainly due to action of the reactive metabolites, whereas benzene neurotoxicity results from benzene itself. In addition, both benzene and toluene have, at least partially, an additive negative effect on immune function via the activated HPA axis.

REFERENCES

ABOU-DONIA, M. B., LAPADULA, D. M., CAMPBELL, G., AND ABODO, K. M. (1985a). The joint neurotoxic action of inhaled methyl butyl ketone vapor and dermally applied *O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate in hens: Potentiating effect. *Toxicol. Appl. Pharmacol.* 79, 69-82.

ABOU-DONIA, M. B., LAPADULA, D. M., CAMPBELL, G., AND TIMMONS, P. R. (1985b). The synergism of *n*-hexane-induced neurotoxicity by methyl isobutyl ketone following subchronic (90 days) inhalation in hens: Induction of hepatic cytochrome p-450. *Toxicol. Appl. Pharmacol.* 81, 1-16.

ADER, R. (1981). *Psychoneuroimmunology*. Academic Press, New York.

ADER, R., AND COHEN, N. (1985). CNS-immune system interactions: Conditioning phenomena. *Behavior Brain Sci.* 8, 379-394.

AKSOY, M. (1985). Benzene as a leukemogenic and carcinogenic agent. *Am. J. Ind. Med.* 8, 9-20.

ALLEN, T. D., AND DEXTER, T. D. (1984). The essential cells of the hemopoietic microenvironment. *Exp. Hematol.* 12, 517-521.

ANDERSSON, K., FUSE, K., TOFTGARD, R., NILSEN, O. G., ENEROTH, P., AND GUSTAFSSON J. A. (1980). Toluene-induced activation of certain hypothalamic and median eminence catecholamine nerve terminal systems of the male rat and its effects on anterior pituitary hormone secretion. *Toxicol. Lett.* 5, 393-398.

ANDERSSON, K., NILSEN, O. G., TOFTGARD, R., ENEROTH, P., GUSTAFSSON, J.A., BATTISTINI, N., AND AGNATI, L. F. (1983). Increased amine turnover in several noradrenaline terminal systems and changes in

prolactin secretion in the male rat by exposure to various concentrations of toluene. *Neurotoxicology* 4, 43-48.

ANDREWS, L. S., LEE, E. W., WITMER, C. M., KOCSIS, J. J., AND SNYDER, R., (1977). Effects of toluene on metabolism, disposition, and hematopoietic toxicity of [^{3}H]-benzene. *Biochem. Pharmacol.* 26, 293-300.

ANDREWS, L. S., SASAME, H. A., AND GILLETTE, J. R. (1979). [^{3}H]-benzene metabolism in rabbit bone marrow. *Life Sci.* 25, 567-572.

AOYAMA, K. (1986). Effects of benzene inhalation on lymphocyte subpopulations and immune response in mice. *Toxicol. Appl. Pharmacol.* 85, 92-101.

ARANYI, C., O'SHEA, W. J., GRAHAM, J. A., AND MILLER, F. J. (1986). The effects of inhalation of organic chemical air contaminants on murine lung host defenses. *Fundam. Appl. Toxicol.* 6, 718-720.

ARANYI, C., O'SHEA, W. J., SHERWOOD, R. L., GRAHAM, J. A., AND MILLER, F. J. (1985). Effects of toluene inhalation on pulmonary host defenses of mice. *Toxicol. Lett.* 25, 103-110.

ARITO, H., TSURUTA, H., AND NAKAGAKI, K. (1984). Acute effects of toluene on circadian rhythms of sleep-wakefulness and brain monoamine metabolism in rats. *Toxicology* 33, 291-301.

ARITO, H., TSURUTA, H., NAKAGAKI, K., AND TANAKA, S. (1985). Partial insomnia, hyperactivity and hyperdipsia induced by repeated administration of toluene in rats: Their relation to brain monoamine metabolism. *Toxicology* 37, 99-110.

ARTELLINOI, G., GRILLI, S., CALACCI, A., MAZZULLO, M., AND PRODI, G. (1985). *In vivo* and *in vitro* binding of benzene to nucleic acids and proteins of various rat and mouse organs. *Cancer Lett.* 28, 159-168.

AXELROD, J., AND REISINE, T. D. (1984). Stress hormones: Their interaction and regulation. *Science* 224, 452-459.

BAARSON, K. A., SNYDER, C. A., AND ALBERT, R. E. (1984). Repeated exposure of C57Bl mice to inhaled benzene at 10 ppm markedly depressed erythropoietic colony. *Toxicol. Lett.* 20, 337-342.

BAKER, E. L., SMITH, T. J., AND LANDRIGAN, P. J. (1985). The neurotoxicity of industrial solvents: A review of the literature. *Am. J. Ind. Med.* 8, 207-217.

BAKKE, O. M., AND SCHELINE, R. R. (1970). Hydroxylation of aromatic hydrocarbons in the rat. *Toxicol. Appl. Pharmacol.* 16, 691-700.

BASLO, A., AND AKSOY, M. (1982). Neurological abnormalities in chronic benzene poisoning. A study of six patients with aplastic anemia and two with preleukemia. *Environ. Res.* 27, 457-465.

BENIGNUS, V. A. (1981a). Health effects of toluene--A review. *Neurotoxicology* 2, 567-588.

BENIGNUS, V. A. (1981b). Neurobehavioral effects of toluene--A review. *Neurobehav. Toxicol. Teratol.* 3, 407-415.

BESEDOVSKY, H. O., DEL REY, A. E., AND SORKIN, E. (1985). Immune neuroendocrine interactions. *J. Immunol.* 135, 750s-754s.

BICK, P. H. (1982). Immune system as a target organ for toxicity, *Environ. Health Perspec.* 43, 3-7.

BICK, P. H., HOLSAPPLE, M. P. AND WHITE, K. L. (1985). Assessment of the effects of chemicals on the immune system. In: Li AP (ed) *New Approaches in Toxicity Testing and Their Application in Human Risk Assessment*. pp. 165-177. Raven Press, New York.

BLECHA, F., AND BAKER, P. E. (1986). Effect of cortisol in *in vitro* and *in vivo* production of bovine interleukin 2. *Am. J. Vet. Res.* 47,

841-845.

BOLCSAK, L. E., AND NERLAND, D. E. (1983). Inhibition of erythropoiesis by benzene metabolites. *Toxicol. Appl. Pharmacol.* 69, 363-368.

BONDY, S. C. (1985). Especial considerations for neurotoxicological research. *CRC Critical Rev. Toxicol.* 14, 381-401.

BONDY, S. C. (1986). The biochemical evaluation of neurotoxic damage. *Fundam. Appl. Toxicol.* 6, 208-216.

BRIEF, R. S., LYNCH, J., BERNATH, T., AND SCALA, R. A. (1980). Benzene in the workplace. *Am. Ind. Hyg. Assoc. J.* 41, 616-623.

CELANI, M. F., FUXE, K., AGNATI, L. F., ANDERSSON, K., HANSSON, T., GUSTAFSSON, J.A., BATTISTINI, N., AND ENEROTH, P. (1983). Effects of subacute treatment with toluene on central monoamine receptors in the rat. Reduced affinity in [^{3}H]5-hydroxytryptamine binding sites and in [3-H]spiperone binding sites linked to dopamine receptors. *Toxicol. Lett.* 17, 275-282.

CLAMAN, H. N. (1972). Corticosteroids and lymphoid cells. *New Engl. J. Med.* 287, 388-397.

COMMISSIONG, J. W. (1985). Monoamine metabolites: their relationship and lack of relationship to monoaminergic neuronal activity. *Biochem. Pharmacol.* 34, 1127-1131.

COOPER, JR., BLOOM, F. E., AND ROTH, R. H. (1986). *The Biochemical Basis of Neuropharmacology*. Oxford University Press, New York.

