Unclassified

AD-A224 022

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS: None
2a. SECURITY CLASSIFICATION AUTHORITY: Not Applicable	3. DISTRIBUTION / AVAILABILITY OF REPORT: Unlimited
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE: Not Applicable	
4. PERFORMING ORGANIZATION REPORT NUMBER(S): Not Applicable	5. MONITORING ORGANIZATION REPORT NUMBER(S): ~~Not Applicable~~ AFOSR-TR-90-0584
6a. NAME OF PERFORMING ORGANIZATION: University of Arkansas for Medical Sciences; 6b. OFFICE SYMBOL (if applicable): n/a	7a. NAME OF MONITORING ORGANIZATION: AFOSR
6c. ADDRESS (City, State, and ZIP Code): 4301 West Markham Street, Little Rock, Arkansas 72205-7199	7b. ADDRESS (City, State, and ZIP Code): Building 410, Bolling AFB, D.C. 20332-6448
8a. NAME OF FUNDING / SPONSORING ORGANIZATION: AFOSR; 8b. OFFICE SYMBOL (if applicable): NL	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: AFOSR-89-0344
8c. ADDRESS (City, State, and ZIP Code): Building 410, Bolling AFB, D.C. 20332-6448	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO: 61102F; PROJECT NO: 2312; TASK NO: A5; WORK UNIT ACCESSION NO: None

11. TITLE (Include Security Classification): Mechanisms of Halocarbon-Induced Hepatotoxicity in the Mouse

12. PERSONAL AUTHOR(S): Mary Alice Smith, Ph.D. and Jay Gandy, Ph.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM 4/1/89 TO 3/31/90	5/30/90	72

16. SUPPLEMENTARY NOTATION:

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Glutathione, Adrenergic antagonist, Phentolamine, Halocarbon, Hepatotoxicity, Chlorobenzene, Iodobenzene, Bromobenzene
Not Applicable			

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Previous studies have shown that high-dose exposure to the halocarbon bromobenzene resulted in hepatotoxicity and lethality that was substantially diminished by co-treatment with the alpha-adrenergic antagonist, phentolamine. The purpose of this study was to compare the hepatotoxicity resulting from exposure to the related halocarbons, chlorobenzene, bromobenzene and iodobenzene, and to determine whether the resulting hepatotoxicity could be antagonized by phentolamine. Halobenzene-induced changes in hepatic glutathione concentrations and serum concentrations of catecholamines were determined as possible mediators of hepatic damage.

Iodobenzene administration resulted in toxicities similar to that seen with bromobenzene. Administration of either iodobenzene or bromobenzene resulted in hepatotoxicity as measured by serum alanine aminotransferase (ALT) activity about 1000 fold above normal values. Chlorobenzene was also capable of producing hepatotoxicity, but not to the same extent as iodobenzene. Chlorobenzene-induced hepatotoxicity resulted in serum ALT values approximately

(continued)

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Lt. Col. T. Jan Cerveny, USAF	202/767-5021	NL

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE: Unclassified

31 MAY 1990

19. (continued)

100 fold above normals. Chlorobenzene, bromobenzene or iodobenzene administration significantly decreased hepatic glutathione concentrations to approximately 20% of control concentrations. Phentolamine co-treatments significantly decreased serum ALT activity for all three compounds suggesting that hepatotoxicity might be mediated through an alpha-adrenergic system. Studies examining serum catecholamine concentrations indicated that at high doses iodobenzene and bromobenzene appeared to increase serum catecholamine concentrations, while chlorobenzene did not. The results indicate that iodobenzene and bromobenzene are similar in their ability to induce hepatotoxicity and that chlorobenzene is less hepatotoxic. Also, phentolamine hepatoprotection is not unique to bromobenzene-induced hepatic injury, and the mechanism of protection for the severe toxicity seen with high dose exposure to iodobenzene and bromobenzene may be correlated with increases in serum catecholamine concentrations. However, phentolamine is also protective against chlorobenzene-induced hepatotoxicity although there is no accompanying increase in serum catecholamine concentrations.

"Mechanisms of Halocarbon-Induced Hepatotoxicity in the Mouse: Chlorobenzene- and Iodobenzene-Induced Hepatotoxicity and Its Antagonism by Phentolomine"

Final Technical Report Prepared for the United States Air Force
Office of Scientific Research
Grant # AFOSR-89-0344

By

MARY ALICE SMITH, Ph.D.
JAY GANDY, Ph.D.