COULOMBE, R. A., AND SHARMA, R. P. (1985). Effect of repeated dietary exposure of aflatoxin B1 on brain biogenic amines and metabolites in rat. *Toxicol. Appl. Pharmacol.* 80, 496-501.

COULOMBE, R. A., AND SHARMA, R. P. (1986). Neurobiochemical alterations

induced by the artificial sweetner aspartame (NutraSweet). *Toxicol. Appl. Pharmacol.* 83, 79-85.

COURTNEY, K. D., ANDREWS, J. E., SPRINGER, J., MENACHE, M., WILLIAMS, T., DALLEY, L., AND GRAHAM, J.A. (1986). A perinatal study of toluene in CD-1 mice. *Fundam. Appl. Toxicol.* 6, 145-154.

CRONKITE, E. P., BULLIS, J. E., INOUE, T., AND DREW, R. T. (1984). Benzene inhalation produces leukemia in mice. *Toxicol. Appl. Pharmacol.* 75, 358-361.

CRONKITE, E. P., DREW, R. T., INOUE, T., AND BULLIS, J. E. (1985). Benzene hematotoxicity and leukemogenesis. *Am. J. Ind. Med.* 7, 447-456.

CRONKITE, E. P., INOUE, T., CARSTEN, A. L., MILLER, M. E., AND BULLIS, J. E. (1982). Effects of benzene inhalation on murine pleuripotential stam cells. *J. Toxicol. Environ. Health* 9, 411- 421.

CUNNINGHAM, A. J., AND SZENBERG, A. (1968). Further improvements in the plaque technique for detecting single antibody producing cells. *Immunology* 14, 599-601.

CUPPS, T. R., GERRARD, T. L., FALKOFF, R. J. M., WHALEN, G., AND FAUCI, A. S. (1985). Effects of *in vitro* corticosteroids on B cell activation, proliferation, and differentiation. *J. Clin. Invest.* 75, 754-761.

DALLMAN, M. F., AKANA, S. F., CASCIO, C. S., DARLINGTON, D. N., JACOBSON, L., AND LEVIN, N. (1987). Regulation of ACTH secretion: variations on a theme of B. *Recent Prog. Horm. Res.* 43, 113-173.

DAYER, J. M., SUNDSTROM, L., POLLA, B. S., AND JUNOD, A. F. (1985). Cultured human alveolar macrophages from smokers with lung cancer: resolution of factors that stimulate fibroblast proliferation,

production of collagenase, or prostaglandin E_2. *J. Leukocyte Biol.* 37, 641-649.

DEAN, B. J. (1985). Recent findings on the genetic toxicology of benzene, toluene, xylenes and phenols. *Mutation Res.* 154, 153-181.

DEAN, J. H., LUSTER, M. I., BOORMAN, G. A., AND LAUER L. D. (1982). Procedures available to examine the immunotoxicity of chemicals and drugs. *Pharmacol. Rev.* 34, 137-151.

DEAN, J. H., LUSTER, M. I., MUNSON, A. E., AND AMOS, H. (1985a). *Immunotoxicology and Immunopharmacology.* Raven Press, New York.

DEAN, J. H., MURRAY, M. J., AND WARD, E. C. (1986). Toxic responses of the immune system. In *Casarett and Doull's Toxicology: The basic science of poisons,* 3rd ed. (C. D. Klaassen, M. O. Amdur and J. Doull, eds.). pp. 245-285. Macmillan, New York.

DEAN, J. H., WARD, E. C., MURRAY, M. J. LAUER, L. D., AND HOUSE, R. V. (1985b). Mechanisms of dimethylbenzanthracene-induced immunotoxicity. *Clin. Physiol. Biochem.* 3, 98-110.

DEMPSTER, A. M., EVANS, H. L., AND SNYDER, C. A. (1984). The temporal relationship between behavioral and hematological effects of inhaled benzene. *Toxicol. Appl. Pharmacol.* 76, 195-203.

DINARELLO, C. A., AND MIER, J. W. (1986). *Interleukins. Ann. Rev. Med.* 37, 173-178.

DOWDY, S., AND WEARDEN, S. (1983). *Statistics for Research.* John Wiley & Sons, New York.

DUNN, A. J., AND KRAMARCY, N. R. (1984). Neurochemical responses in stress: Relationships between the hypopthalamic-pituitary-adrenal and catecholamine systems. In *Handbook of Psychopharmacology* (L. L. Iversen, S. D. Iversen, S. H. Snyder, Eds.). Vol. 18, pp. 455-515.

Plenum, New York.

DYER, R. S., MULLER, K. E., JANSSEN, R., BARTON, C., BOYES, W. K., AND BENIGNUS, V. A. (1984). Neurophysiological effects of 30 day chronic exposure to toluene in rats. *Neurobehav. Toxicol. Teratol.* 6, 363-368.

EASTMOND, D. A., FRENCH, R. C., ROSS, D., AND SMITH, M. T. (1987a). Metabolic activation of 1-naphthol and phenol by a simple superoxide-generating system and human leukocytes. *Chem.-Biol. Interact.* 63, 47-62.

EASTMOND, D. A., SMITH, M. T., AND IRONS, R. D. (1987b). An interaction of benzene metabolites reproduces the myelotoxicity observed with benzene exposure. *Toxicol. Appl. Pharmacol.* 91, 85-95.

EASTMOND, D. A., SMITH, M. T., RUZO, L. O., AND ROSS, D. (1986). Metabolic activation of phenol by human myeloperoxidase and horseradish peroxidase. *Mol. Pharmacol.* 30, 674-679.

ERESON, G. L., WILMER, J. L., AND KLIGERMAN, A. W. (1985). Sister chromatid exchange induction in human lymphocytes exposed to benzene and its metabolites *in vitro*. *Cancer Res.* 45, 2471-2477.

ESTERLING, B., AND RABIN, B. S. (1987). Stress-induced alteration of T-lymphocyte subsets and humoral immunity in mice. *Behavior. Neurosci.* 101, 115-119.

EVANS, H. L., DEMPSTER, A. M., AND SNYDER, C. A. (1981). Behavioral changes in mice following benzene inhalation. *Neurobehav. Toxicol. Teratol.* 3, 481-485.

EXON, J. H., TALCOTT, P. A., AND KOLLER, L. D. (1983). Effect of lead, polychlorinated biphenyls, and cyclophosphamide on rat natural killer cells, interleukin 2, and antibody synthesis. *Fundam. Appl. Toxicol.*

5, 158-164.

FAIMAN, M. D., MYERS, N. B., AND SCHOWEN, R. L. (1973). Post-mortem degradation kinetics of norepinephrine. *Biochem. Pharmacol.* 22, 2171-2181.

FAITH, R. E., LUSTER, M. I., AND VOS, J. G. (1980). Effects on immunocompetence by chemicals of environmental concern. *Ann. Rev. Biochem. Toxicol.* 2, 173-211.

FARRAR, J. J., BENJAMIN, W. R., HILFIKER, M. L., HOWARD, M., FARRAR, W. L., AND FULLER-FARRAR, J. (1982). The biochemistry, biology, and role of interleukin 2 in the induction of cytotoxic T cell and antibody-forming B cell responses. *Immunol. Rev.* 63, 129-166.

FARRAR, W. L., CLEVELAND, J. L., BECKNER, S. K., BONVINI, E., AND EVANS, S. W. (1986). Biochemical and molecular events associated with interleukin 2 regulation of lymphocyte proliferation. *Immunol. Rev.* 92, 49-65.

FISHBEIN, L. (1984). An overview of environmental and toxicological aspects of aromatic hydrocarbons. I. Benzene. *Sci. Total Environ.* 40, 189-218.

FISHBEIN, L. (1985). An overview of environmental and toxicological aspects of aromatic hydrocarbons II. toluene. *Sci. Total Environ.* 42, 267-288.

FRANZ, T. J. (1983). Percutaneous absorption of benzene. *Adv. Mod. Environ. Toxicol.* 7, 61-70.