MAY, 1990
The University of Arkansas for Medical Sciences

The contents of this report were sub ed by M.A. Smith as a portion of a dissertation for the completion of the Ph.D. degree.

INTRODUCTION

1.1 Halocarbon Occurrence and Use

The major focus of this research was to examine the hepatotoxic potential of two halobenzenes, chlorobenzene and iodobenzene and compare them with the more-studied bromo-compound. Administration of these compounds has been shown to result in hepatotoxicity in experimental animals (NTP, 1985; Casini *et al.*, 1985; Klawe *et al.*, 1985; Reid *et al.*, 1973; Brodie *et al.*, 1971; Reid *et al.*, 1971). Since chlorobenzene, bromobenzene and iodobenzene are used principally as solvents, chemical intermediates or in research, there is potential for occupational exposure to personnel engaged in the manufacture and use of these substances (Diechmann, 1981; Torkelson and Rowe, 1981). Also, chlorobenzene has been detected in drinking water supplies (Dowty *et al.*, 1975). Thus, workers and the general public are at risk, at least theoretically, from exposure to these compounds.

1.2 Halocarbon Hepatotoxicity

The specific mechanisms by which halocarbons induce hepatotoxicity remain unknown. However, many halocarbons undergo oxidative metabolism in the liver producing intermediates that are water soluble and often highly reactive.

Liver injury induced by compounds which are electrophilic substrates for glutathione-S-transferase, such as bromobenzene and acetaminophen, has been attributed to the depletion of hepatic glutathione leaving reactive metabolites free to bind to target macromolecules (Mitchell *et al.*, 1973). These compounds exhibit a dose-threshold for toxicity. If low doses of these compounds are given, hepatotoxicity is not seen. This is probably due to the availability of hepatic glutathione to conjugate to the compound, thereby leading to the excretion of the toxic metabolite. (Mitchell and Jollow, 1974).

However, disturbances in hepatic glutathione concentration have been correlated with halocarbon-induced hepatotoxicity in several studies (Jollow *et al.*, 1974; Docks and Krishna, 1976; MacDonald *et al.*, 1982a,b; Sipes *et al.*, 1986; Kerger *et al.*, 1988a).