FRASH, V. N., YUSHKOV, B. G., KARAULOV, A. V., AND SKURATOV, V. L. (1976). Mechanism of action of benzene on hematopoiesis. Investigation of hematopoietic stem cells. *Bull. Exp. Biol. Med.* 82, 985-987.

GAD-EL-KARIM, M. M., HARPER, B. L., AND LEGATOR, M. S. (1984). Modifications in the myeloclastogenic effect of benzene in mice with toluene, phenobarbital, 3-methylcholanthrene, aroclor 1254 and SKF-525A. *Mutation Res.* 135, 225-243.

GAD-EL-KARIM, M. M., SADAGOPA RAMANUJAM, V. M., AHMED, A. E., AND ANDLEGATOR, M. S. (1985a). Benzene myeloclastogenicity: a function of tis metabolism. *Amer. J. Ind. Med.* 7, 475-484.

GAD-EL-KARIM, M. M., SADAGOPA RAMANUJAM, V. M., AND LEGATOR, M. S. (1985b). Ironstrans-Muconic acid, an open-chair urinary metabolite of benzene in mice. Quantification by high-pressure liquid chromatogtraphy. *Xenobiotica* 15, 211-220.

GAD-EL-KARIM, M. M., SADAGOPA RAMANUJAM, V. M., AND LEGATOR, M. S. (1986). Correlation between the induction of micronuclei in bone marrow by benzene exposure and the excretion of metabolites in urine of CD-1 mice. *Toxicol. Appl. Pharmacol.* 85, 464-477.

GAIDO, K., AND WIERDA, D. (1984). *In vitro* effects of benzene metabolites on mouse bone marrow stomal cells. *Toxicol. Appl. Pharmacol.* 76, 45-55.

GAIDO, K., AND WIERDA, D. (1985). Modulation of stromal cell function in DBA/2J and B6C3F1 mice exposed to benzene or phenol. *Toxicol. Appl. Pharmacol.* 81, 469-475.

GAIDO K. W., AND WIERDA, D. (1987). Suppression of bone marrow stromal cell function by indomethacin. *Toxicol. Appl. Pharmcol.* 89, 378-390.

GANONG, W. F. (1980). Neurotransmitters and pituitary function: Regulation of ACTH secretion. *Fed. Proc.* 39, 2923-2930.

GARNETT, H. M., CRONKITE, E. P., AND DREW, R. T. (1983). Effect of *in vivo* exposure to benzene on the characteristics of bone marrow

adherent cells. *Leukemia Res.* 7, 803-810.

GENTILE, P. S., AND PELUS, L. M. (1987). *In vivo* modulation of myelopoiesis by prostaglandin E_2. II. Inhibition of granulocyte-monocyte progenitor cell (CFU-GM) cycle rate. *Exp. Hematol.* 15, 119-126.

GILL, D. P., AND AHMED, A. (1981). Covalent binding of [^{14}C]benzene to cellular organelles and bone marrow nucleic acids. *Biochem. Pharmacol.* 30, 1127-1131.

GILL, D. P., JENKINS, V. R., KEMPER, R. R., AND ELLIS, S. (1980). The importance of pluripotential stem cells in benzene toxicity. *Toxicology* 16, 163-171.

GILLIS, S., CRABTREE, G. R., AND SMITH, K. A. (1979a). Glucocorticoid-induced inhibition of T cell growth factor production. I. The effect on mitogen-induced lymphocyte proliferation. *J. Immunol.* 123, 1624-1631.

GILLIS, S., CRABTREE, G. R., AND SMITH, K. A. (1979b). Glucocorticoid-induced inhibition of T cell growth factor production. II. The effect on the *in vitro* generation of cytolytic T cells. *J. Immunol.* 123, 1632-1638.

GILLIS, S., FERM, M. M., WINNY, O., AND SMITH, K. A. (1978). T cell growth factor: Parameters of production and a quantitative microassay for activity. *J. Immunol.* 120, 2027-2032.

GILMOUR, S. K., KALF, G. F., AND SNYDER, R. (1986). Comparison of the metabolism of benzene and its metabolite phenol in rat liver microsomes. *Adv. Exp. Med. Biol.* 197, 223-235.

GLOWINSKI, J., AND IVERSEN, L. L. (1966). Regional studies of catecholamines in the rat brain. *J. Neurochem.* 13, 655-669.

GOLDSTEIN, B. D. (1983). Clinical hematoxicity of benzene. *Adv. Mod. Environ. Toxicol.* 4, 51-61.

GOLLMER, L., GRAF, H., AND ULLRICH, V. (1984). Characterization of the benzene monooxygenase system in rabbit bone marrow. *Biochem. Pharmacol.* 33, 3597-3602.

GONASUN, L. M., WITMER, C. M., KOCSIS, J. J., AND SNYDER, R. (1973). Benzene metabolism in mouse liver microsomes. *Toxicol. Appl. Pharmacol.* 26, 398-406.

GOODWIN, J. S., ATLURU, D., SIERAKOWSKI, S., AND LIANOS, E. A. (1986). Mechanism of action of glucocorticosteroids: Inhibition of T cell proliferation and interleukin 2 production by hydrocortisone is reversed by leukotriene B_4. *J. Clin. Invest.* 77, 1244-1250.

GORSKY, L. D., AND COON, M. J. (1985). Evaluation of the role of free hydroxyl radicals in the cytochrome p-450 catalyzed oxidation of benzene and cyclohexanol. *Drug Metab. Dispos.* 13, 169-174.

GRABSTEIN, K. (1980). Cell-mediated cytolytic responses, In *Selected Methods in Cellular Immunology* (B. B., Mishell and S. M. Shiigi, Eds.). pp. 124-137, W. H. Freeman and Company. San Francisco.

GRASSO, P., SHARRATT, M., DAVIES, D. M., AND IRVINE, D. (1984). Neurological and psychological disorders and occupational exposure to organic solvents. *Fd. Chem. Toxicol.* 22, 819-852.

GREEN, J. D., LEONG B. K. J., AND SIENDY, L. (1978). Inhaled benzene fetotoxicity in rats. *Toxicol. Appl. Pharmacol.* 46, 9-18.

GREEN, J. D., SNYDER, C. A., LOBUE, J., GOLDSTEIN, B. D., AND ALBERT, R. E. (1981a). Acute and chronic dose/response effect of benzene inhalation on the peripheral blood, bone marrow, and spleen cells of CD-1 male mice. *Toxicol. Appl. Pharmacol.* 59, 204-214.

GREEN, J. D., SNYDER, C. A., LOBUE, J., GOLDSTEIN, B. D., AND ALBERT, R. E. (1981b). Acute and chronic dose/response effects of inhaled benzene on multipotential hemopoietic stem (CFU-S) and granulocyte/macrophage progenitor (GM-CFU-C) cells in CD-1 mice. *Toxicol. Appl. Pharmacol.* 58, 492-503.

GREENLEE, W. F., GROSS, E. A., AND IRONS, R. D. (1981a). Relationship between benzene toxicity and the disposition of ^{14}C-labeled benzene metabolites in the rat. *Chem.-Biol. Interact.* 33, 285-299.

GREENLEE, W. F., SUN, J. D., AND BUS, J. S. (1981b). A proposed mechanism of benzene toxicity: formation of reactive intermediates from polyphenol metabolites. *Toxicol. Appl. Pharmacol.* 59, 187-195.

HALEY, T. (1977). Evaluation of the health effects of benzene inhalation. *Clin. Toxicol.* 11, 531-548.

HANAHAN, D. J., AND EKHOLM, J. E. (1974). The preparation of red cell ghosts (membranes). *Meth. Enzm. vol.* XXXI, 168-172.

HARIGAYA, K., MILLER, M. E., CRONKITE, E. P., AND DRIW, R. T. (1981). The detection of *in vivo* hematotoxicity of benzene by *in vitro* liquid bone marrow cultures. *Toxicol. Appl. Pharmacol.* 60, 346-353.