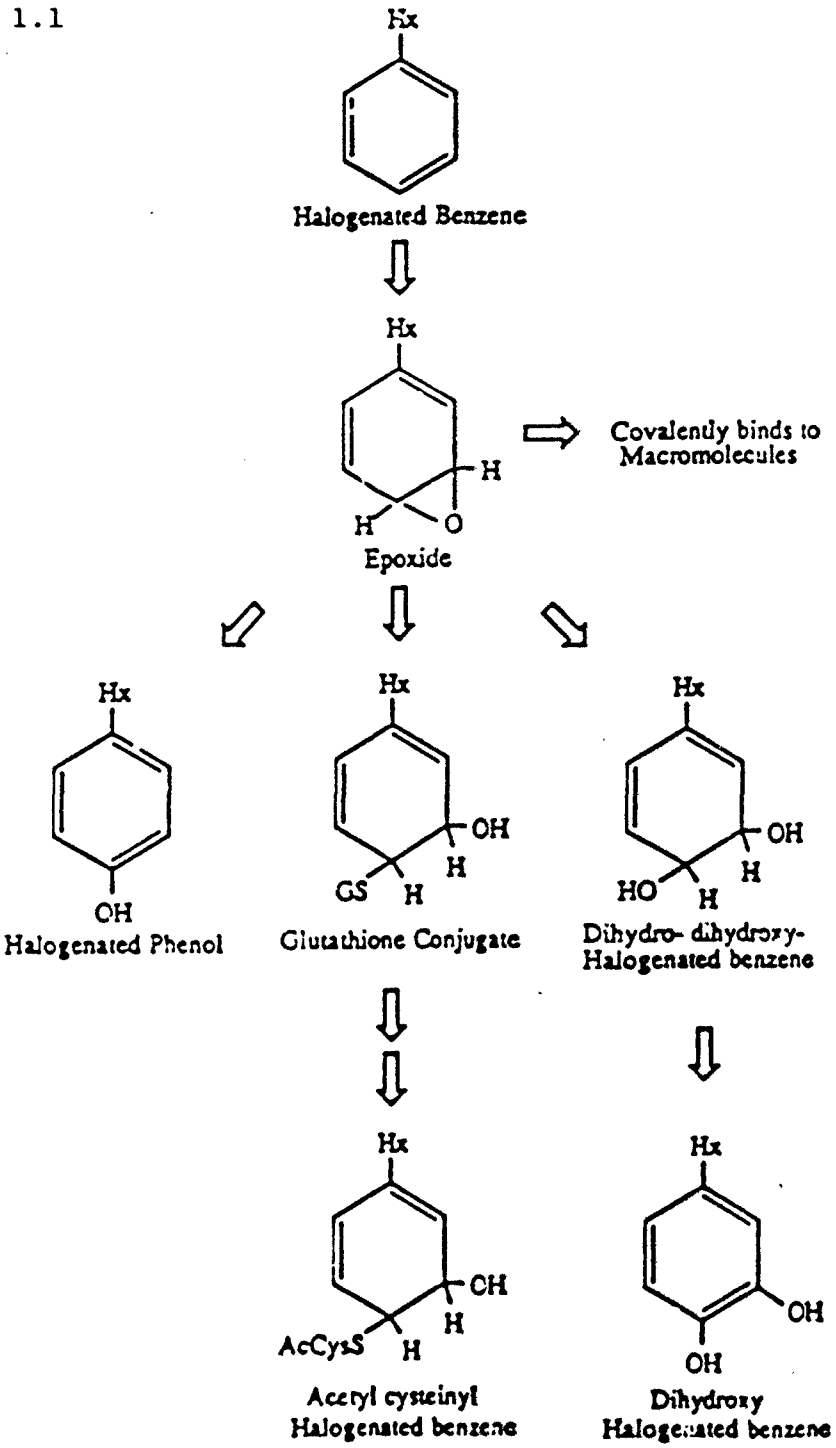
Although much more published literature exists for bromobenzene-induced hepatotoxicity, chlorobenzene and iodobenzene appear to be similar to bromobenzene (Casini *et al.*, 1985; Reid *et al.*, 1973; Brodie *et al.*, 1971). In the liver, bromobenzene is activated by the cytochrome P-450 system to a reactive epoxide. Brodie *et al.* (1971) proposed that bromobenzene-induced centrilobular necrosis was produced by an active metabolite of bromobenzene, probably an epoxide, that reacted covalently with macromolecules in hepatocytes. Support for the proposal that hepatotoxicity seen after bromobenzene treatment was a result of an active metabolite was published that same year when Reid *et al.* (1971) showed that two inhibitors of microsomal enzymes, SKF-525A and piperonyl butoxide, prevented necrosis and that phenobarbital induction enhanced liver injury and increased

mercapturic acid excretion. Jollow *et al.* (1974) showed that bromobenzene was metabolized in the liver to a 3,4-bromobenzene oxide which is the reactive metabolite and that the metabolite is preferentially conjugated with glutathione resulting in the depletion of glutathione from the liver. They concluded that there is a dose threshold for bromobenzene-induced hepatic necrosis that occurs when glutathione is no longer available for conjugation to the reactive metabolite. Furthermore, they suggested that this results in liver necrosis and arylation of cellular macromolecules. Diethyl maleate, which depletes hepatic glutathione, increased hepatic lesions that were produced by low doses of bromobenzene. All evidence thus far indicates that the toxic metabolite is bromobenzene epoxide which can be further degraded by glutathione transferase and epoxide hydrolase (Mitchell *et al.*, 1976).

The metabolic pathway appears to be the same for all of the hepatotoxic halobenzenes and is shown in Figure 1.1. The halobenzene (Hx) is converted to an epoxide by the endoplasmic reticulum in the liver, the epoxides are then converted nonenzymatically to phenols or enzymatically to their glutathione conjugates or dihydrodiol derivatives, and subsequently, the glutathione conjugates are transformed and excreted in the urine as mercapturic acids while the dihydrodiol derivatives are converted to catechols (Brodie *et al.*, 1971; Jollow *et al.*, 1974). According to Brodie *et al* (1971), the epoxides of the hepatotoxic aromatic hydrocarbons produce necrosis by alkylation of macromolecules in the hepatocytes. The conversion of the epoxides to glutathione conjugates, phenols, and dihydrodiols are alternate pathways that compete with the reaction of epoxides and macromolecules in the hepatocyte. If it is difficult for the compound to be converted to the epoxide, as is probably the case for 1,4-dichlorobenzene, or if the epoxide is rapidly converted to the conjugate, then hepatotoxicity is probably avoided.

Previous work in our laboratory has shown that the alpha-adrenoreceptor antagonists drugs, phentolamine or yohimbine, significantly reduce the hepatic glutathione depletion and liver injury induced by cocaine administration to mice (James *et al.*,1987). Similarly, bromobenzene depletes glutathione to approximately 10% of control values and elevates serum catecholamine concentrations. Repeated administration of alpha-adrenergic antagonists prevents a small part of this depletion. However, the alpha-adrenergic blockers have a more dramatic effect on protecting against the hepatotoxicity and lethality of bromobenzene (Kerger *et al.*, 1988a). This evidence led to the suggestion that large doses of xenobiotics cause a "stress-like" response that is manifested by increases in serum catecholamine concentrations which cause a further depletion of hepatic glutathione. The mechanism of action is suspected to be mediated through an alpha-2-adrenoreceptor since administration of the specific alpha-2-adrenoreceptor antagonist, yohimbine, blocks the glutathione depletion induced by catecholamines (James *et al.*, 1983). The simultaneous or sequential exposure to more than one compound can alter the pharmacologic or toxicologic responses to those compounds. In general, if a compound is dependent on its metabolism to exert its effect then changing the rate of metabolism will also change the duration or intensity of its action (Fouts, 1964). This change

Figure 1.1


Hx
Halogenated Benzene
Hx
H
H
O
Epoxide
Covalently binds to Macromolecules
Hx
OH
Halogenated Phenol
Hx
OH
GS
H
H
Glutathione Conjugate
Hx
OH
HO
H
H
Dihydro- dihydroxy- Halogenated benzene
Hx
CH
AcCysS
H
H
Acetyl cysteinyl Halogenated benzene
Hx
OH
OH
Dihydroxy Halogenated benzene

may be in the form of enhancing or inhibiting the metabolism of the compound. In humans the enhanced effects of alcohol on other hepatotoxins has been recognized for many years (Zimmerman, 1986). By pretreating animals with the alpha-adrenoreceptor antagonist drugs, phentolamine or yohimbine, James *et al.* (1983) showed that catecholamine-induced depression of hepatic glutathione could be abolished. Utilizing this tool in a follow-up study, James *et al.* (1987) showed that administration of phentolamine or yohimbine could reduce the hepatotoxicity produced by administration of cocaine. Subsequently, James and co-workers studied catecholamine involvement of bromobenzene-induced hepatotoxicity (Kerger *et al.*, 1988a,b; 1989). They found that four hours after high dose bromobenzene administration, serum epinephrine concentrations were elevated significantly above controls (Kerger *et al.*, 1988a). Additionally, treatment with the alpha-adrenergic antagonists, phentolamine or yohimbine, significantly decreased the bromobenzene-induced hepatotoxicity (Kerger *et al.*, 1988a). This suggested that a high dose exposure to bromobenzene results in the release of catecholamines which subsequently potentiates hepatotoxicity. An important implication of this work is that if risk assessments are made using extrapolations from high dose to low dose exposure, then a large error may be introduced into the calculations (Kerger *et al.*, 1988a). Despite an extensive amount of research into the potentiation of hepatotoxicity, the exact mechanism by which these occur are still unclear (Plaa, 1986).

1.3 Halobenzene Hepatotoxicity

Few studies have systematically compared the hepatotoxicity of the halobenzenes. In a histological study of some aromatic organic compounds Brodie *et al.* (1971) found that bromobenzene, iodobenzene and chlorobenzene were capable of producing minimal necrosis at low doses. However, fluorobenzene administered at a dose five times greater than chlorobenzene produced no specific lesions. Furthermore, when rats were pretreated with phenobarbital, marked increases in toxicity were seen in all the aforementioned halobenzenes as well as 1,2- and 1,3-dichlorobenzene. Neither benzene nor 1,4-dichlorobenzene produce liver lesions either in normal or in phenobarbital-induced rats. To determine whether these halobenzenes were conjugated to glutathione, Brodie *et al.* (1971) completed *in vitro* studies with liver microsomes from both normal and phenobarbital induced rats. Glutathione conjugates were formed with all the compounds that produced liver necrosis but not with compounds that did not produce necrosis. Thus, glutathione conjugates were formed with iodobenzene, bromobenzene, chlorobenzene and 1,2- and 1,3-dichlorobenzene but not with 1,4-dichlorobenzene, fluorobenzene or benzene.