HARY, L., DUPOUY, J. P., AND CHATELAIN, A. (1984). Effect of norepinephrine on the pituitary adrenocorticotrophic activation by ether stress and on the *in vitro* release of ACTH by the adenohypophysis of male and female newborn rats. *Neuroendocrinology* 39, 105-113.

HAYDEN, J. W., PETERSON, R. J., AND BRUCKNER, J. V. (1977). Toxicology of toluene (methylbenzene): review of current literature. *Clin. Toxicol.* 114, 549-559.

HEDGE, G. A., VAN REE, J. M., AND VERSTEEG, D. H. G. (1976).

Correlation between hypothalamic catecholamine synthesis and ether stress-induced ACTH secretion. *Neuroendocrinology* 21, 236-246.

HEMLER, M. E., AND LANDS, E. M. (1980). Evidence for a peroxide-initiated free radical mechanism of prostaglandin biosynthesis. *J. Biol. Chem.* 255, 6253-6261.

HINSON, J. A., FREEMAN, J. P., POTTER, D. W., AND MITCHUM, R. K. (1984). Evidence for NIH shift in the microsomal metabolism of benzene to phenol. *Fed. Proc.* 43, 544.

HINSON, J. A., FREEMAN, J. P., POTTER, D. W., MITCHUM, R. K., AND EVANS, F. E. (1985). Mechanism of the microsomal metabolism of benzene to phenol. *Mol. Pharmacol.* 27, 574-577.

HIRAFUGI, M., AND OGURA, Y. (1985). Lipid peroxidation modifies the effect of phenolic anti-inflammatory drugs on prostaglandin biosynthesis. *Biochem. Pharmacol.* 34, 933-936.

HOBARA, T., KOBAYASHI, H., HIGASHIHARA, E., KAWAMOTO, T., AND SAKAI, T. (1984). Acute effects of 1,1,1-trichloroethane, trichloroethylene, and toluene on the hematologic parameters in dogs. *Arch. Environ. Contam. Toxicol.* 13, 589-593.

HOLMBERG, B., AND LUNDBERG, P. (1985). Benzene: standards, occurrence, and exposure. *Am. J. Ind. Med.* 7, 375-384.

HSIEH, G. C., SHARMA, R. P. AND PARKER, R. D. R. (1988). Effects of phenol on regional brain catecholamine and indoleamine metabolisms (unpublished data).

IKEDA, M., OHTSUJI, H., AND IMAMURA, T. (1972). *In vivo* suppression of benzene and styrene oxidation by co-administered toluene in rats and effects of phenobarbitol. *Xenobiotica* 2, 101-106.

INFANTE, P. F., AND WHITE, M. C. (1985). Projections of leukemia risk

associated with occupational exposure to benzene. *Am. J. Ind. Med.* 7, 403-414.

IRONS, R. D. (1985). Quinones as toxic metabolites of benzene. *J. Toxicol. Environ. Health* 16, 673-678.

IRONS, R. D., DENT, J. G., BAKER, T. S., AND RICKERT, D. E. (1980). Benzene is metabolized and covalently bound in bone marrow in situ. *Chem.-Biol. Interact.* 30, 241-245.

IRONS, R. D., HECK, H. D'A., MOORE, B. J., AND MUIRHEAD, K. A. (1979). Effects of short-term benzene administration on bone marrow cell cycle kinetics in the rat. *Toxicol. Appl. Pharmacol.* 51, 399-409.

IRONS, R. D., NEPTUN, D. A., AND PFEIFER, R. W. (1981). Inhibition of lymphocyte transformation and microtubule assembly by quinone metabolites of benzene: evidence for a common mechanism. *J. Reticuloendothel. Soc.* 30, 359-372.

IRONS, R. D., PFEIFER, R. W., AUNE, T. M., AND PIERCE, C. W. (1984). Soluble immune response suppressor inhibits microtubule function *in vivo* and microtubule assembly *in vitro*. *J. Immunol.* 133, 2032-2036.

JERINA, D., DALY, J., WITKOP, B., ZALTZMAN-NIRENBERG, P., AND UNDERFRIEND, S. (1968). Role of the arene oxide-oxepin system in the metabolism of aromatic substrates. I. *In vitro* conversion of benzene oxide to a premercapturic acid and a dihydrodiol. *Arch. Biochem. Biophys.* 128, 176-183.

JERNE, N. K., AND NORDIN, A. A. (1963). Plaque formation in agar by single antibody-producing cells. *Science* 140, 405-407.

JOHANSSON, I., AND INGELMAN-SUNDBERG, M. (1983). Hydroxyl radical-mediated cytochrome p-450 dependent metabolic activation of benzene in microsomes and reconstituted enzyme systems from rabbit liver. *J.*

Biol. Chem. 258, 7311-7316.

JOHNSTON, C. A., SPINEDI, E. J., AND NEGRO-VILAR, A. (1985). Effect of acute ether stress on monoamine metabolism in median eminence and discrete hypothalamic nuclei of the rat brain and on anterior pituitary hormone secretion. *Neuroendocrinology* 41, 83-88.

JUORIO, A. V., AND YU, P. H. (1985a). Effects of benzene and pyridine on the concentration of mouse striatal tryptamine and 5-hydroxytryptamine. *Biochem. Pharmacol.* 34, 3774-3776.

JUORIO, A. V., AND YU, P. H. (1985b). Effects of benzene and other organic solvents on the decarboxylation of some brain aromatic-L-aminoacids. *Biochem. Pharmacol.* 34, 1381-1387.

KALF, G. F., POST, G. B., AND SNYDER, R. (1987). Solvent toxicology: recently advances in the toxicology of benzene, the glycol ethers, and corbon tetrachloride. *Ann. Rev. Pharmacol. Toxicol.* 27, 399-427.

KALF, G. F., RUSHMORE, T. R., AND SNYDER, R. (1982). Benzene inhibits RNA synthesis in mitochondria from liver and bone marrow. *Chem.-Biol. Interact.* 42, 353-370.

KALF, G. F., SNYDER, R., AND RUSHMORE, T. R. (1985). Inhibition of RNA synthesis by benzene metabolites and their covalent binding to DNA in rabbit bone marrow mitochondria *in vitro*. *Am. J. Int. Med.* 7, 485-492.

KAPLAN, H. S. (1967). On the natural history of the murine leukemias. *Cancer Res.* 27, 1325-1340.

KELLER, K. A., AND SNYDER, C. A. (1986). Mice exposed in utero to low concentrations of benzene exhibit enduring changes in their colony forming hematopoietic cells. *Toxicology* 42, 171-181.

KHAN, W. A., GUPTA, A., SHANKER, U., AND PANDYA, K. P. (1984).

Involvement of iron and free radicals in benzene toxicity. *Biochem. Pharmacol.* 33, 2009-2012.

KING, A. G., LANDRETH, K. S., AND WIERDA, D. (1986). Hydroquinone inhibits bone marrow pre-B cell maturation *in vitro*. *Toxicologist* 6, 169.

KORTE, F., AND KLEIN, W. (1982). Degradation of benzene in environment. *Ecotoxicol. Environ. Safety* 6, 311-327.

KRIEGLER, A. B., BRADLEY, T. R., AND HODGSON, G. S. (1984). The effect of prostaglandins E_1 and E_2 on macrophage progenitor cells with high proliferative potential in mouse bone marrow. *In vitro Blood* 63, 1348-1352.

KUNA, R. A., AND KAPP, R. W. JR. (1981). The embryotoxic/teratogenic potential of benzene vapor in rats. *Toxicol. Appl. Pharmacol.* 57, 1-7.

KURLAND, J. I., AND MOORE, M. A. S. (1977). Modulation of hemopoiesis by prostaglandins. *Exp. Hematol.* 5, 357-373.