Nearly all information concerning halobenzene toxicity is derived from studies of bromobenzene. Many studies have examined the protective role of glutathione in bromobenzene toxicity, the role of metabolism in bromobenzene toxicity and the mechanism of cellular injury (covalent binding or lipid peroxidation (see Mitchell *et al.*, 1982 and Casini *et al.*, 1985).

1.4 Statement of Problem

Chlorobenzene and iodobenzene were examined in this dissertation research for their ability to potentiate their own toxicity through an alpha-adrenergic mechanism and these results were compared to those obtained using bromobenzene. These halocarbons were selected because we know very little about their comparative potencies and whether or not they consume glutathione. Since decreases in hepatic glutathione have been correlated with halocarbon-induced hepatotoxicity in rodents (Casini *et al.*, 1985; Kerger *et al.*, 1988a), a study was needed to examine the dose response and temporal patterns of glutathione concentrations and to compare this depletion with the ensuing hepatotoxicity. Although fluorobenzene was another halobenzene of potential interest, all previous studies indicated that fluorobenzene was not hepatotoxic in uninduced animals and, therefore, was not appropriate for this study (Brodie et al., 1971; Reid et al., 1973). Also, since bromobenzene possesses self-potentiation properties, it was of interest to see if this property would be shared by other halobenzenes or was unique to bromobenzene.

Table 1.1 compares the basic chemical properties and toxicity of fluorobenzene, chlorobenzene, bromobenzene and iodobenzene. This table shows that although the chemical structures are the same except for the halobenzene attached to the benzene ring, the toxicity as measured by the rat LD_{50} (oral) increases with increasing molecular weight. It was hoped that a systematic study examining basic hepatotoxic effects and comparing these effects among chlorobenzene, bromobenzene and iodobenzene would give clues to basic structure-activity relationships among the halobenzenes.

Presently there is no way to predict which halobenzenes might be more potent in producing toxicity. A better understanding of the correlation between hepatotoxicity and structure-activity relationships of this group of halobenzenes may provide some evidence needed to make predictions concerning the hepatotoxic potential of other halocarbons. Therefore, a study was needed to examine the relationship between hepatotoxicity and one suspected mechanism by which this hepatotoxicity might occur, that of hepatic glutathione depletion.

The purpose of this dissertation research was two-fold. Initially, studies were designed to systematically describe and compare the hepatic effects of chlorobenzene and iodobenzene in mice and to compare these findings with those using the classic hepatotoxin, bromobenzene. Additionally, studies were designed to test the hypothesis that the alpha adrenergic antagonist, phentolamine, diminishes the hepatotoxic effects produced from administration of chlorobenzene or iodobenzene to mice, and that this protection is mediated through increases in endogenous catecholamines. The following were the specific aims to accomplish these goals:

1. The first specific aim was to describe the hepatic effects resulting from the administration of chlorobenzene and iodobenzene to B6C3F1 male mice and to compare those results with that of the classic hepatotoxin, bromobenzene. After halobenzene

administration, the extent of hepatotoxicity was determined by measuring the dose-response and temporal changes of serum alanine aminotransferase activity. The effects on hepatic glutathione concentrations were determined by measuring the dose-response and temporal changes of hepatic glutathione.

2. The second specific aim was to determine if co-administration of an alpha-adrenoreceptor antagonist with each halobenzene provided protection against some or all of the hepatotoxicity and whether hepatic glutathione concentrations were affected. This was determined by measuring serum ALT activity and hepatic glutathione concentrations for those halobenzenes capable of producing hepatotoxicity.

3. The third specific aim was to determine whether administration of high doses of halobenzene resulted in increases in endogenous catecholamines. This was determined by measuring temporal changes in serum catecholamine concentrations after the administration of each halobenzene.