LATRIANO, L., GOLDSTEIN, B. D., AND WITZ, G. (1986). Formation of muconaldehyde, an open-ring metabolite of benzene, in mouse liver microsomes: an additional pathway for toxic metabolites. *Proc. Natl. Cad. Sci.* 83, 8356-8360.

LATRIANO, L., ZACCARIA, A., GOLDSTEIN, B. D., AND WITZ, G. (1985). Muconaldehyde formation from ^{14}C-benzene in a hydroxyl radical generating system. *J. Free Radicals Biol. Med.* 1, 363-371.

LEE, E. W., KOCSIS, J. J., AND SNYDER, R. (1974). Acute effect of benzene on ^{59}Fe incorporation into circulating erythrocytes. *Toxicol. Appl. Pharmacol.* 27, 431-436.

LEE, S. D., DOURSON, M., MUKERJEE, D., STARA, J. F., AND KASECKI, J.

(1983). Assessment of benzene health effects in ambient water. *Adv. Mod. Environ. Toxicol.* 4, 91-126.

LEIBOWITZ, S. F. (1986). Brain monoamines and peptides: role in the control of eating behavior. *Federation Proc.* 45, 1396-1403.

LEWIS, S. C., AND HOLDSWORTH, C. E. (1982). Subchronic inhalation toxicity studies of n-heptane and toluene in the rat. *The Toxicologist* 2, 11.

LEWTAS, J. (1985). Development of a comparative potency method for cancer risk assessment of complex mixtures using short-term *in vivo* and *in vitro* bioassays. *Toxicol. Ind. Health* 1, 193-203.

LI, G. L., YIN, S. N., WATANABE, T., NAKATSUKA, H., KASAHARA, M., ABE, H., AND IKEDA, M. (1986). Benzene-specific increase in leukocyte alkaline phosphatase activity in rats exposed to vapors of various organic solvents. *J. Toxicol. Environ. Health* 19, 581-589.

LICHTMAN, M. A. (1981). The ultrastructure of the hemopoietic environment of the marrow: a review. *Exp. Hematol.* 9, 391-410.

LONGACRE, S. L., KOCSIS, J. J., AND SNYDER R. (1981). Influence of strain differences in mice on the metabolism and toxicity of benzene. *Toxicol. Appl. Pharmacol.* 60, 398-409.

LUMPKIN, M. D. (1987). The regulation of ACTH secretion by IL-1. *Science* 238, 452-454.

LUNTE, S., AND KISSINGER, P. (1983). Detection and identification of sulfhydryl conjugates of p-benzoquinone in microsomal incubations of benzene and phenol. *Chem.-Biol. Interact.* 47, 195- 212.

LUTZ, W. K., AND SCHLATTER, C. H. (1977). Mechanism of carcinogenic action of benzene: irreversible binding to rat liver DNA. *Chem.-Biol. Interact.* 18, 241-245.

MAILMAN, R. B., AND DEHAVEN D. L. (1984). Responses of neurotransmitter systems to toxicant exposure. In *Cellular and Molecular Neurotoxicology* (T. Narahashi, ed.). Raven Press, New York.

MAKARA, G. B., PALKOVITS, M., AND SZENTAGOTHAI (1980). The endocrine hypothalamus and the hormonal response to stress. In: *Selye's Guide to Stress Research* (H. Selye, ed.). pp. 280-337. Van Nonstrand Reinhold, New York.

MALTONI, C. (1983). Myths and facts in the history of benzene carcinogenicity. *Adv. Mod. Environ. Toxicol.* 4, 1-16.

MALTONI, C., CONTI, B., AND COTTI, G. (1983). Benzene: a multipotential carcinogen. Result of long-term bioassays performed at the Bologna Institute of Oncology. *Am. J. Ind. Med.* 4, 589-630.

MALTONI, C., CONTI, B., COTTI, G., AND BELPOGGI. F. (1985). Experimental studies on benzene carcinogenicity at the Bologna Institute of Oncology: current results and ongoing research. *Am. J. Ind. Med.* 7, 415-446.

MANN, K., GIESEL, M., FASOLD, H., AND HAASE, W. (1974). Isolation of native microtubules from porcine brain and characterization of SH groups essential for polymerization at the GTP binding sites. *FFBS Lett.* 92, 45-48.

MATSUMOTO, M., KIMURA, K., FUJISAWA, A., UYAMA, O., YONEDA, S., IMAIZUMI, M., WADA, H., AND ABE, H. (1981). Diurnal variations in monoamine contents in discrete brain regions of the mongolian gerbil. *J. Neurochem.* 37: 792-794.

MAYER, G. S., AND SHOUP, R. E. (1983). Simultaneous multiple electrode liquid chromatographic-electrochemical assay for catecholamines, indoleamines and metabolites in brain tissue. *J. Chromatogr.* 255,

533-544.

MERIAN, E., AND ZANDER, M. (1982). Volatile aromatics. In *Handbook of Environmental Chemistry. Vol. 3. Part B. Anthropogenic Compounds* (O. Hutzinger, ed.). pp. 117-161. Springer-Verlag, Berlin.

MITCHELL, C. L. (1976). The design and analysis of experiments for the assessment of drug interactions. *Ann. N.Y. Acad. Sci.* 281, 118.

MITCHELL, C. L. (1982). *Nervous System Toxicology*. Raven Press, New York.

MOHTASHAMIPUR, E., NORPOTH, K., AND HUBER, P. (1985). Effects of ethylbenzene, toluene, and xylene on the induction of micronuclei in bone marrow polychromatic erythrocytes of mice. *Arch. Toxicol.* 58, 106-109.

MOHTASHAMIPUR, E., STRATER, H., TRIEBEL, R., AND NORPOTH, K. (1987). Effects of pretreatment of male NMRI mice with enzyme inducers or inhibitors on clastogenicity of toluene. *Arch. Toxicol.* 60, 460-463.

MOSZCZYNSKY, P. (1980). Cytoenzymatic studies on neutrophils in workers having contact with organic solvents containing benzene, toluene and xylene. *Folia Haematol.* (Leipz). 107, 747-756.

MOSZCZYNSKY, P., AND LISIEWICZ, J. (1984). Occupational exposure to benzene, toluene and xylene and the T lymphocyte functions. *Haematologia* 17, 449-453.

NAALSUND, L. U. (1986). Hippocampal EEG in rats after chronic toluene inhalation. *Acta. Pharmacol. Toxicol.* 59, 325-331.

NAKAJIMA, T., OKUYAMA, S., YONEKURA, I., AND SATO, A. (1985). Effects of ethanol and phenobarbital administration on the metabolism and toxicity of benzene. *Chem.-Biol. Interact.* 55, 23-38.

NARAHASHI, T. (1984). *Cellular and Molecular Neurotoxicology*. Raven

Press, New York.

NAWROT, P. S., AND STAPLES, R. E. (1979). Embryo fetal toxicity and teratogenicity of benzene and toluene in the mouse. *Teratology* 19, 41.

NORTON, S. (1986). Toxic responses of the central nervous system. In *Casarett and Doull's Toxicology: The basic science of poisons*, 3rd ed. (C. D. Klaassen, M. O. Amdur and J. Doull, eds.). pp. 359-386. Macmillan, New York.

O'DONOGHUE, J. L. (1985). *Neurotoxity of Industrial and Commercial Chemicals*, vol. I and vol. II, CRC Press, Boca Raton, FL.

PANDYA, K. P., SHANKER, R., GUPTA, A. KHAN, W. A., AND RAY, P. K. (1986). Modulation of benzene toxicity by an interferon inducer (6MFA). *Toxicology* 39, 291-305.

PARADOWSKI, M., HEIMBURGER, M., COHEN, Y., AND ANDRZEJEWSKI, S. W. (1984). The effects of a single dose and prolonged benzene administration on 5-hydroxytryptamine and 5-hydroxyindoleacetic acid content in rat brain. *Xenobiotica* 14, 781-784.

PARADOWSKI, M., PRIOUX-GUYONNEAU, M., HEIMBURGER, M., ANDRZEJEWSKI, S. W., AND COHEN, Y. (1985). Effects of single and prolonged benzene administration on noradrenaline and dopamine content of the rat brain and internal organs. *Biogenic Amines* 2, 191-196.

PATHIRATNE, A., PUYEAR, R. L., AND BRAMMER, J. D. (1986a). A comparative study of the effects of benzene, toluene, and xylenes on their *in vitro* metabolism and drug-metabolizing enzymes in rat liver. *Toxicol. Appl. Pharmacol.* 82, 272-280.

PATHIRATNE, A., PUYEAR, R. L., AND BRAMMER, J. D. (1986b). Activation of ^{14}C-toluene to covalently binding metabolites by rat liver

microsomes. *Drug. Metab. Disp.* 14, 386-391.

PELLACK-WALKER, P., AND BLUMER, J. (1986). DNA damage in L5178YS cells following exposure to benzene metabolites. *Mol. Pharmacol.* 30, 42-47.

PELLACK-WALKER, P., WALKER, J. K., EVANS, H. H., AND BLUMER, J. L. (1985). Relationship between the oxidation potential of benzene metabolites and their inhibitory effect on DNA synthesis in L5178YS cells. *Mol. Pharmacol.* 28, 560-566.

PFEIFER, R. W., AND IRONS, R. D. (1981). Inhibition of lectin stimulated agglutination and mitosis by hydroquinone: reactivity with intracellular sulfahydryl groups. *Exp. Mol. Pathol.* 35, 189-198.

PFEIFER, R. W., AND IRONS, R. D. (1982). Effect of benzene metabolites on phytohemagglutinin-stimulated lymphopoiesis in rat bone marrow. *J. Reticuloendothel. Soc.* 31, 155-170.

PFEIFER, R. W., AND IRONS, R. D. (1983). Alteration of lymphocyte function by quinones through sulfahydryl-dependent disruption of microtubule assembly. *Int. J. Immunopharmacol.* 5, 463-470.

POIRIER, L. J., AND BEDARD, P. J. (1984). Behavior correlates of neurotransmitter activity. *Can. J. Neurol. Sci.* 11, 100-104.

POLSKY-CYNKIN, R., HONG, S., AND LEVINE, L. (1976). The effects of hydroquinone, hematin and heme containing proteins on prostaglandin biosynthesis by methylcholanthrene-transformed mouse BALB/3T3 Fibroblasts. *J. Pharmacol. Exp. Ther.* 197, 567-574.

POST, G. B., AND SNYDER, R. (1983). Effects of enzyme induction on microsomal benzene metabolism. *J. Toxicol. Environ. Health* 11, 811-825.

POST, G. B., SNYDER, R., AND KALF, G. F. (1984). Inhibition of RNA synthesis in rabbit bone marrow nuclei *in vitro* by quinone

metabolites of benzene. *Chem.-Biol. Interact.* 50, 203-211.

POST, G. B., SNYDER, R., AND KALF, G. F. (1985). Inhibition of RNA synthesis and interleukin-2 production in lymphocytes *in vitro* by benzene and its metabolites, hydroquinone and p-benzoquinone. *Toxicol. Lett.* 29, 161-168.

POST, G. B., SNYDER, R., AND KALF, G. F. (1986). Metabolism of benzene in macrophages *in vitro* and the inhibition of RNA synthesis by benzene metabolites. *Cell Biol. Toxicol.* 2, 231-246.

PYYKKO, K. (1980). Effects of methylbenzenes on microsomal enzymes in rat liver, kidney and lung. *Biochem. Biophys. Acta.* 633, 1-9.

PYYKKO, K. (1983). Time-course of effects of toluene on microsomal enzymes in rat liver, kidney and lung during and after inhalation exposure. *Chem.-Biol. Interact.* 44, 299-310.

REA, T. M., NASH, J. F., ZABIK, J. E., BORN, G. S., AND KESSLER, W. V. (1984). Effects of toluene inhalation on brain biogenic amines in the rat. *Toxicology* 31, 143-150.

RICKERT, D. E., BAKER, T. S., BUS, J. S., BARROW, C. S., AND IRONS, R. D. (1979). Benzene disposition in the rat after exposure by inhalation. *Toxicol. Appl. Pharmacol.* 49, 417-423.

RINSKY, R. A., SMITH, A. B., HORNUNG, R., FILLOON, T. G., YOUNG, R. J., OKUN, A. H., AND LANDRIGAN, P. J. (1987). Benzene and leukemia an epidemiologic risk assessment. *New Engl. J. Med.* 316, 1044-1050.

ROGAWSKI, M. A., AND BAKER, J. L. (1985). *Neurotransmitter actions in the vertebrate nervous systems.* Plenun Press, New York

ROGHANI, M., SILVA, C. D., GUVELLI, D., AND CASTAGNA, C. (1987). Benzene and toluene activate protein kinase C. *Carcinogenesis* 8, 1105-1107.

ROSENTHAL, G. J., AND SNYDER, C. A. (1985). Modulation of the immune

response to *Listeria monocytogenes* by benzene inhalation. *Toxicol. Appl. Pharmacol.* 80, 502-510.

ROSENTHAL, G. J., AND SNYDER, C. A. (1987). Inhaled benzene reduces aspects of cell-mediated tumor surveillance in mice. *Toxicol. Appl. Pharmacol.* 88, 35-43.

ROZEN, M. G. AND SNYDER, C. A. (1985). Protracted exposure of C57BL/6 mice to 300 ppm benzene depresses B- and T-lymphocyte numbers and mitogen responses. Evidence for thymic and bone marrow proliferation in response to the exposures. *Toxicology* 37, 13-26.

ROZEN, M. G., SNYDER, C. A., AND ALBERT, R. E. (1984). Depression in B- and T-lymphocyte mitogen-induced blastogenesis in mice exposed to low concentrations of benzene. *Toxicol. Lett.* 20, 343-349.

RUSHMORE, T. R., SNYDER, R., AND KALF, G. F. (1984). Covalent binding of benzene and its metabolites to DNA in rabbit bone marrow mitochondria *in vitro*. *Chem.-Biol. Interact.* 49, 133-154.

SAMMETT, D., LEE, E. W., KOCSIS, J. J., AND SNYDER, R. (1979). Partial hepatectomy reduces both metabolism and toxicity of benzene. *J. Toxicol. Environ. Health* 5, 785-792.

SATO, A., AND NAKAJIMA, T. (1979). Dose-dependent metabolic interaction between benzene and toluene *in vivo* and *in vitro*. *Toxicol. Appl. Pharmacol.* 48, 249-250.

SAVOLAINEN, H. (1977). Some aspects of the mechanisms by which industrial solvents produce neurotoxic effects. *Chem.-Biol. Interact.* 18, 1-10.

SAVOLAINEN, H. (1978). Distribution and nervous system binding of intraperitoneally injected toluene. *Acta. Pharmacol. Toxicol.* 43, 78-80.

SAWAHATA, T., AND NEAL, R. A. (1983). Biotransformation of phenol to hydroquinone and catechol by rat liver microsomes. *Mol. Pharmacol.* 23, 453-460.

SAWAHATA, T., RICKERT, D. E., AND GREENLEE, W. F. (1985). Metabolism of benzene and its metabolites in bone marrow. In *Toxicology of the Blood and Bone Marrow* (R. D. Irons, ed.). pp. 141-148. Raven Press, New York.

SCHMID, E., BAUCHINGER, M., AND HAUF, R. (1985). Chromosome changes with time in lymphocytes after occupational exposure to toluene. *Mutation Res.* 142, 37-39.

SCHWARTZ, C. S., SNYDER, R., AND KALF, G. F. (1985). The inhibition of mitochondrial DNA replication *in vitro* by the metabolites of benzene, hydroquinone and p-benzoquinone. *Chem.-Biol. Interact.* 53, 327-350.