Table 1.1

Chemical Characteristics of Halobenzenes

	Molecular Weight[1]	Molecular Formula[1]	Specific Gravity[1]	Oral Rat LD_{50}[2]
Fluorobenzene	96.10	C_6H_5F	1.024	4400 mg/kg
Chlorobenzene	112.56	C_6H_5Cl	1.107	2910 mg/kg
Bromobenzene	157.02	C_6H_5Br	1.495	2699 mg/kg
Iodobenzene	204.01	C_6H_5I	1.824	1799 mg/kg

[1]Von Oettingen, 1955
[2]Material Safety Data Sheet, Aldrich Chemical Company

MATERIALS AND METHODS

2.1 Experimental Animals and Treatments

The experimental animal chosen for this study was the male B6C3F1 hybrid mouse. Previous relevant studies used this test animal and comparisons were needed with these studies. Also, since the National Toxicology Program has chosen the B6C3F1 mouse as the experimental animal for use in its studies, the B6C3F1 mouse has become a standard experimental animal for toxicological studies. Preliminary experiments showed the B6C3F1 male mouse responded consistently to the test compounds. Weanling male B6C3F1 hybrid mice were obtained from the National Center for Toxicological Research, Jefferson, AR. The animals were used in experiments when they attained a weight of at least 24 grams and when they were approximately 7-8 weeks old.

All animals were housed, 4 mice per cage, in polycarbonate cages on a bedding of hardwood chips. The animal room was located in the University of Arkansas for Medical Sciences (UAMS) Laboratory Animal Care facilities in an environmentally-controlled building with a 12 hour light/12 hour dark cycle, a temperature of 20-24°C and relative humidity ranging from 40 to 60%. Food (Purina Lab Chow) and water were provided *ad libitum*. Treatment and control groups ranged in size from 6-10 animals/group depending on the estimated number needed to obtain statistical significance for each specific experiment. For any given experiment, only animals with the same birth date were used. All animals were sacrificed by carbon dioxide asphyxiation except for those used in the catecholamine studies (see Section 2.5).

All treatments were administered as an intraperitoneal injection (ip) in a volume of 0.1 milliliters (ml)/10 grams (g) body weight. Treatments began between 8:30 and 10:30 a.m. and sacrifice times depended on the design of each experiment. Two experimental designs were used for the treatment of the animals. If animals received only the test halobenzene, then each animal in the treatment group received a single ip injection of that test solution, and the concurrent control group animals received a single ip injection of vehicle (corn oil). In experiments where phentolamine was given as a co-treatment with the halobenzene, a phentolamine injection was given as a 15 minute pretreatment, the halobenzene was given at time zero, and up to four more injections of phentolamine were given at 90 minute intervals after the halobenzene.

2.2 Chemicals, Reagents and Supplies

The halobenzenes, iodobenzene, chlorobenzene and bromobenzene, were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI) and were Gold Label® or the highest purity

available. These test compounds were diluted with and administered in a corn oil vehicle. The solutions were prepared immediately before each experiment to prevent volatilization or degradation of the test compounds. Phentolamine (Regitine HCL) was a generous gift of Ciba-Geigy (Summit, NJ) and was dissolved in distilled, deionized water for injections.

Chemicals for the glutathione assay, reduced glutathione, 5,5'-dithiobis-2-nitrobenzoic acid and disodium ethylenediamine tetraacetate (EDTA), were purchased from Sigma Chemical Company (St Louis, MO). Monobasic sodium phosphate, dibasic sodium phosphate and trichloroacetic acid were purchased from Fisher Scientific (Pittsburg, PA). Liver tissue was processed in 15 ml graduated disposable centrifuge tubes obtained from Sarstedt, Inc. (St. Louis, MO). Enzymatic reactions were carried out in glass disposable 13 x 100 millimeter (mm) culture tubes and spectrophotometric measurements were completed in 3 ml disposable polystyrene cuvets, both purchased from Fisher Scientific (Pittsburg, PA).

Blood for the determination of alanine aminotransferase activity or catecholamines was processed in 1.5 ml microcentrifuge tubes obtained from Fisher Scientific (Pittsburg, PA). The alanine aminotransferase activity was determined using a commercially available kit from Sigma Diagnostics (St. Louis, MO). Spectrophotometric measurements were completed in polystyrene 1.6 ml semimicro-cuvets obtained from Fisher Scientific (Pittsburg, PA).