SELYE, H. (1950). Stress and the general adaptation syndrome. *Br. Med. J.* 1, 1383-1392.

SHARMA, R. P. (1981). *Immunologic Considerations in Toxicology.* CRC Press, Inc., Boca Raton, Fl.

SHARMA, R. P., COULOMBE, R. A., AND SRISUCHART, B. (1986). Effects of dietary vanadium exposure on levels of regional brain neurotransmitters and their metabolites. *Biochem. Pharmacol.* 35, 461-465.

SHARMA, R. P., AND GEHRING, P. J. (1979). Immunologic effects of vinyl chloride in mice. *Ann. N. Y. Acad. Sci.* 320, 551-563.

SHARMA, R. P., AND ZEEMAN, M. G. (1980). Immunological alterations by environmental chemicals. Relevance of studying mechanisms versus effects. *J. Immunopharmacol.* 2, 285-307.

SIPES, I. G., AND GANDOLFI, A. J. (1986). Biotransformation of

toxicants. In *Casarett and Doull's Toxicology: The basic science of poisons*, 3rd ed. (C. D. Klaassen, M. O. Amdur and J. Doull, eds.). pp. 64-98. Macmillan, New York.

SMART, R. C., AND ZANNONI, V. G. (1984). DT-Diaphorase and peroxidase influence the covalent binding of the metabolites of phenol, the major metabolite of benzene. *Mol. Pharmacol.* 26, 105-111.

SMART, R. C., AND ZANNONI, V. G. (1985). Effect of ascorbate on covalent binding of benzene and phenol metabolites to isolated tissue preparations. *Toxicol. Appl. Pharmacol.* 77, 334-343.

SMYTHE, G. A., BRADSHAW, J. E., AND VINING, R. F. (1983). Hypothalamic monoamine control of stress-induced adrenocorticotropin release in the rat. *Endocrinology* 113, 1062-1071.

SMYTHE, G. A., GLEESON, R. M., AND STEAD, B. H. (1987). Stimulation of the hypothalamic-pituitary-adrenal axis and inhibition of growth hormone release via increased central noradrenaline neuronal activity by urethane anesthesia in the rat: Blockade by clonidine. *Aust. J. Biol. Sci.* 40, 91-96.

SNYDER, C. A., ERLICHMAN, M. N., LASKIN, S., GOLDSTEIN, B. D., AND ALBERT R. E. (1981a). The pharmacokinetics of repetitive benzene exposure at 300 and 100 ppm in AKR mice Spraque-Dawley rats. *Toxicol. Appl. Pharmacol.* 57, 164-171.

SNYDER, C. A., GOLDSTEIN, B. D., SELLAKUMAR, A. R., BROMBERG, I., LASKIN, S., AND ALBERT, R. E. (1980). The inhalation toxicology of benzene: incidence of hematopoietic neoplasms and hematotoxicity in AKR/J and C57BL/6J mice. *Toxicol. Appl. Pharmacol.* 54, 323-331.

SNYDER, C. A., GOLDSTEIN, B. D., SELLAKUMAR, A., BROMBERG, I., LASKIN, S., AND ALBERT R. E. (1982). Toxicity of chronic benzene inhalation:

CD-1 mice exposed to 300 ppm. *Bull. Environm. Contam. Toxicol.* 29, 385-391.

SNYDER, C. A., GREEN, J. D., LOBUE, J., GOLDSTEIN, B. D., BALLEL, C. D., AND ALBERT, R. E. (1981b). Protracted benzene exposure causes a proliferation of myeloblasts and/or promyelocytes in CD-1 mice. *Bull. Environ. Contam. Toxicol.* 27, 17-22.

SNYDER, D. S., AND UNANUE, E. R. (1982). Corticosteroids inhibit murine macrophage Ia expression and interleukin 1 production. *J. Immunol.* 129, 1803-1805.

SNYDER, R. (1984). The benzene problem in historical perspective. *Fundam. Appl. Toxicol.* 4, 692-699.

SNYDER, R., LEE, E. S., KOCSIS, J. J., AND WITMER, C. M. (1977). Bone marrow depressant and leukemogenic actions of benzene. *Life Sci.* 21, 1709-1722.

SNYDER, R., LONGACRE, S. L., SAMMETT, D., WITMER, C. M., AND KOCSIS, J. J. (1983). Relationship between the toxicity and metabolism of benzene. *Adv. Mod. Environ. Toxicol.* 4, 23-36.

SNYDER, R., LONGACRE, S. L., WITMER, C. M., KOCSIIS, J. J., ANDREWS, L. S., AND LEE, E. W. (1981c). Biochemical toxicology of benzene. *Rev. Biochem. Toxicol.* 3, 123-154.

SNYDER, R., JOWA, L., KALF, G., AND RUSHMORE, T. (1987). Formation of reactive metabolites from benzene. *Arch. Toxicol.* 60, 61-64.

SULEIMAN, S. A. (1987). Petroleum hydrocarbon toxicity *in vitro*: effect of n-alkanes, benzene and toluene on pulmonary alveolar macrophages and lysosomal enzymes of the lung. *Arch. Toxicol.* 59, 402-407.

TAKAHASHI, S., KAGAWA, M., INAGAKI, O., AKANE, A., AND KUKUI, Y. (1987). Metabolic interaction between toluene and ethanol in rabbits. *Arch.*

Toxicol. 59, 307-310.

TARDIFF, R. G., AND YOUNGREN, S.H. (1986). Public health significance of organic substances in drinking water. In *Organic Carcinogens in Drinking Water* (N. M. Ram, E. J. Calabrese and R. F. Christman, eds.). pp. 405-436. John Wiley & Sons, New York.

TAVASSOLI, M., AND FRIEDENSTEIN, A. (1983). Hemopoietic stromal microenvironment. *Am. J. Hematol.* 15, 195-203.

THOMAS, P. T., FUGMANN, R. A., ARANYI, C., AND FENTERS, J. D. (1985). Development and velidation of a panel of host resistance and immune function assays designed to detect chemical-induced immunomodulation. In *New Approaches in Toxicity Testing and Their Application in Human Risk Assessment* (A. P. Li, ed.). pp. 165-177. Raven Press, New York.

TICE, R. R., VOGT, T. F., AND COSTA, D. L. (1982). Cytogenetic effects of inhaled benzene in murine bone marrow. *Environ. Sci. Res.* 25, 257-275.

TOFTGARD, R., NILSEN, O. G., AND GUSTAFSSON, J. (1982). Dose dependent induction of rat liver microsomal cytochrome p-450 and microsomal enzymatic activities after inhalation of toluene and dichloromethane. *Acta Pharmacol. Toxicol.* 51, 108-114.

TOMASZEWSKI, J. E., JERINA, D. M., AND DALY, J. W. (1975). Deuterium isotope effects during formation of phenols by hepatic monooxygenases. Evidence for an alternative to the arene oxide pathway. *Biochemistry* 14, 2024-2031.

TUNEK, A., HOGSTEDT, D., AND OLOFSSON, T. (1982). Mechanism of benzene toxicity. Effects of benzene and benzene metabolites on bone marrow cellularity number of granulopoietic stem cells and frequency of mironuclei in mice. *Chem.-Biol. Interact.* 39, 129-138.

TUNEK, A., OLOFSSON, T., AND BERLIN, M. (1981). Toxic effects of benzene and benzene metabolites on granulopoietic stem cells and bone marrow cellularity in mice. *Toxicol. Appl. Pharmacol.* 59, 149-156.

TUNEK, A., PLATT, K. L., BENTLEY, P., AND OESCH, F. (1978). Microsomal metabolism of benzene to species irreversibly binding to microsomal protein and effects of modifications of their mechanism. *Mol. Pharmacol.* 14, 920-929.

TUNEK, A., PLATT, K. L., PRYZYBYLSKI, M., AND OESCH, F. (1980). Multi-step metabolic activation of benzene. Effect of superoxide dismutase on covalent binding to microsomal macromolecules, and identification of glutathione conjugates using high pressure liquid chromatography and field desorption mass spectrometry. *Chem.-Biol. Interact.* 33, 1-17.

UNGVARY, G., AND TATRAI, E. (1985). On the embryotoxic effects of benzene and its alkyl derivatives in mice, rats and rabbits. *Arch. Toxicol. Suppl.* 8, 425-430.

UNGVARY, G. Y., TATRAI, E., LORINCZ, M., AND BARCZA, G. (1983). Combined embryotoxic action of toluene, a widely used industrial chemical and acetylsalicylic acid (aspirin). *Teratology* 27, 261-269.

U.S. ENVIRONMENTAL PROTECTION AGENCY (1980a). *Ambient Water Quality Criteria for Benzene.* 440/5-80-018. U.S. Government Printing Office. Washington D.C.

U.S. ENVIRONMENTAL PROTECTION AGENCY (1980b). *Ambient Water Quality Criteria for Toluene.* EPA 440/5-80-075. U.S. Government Printing Office, Washington D.C.

U.S. ENVIRONMENTAL PROTECTION AGENCY (1986a). *Quality Criteria for Water.* EPA 440/5-86-001. U.S. Government Printing Office, Washington

D.C.

U.S. ENVIRONMENTAL PROTECTION AGENCY (1986b). *Test Methods for Evaluating Solid Waste.* Volume 1B: Laboratory Manual, Physical/Chemical Methods. 3rd Edition. SW-846. pp.5030-1-5030-21. U.S. Government Printing Office, Washington D.C.

U.S. NATIONAL ACADEMY OF SCIENCES (1982). *Alkylbenzene.* Washington D.C.

U.S. NATIONAL INSTITUTE FOR OCCUPATIONAL SAFETY AND HEALTH (1973). *Criteria for a Recommended Standard: Occupational Exposure to Toluene.* NIOSH Doc. No. 73-11023.

U.S. NATIONAL TOXICOLOGY PROGRAM (1981). *Benzene.* In 2nd annual report on carcinogens report NTP81-43. U.S. Dept. of Health and Human Services, December. pp. 49-52.

U.S. NATIONAL TOXICOLOGY PROGRAM (1987). *Review of the Immunotoxicology Program.* U.S. Dept. of Health and Human Service, December. pp. 1-6.

U.S. OFFICE OF TECHNOLOGY ASSESSMENT (1984). *Protecting the Nation's Groundwater from Contamination*: Volume I. OTA-0-233. U.S. Government Printing Office, Washington D.C.

UYEKI, E. M., ELASKAR, A., SHOEMAN, D. W., AND BISEL, T. U. (1977). Acute toxicity of benzene inhalation to hemopoietic precursor cells. *Toxicol. Appl. Pharmacol.* 40, 49-57.

VALE, W., SPIESS, J., RIVER, C., AND RIVER, J. (1981). Characterization of a 41-residue ovine hypothalamic peptide that stimulates the secretion of corticotropin and alpha-endorphin. *Science* 213, 1394-1396.

VAN DOORN, R., BOS, R. P., BROUNS, R. M. E., LEIJDEKKERS, C. M., AND HENDERSON, P. T. (1980). Effect of toluene and xylene on live glutathione and their urinary excretion as mercapturic acids in the

rat. *Arch. Toxicol.* 43, 293-304.

VERBRUGGEN, G., HERMAN, L., ACKERMAN, C., MIELANTS, H., AND VEYS, E. M. (1987). The effect of low doses of prednisolone on T-cell subsets in rheumatoid arthritis. *Int. J. Immunopharmac.* 9, 61-67.

VIGLIANI, E. C. (1976). Leukemia associated with benzene exposure. *Ann. N.Y. Acad. Sci.* 271, 143-151.

VOLLER, A., BIDWELL, D. E., AND BARTLETT, A. (1979). *The Enzyme Linked Immunosorbent Assay (ELISA).* A Guide with Abstracts of Microplate Applications. Dynatech laboratories, Alexandria, VA.

WALLIN, H., MELIN, P., SCHELIN, C., AND JERGIL, D. (1985). Evidence that covalent binding of metabolically activated phenol to microsomal proteins is caused by oxidized products of hydroquinone and catechol. *Chem.-Biol. Interact.* 55, 335-346.

WATSON, J. (1979). Continuous proliferation of murine antigen-specific helper T lymphocytes in culture. *J. Exp. Med.* 150, 1510-1519.

WERMUTH, B., PLATTS, K., SEIDEL, A., AND OESCH, F. (1986). Carbonyl reductase provides the enzymatic basis of quinone reduction in man. *Biochem. Pharmacol.* 35, 1277-1282.

WIERDA, D., AND IRONS, R. D. (1982). Hydroquinone and catechol reduce the frequency of progenitor B-lymphocytes in mouse spleen and bone marrow. *Immunopharmacology* 4, 41-54.

WIERDA, D., IRONS, R. D., AND GREENLEE, W. F. (1981). Immunotoxity in C57BL/6 mice exposed to benzene and aroclor 1254. *Toxicol. Appl. Pharmacol.* 60, 410-417.

WILLIS, G. L., AND SMITH, G. C. (1985). Amine accumulation in behavioural pathology. *Brain Res. Rev.* 9, 109-132.

WITZ, G., RAO, G. S., AND GOLDSTEIN, B. D. (1985). Short-term toxicity

of trans,trans mucondialdehyde. *Toxicol. Appl. Pharmacol.* 80, 511-516.

WOLF, M. A. ROWE, V. K., MCCOLLISTER, D. D., HOLLINGSWORTH, R. L., AND OYEN, F. (1956). Toxicological studies of certain alkylated benzenes and benzene. Experiments on laboratory animals. *Arch. Ind. Health* 14, 387-398.

YAMAWAKI, S., SEGAWA, T., AND SARAI, K. (1982). Effects of acute and chronic toluene inhalation on behavior and [^{3}H]-serotonin binding in rat. *Life Sci.* 30, 1997-2002.

YIN, S., LI, G., AND LIANG, Y. (1982). Significance of leukocyte alkaline phosphatase in the diagnosis of chronic benzene poisoning. *Regul. Toxicol. Pharmacol.* 2, 209-212.

VITA

Gin-Chang Hsieh

Candidate for the Degree of

Doctor of Philosophy

Dissertation: The Immunological and Neurochemical Toxicity of Benzene and Its Interaction with Toluene in Mice

Major Field: Toxicology

Biographical Information:

Personal Data: Born in Taipei, Taiwan, November 17, 1953; Son of Tung H. and S. May Hsieh; Married June L. Chen December 12, 1982.

Education: Attended elementary and high school in Taipei, Taiwan; received the B.V.M. degree from National Taiwan University, Taipei, Taiwan, with a major in veterinary medicine in 1977; 1983 completed the requirements for the M.V.M. degree at National Taiwan University, Taipei, Taiwan, with a major in veterinary immunology and public health; 1988 completed the requirements for the Ph.D. degree at Utah State University, Logan, UT, with a major in Toxicology.

Professional Experience: 1977-1979, 2nd-lieutenant officer of veterinary medicine, ROTC program, Pen-Hu, Taiwan; 1979-1980 veterinarian, International Laboratory for Zoonoses, Taipei, Taiwan; 1980-1983, graduate teaching assistant and lab instructor, Department of Veterinary Medicine, National Taiwan University, Taipei, Taiwan; 1983-1984, veterinary research associate, International Laboratory for Zoonoses, Taipei, Taiwan; 1981-1984, consultant of veterinary medicine, Tai Roun Product Co., Ltd., Taipei, Taiwan; 1985-1987, graduate research assistant, Toxicology Program, Utah State University, Logan, UT.