Other general laboratory chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO). Distilled, deionized water was used in preparing solutions.

2.3 Glutathione Determinations

For glutathione determinations, the left lobe of the liver was removed, rinsed in cold saline and kept on ice until processed. At that time, the liver section was blotted dry, weighed and the weights were recorded for further calculations. The weights ranged from 0.3-0.6 grams. The liver sections were homogenized in 5 ml of 5% trichloroacetic acid (TCA) with 1 mM EDTA for 15 seconds with a Tissumizer® (Tekmar Company, Cincinnati, OH). Cell debris and precipitated protein were pelleted by spinning at 10000xg for 20 minutes in a refrigerated centrifuge (4°C). The volume of the supernatant was recorded and 0.2 ml was removed for glutathione determination.

Non-protein sulfhydryl (GSH) was determined according to a modified method of Sedlak and Lindsay (1968), utilizing Ellman's reagent [5,-5'-dithiobis-(2-nitrobenzoic acid)] (DTNB) (Ellman, 1959). The basis for this procedure is that non-protein sulfhydryl groups react with thiol moieties of DTNB to form 1 mole of yellow colored anion per mole of sulfhydryl (Ellman, 1959). Within 5 minutes, the intensity of the yellow color formed from this reaction is proportional to the non-protein sulfhydryl content of the sample. The anion has a molar extinction coefficient of 13100 at 412 nm (Sedlak and Lindsay, 1968). Specifically, 5 ml of 0.1 M phosphate buffer (pH 8.0) was added to each 0.2 ml aliquot of liver supernatant. Total non-protein sulfhydryl was measured by adding 0.1 ml of 0.01 M DTNB and gently vortex-mixing. The tubes were allowed to stand for 5 minutes at room temperature to assure

the completion of the reaction before the absorbance was read at 412 nm on a Varian Cary 210 dual beam spectrophotometer. Samples were read against a DTNB/buffer blank.

Reduced GSH was used to generate a standard curve. The absorbance from the known concentrations of the standard curve were used to compute a linear regression curve from which the absorbances of the unknown samples were compared and concentrations determined. The hepatic concentration of GSH for each sample was calculated using the following formula:

$$\frac{\text{(Concentration of GSH) x (total volume of supernatant)}}{\text{(weight of liver sample)}}$$

Final concentrations were expressed in milligrams (mg) GSH per gram (g) of liver tissue. Intraassay coefficient of variation was 2%.

2.4 Serum Alanine Aminotransferase (ALT) Activity Determinations

Serum alanine aminotransferase (ALT; also known as SGPT) activity was the serum enzyme examined in this study. Serum ALT is an enzyme highly specific to the liver, and its concentration in the bloodstream is directly proportional to liver damage (Wroblewski and LaDue, 1956). For the measurement of serum ALT activity, blood was collected by cardiac puncture immediately after sacrifice. Tuberculin syringes (1 ml) with a 26 gauge needle were used for the punctures. In order to prevent hemolysis of erythrocytes, needles were always removed before draining blood into the collection tubes. Serum was separated by spinning at 13000xg for 4 minutes in a tabletop microcentrifuge at ambient temperature. Serum was refrigerated for no more than 48 hours prior to analysis.

The procedure and the reagent for the determination of serum ALT activity were obtained from Sigma Diagnostics (St. Louis, MO) and was based on the method of Bergmeyer *et al.* (1978). The alanine aminotransferase reagent contained L-alanine (400 mmoles/L), 2 oxoglutarate (12 mmoles/L), lactate dehydrogenase (2000 U/L), nicotinamide adenine dinucleotide (NADH; 0.25 mmoles/L) and phosphate buffer (pH 7.4). To determine the concentration of ALT in the serum samples, 1 ml of ALT reagent was added to the appropriate number of microcuvets and the cuvets were placed in a 30°C water bath. A 0.1 ml sample of serum was added to the reagent and mixed by inversion of the cuvet. The reaction mixture was incubated for 90 seconds at 30°C. The absorbance was recorded at 0, 30 and 60 seconds at a wavelength of 340 nm. The enzymatic reactions that are the basis for this assay were first described by Wroblewski and LaDue (1956). First, alanine aminotransferase catalyzes the transfer of an amino group from alanine to oxoglutarate to form glutamate and pyruvate. Then the pyruvate is reduced to lactate by lactate dehydrogenase with the simultaneous oxidation of NADH. As the NADH is oxidized, a change in absorbance can be detected. Thus, the amount of NADH oxidized and the rate of the decrease in absorbance at 340 nm is directly proportional to

the ALT activity. The concentration of ALT was determined by the following calculations:

(Change in absorbance per minute) x (total volume in cuvet) /
(6.22 [mM absorptivity of NADH]) x (light path) x (sample volume)

The intraassay coefficient of variation was 4.4% which was comparable to that reported by Sigma Diagnostics for within-run and day-to-day precision of 2.1 to 3.7%. Representative liver samples were preserved for histological confirmation of necrosis. For these studies the left lobe of the liver was excised, rinsed in saline and preserved in 10% neutral buffered formalin. Further processing, staining, microscopic examinations and confirmations of necrosis were done in the pathology laboratory of Dr. Louis Chang.

2.5 Serum Catecholamine Determinations

For these studies special care was taken to minimize any trauma to the animal since stress could result in increases in catecholamines and adversely affect the results of the experiment. To accomplish this, the animals were kept in an adjacent room until time of sacrifice when they were brought singly into the room and sacrificed by swift decapitation. The time elapsing between removal from the adjacent room and decapitiation was less than 20 seconds. Blood for the determination of catecholamines was collected immediately after decapitation into microcentrifuge tubes to which 5 µl of heparin, 1000 units/ml, (Upjohn Company, Kalamazoo, MI) had been added. The blood was drained into a funnel and directly into the microcentrifuge tubes which were immediately capped and inverted several times to prevent the blood from clotting. The plasma was separated by spinning for 4 minutes at 13000xg in a tabletop microcentrifuge. The plasma was pipetted into another set of microcentrifuge tubes. Catecholamine samples were frozen immediately at -80°C. The samples were packed in dry ice and shipped overnight in an insulated container to Dr. D. G. Patel in Cincinnati, OH, for catecholamine determinations. On arrival, the samples were inspected for any evidence of thawing and were stored at -80°C until time of analysis.

The catecholamines were extracted as described by Hallman *et al.* (1978) and by Patel (1983). On the day of analyses, plasma samples were thawed and a 0.1 ml aliquot was diluted with 1.5 ml deionized water, 40 µl of 5 mM sodium bisulfite (an antioxidant) and 2.5 ng of 3,4-dihydroxybenzylamine [DHBA] (an internal standard). This solution was added to dried, acid washed alumina (15 mg), and 0.2 ml of 1 M TRIS buffer with 2.5% EDTA added to the mixture. This mixture was shaken for 15 minutes at room temperature. After the alumina had settled, the supernatant was aspirated and discarded. The alumina was washed two times with distilled water and the supernatant aspirated and discarded. The catecholamines were eluted from the alumina by the addition of 0.05 ml of 0.1 M perchloric acid ($HClO_4$) and shaking for 15 minutes. The catecholamines were contained in the supernatant. A 0.02 ml aliquot of the

supernatant was injected directly onto an HPLC system for the separation of the catecholamines. Epinephrine, norepinephrine and dopamine concentrations were determined by high performance liquid chromatography plus electrochemical detection (LCEC) (Hallman *et al.*, 1978; Patel, 1983; Ali *et al.*, 1983). The mobile phase was 0.15 M monochloroacetate buffer, pH 3.0, containing Na_2EDTA and sodium octyl sulfate.

The analytical system consisted of a Waters Associates M-6000A pump (Milford, MA), a Rheodyne 7125 injector (Rainin Instrument, Woburn, MA), a phase-2 ODS, 3 micron (100 x 3.2 mm) analytical column, an LC-4B amperometric detector and an LC-17 oxidative flowcell consisting of a glassy carbon electrode (TL-5) versus Ag-AgCl reference electrode maintained at a potential of 0.65 V (Bioanalytical Systems Inc., West Lafayette, IN). Peak heights were measured from chromatograms recorded on a Fisher 5000 Series chart recorder. These peak heights were used to calculate ratios of each catecholamine/DHBA.

Standard curves were generated by determining the ratio between four different known amounts of each catecholamine (1.0, 2.0, 4.0 and 8.0 pmol) and a constant amount of DHBA. The standards were extracted in the same manner as the plasma samples. Separate standard curves were generated for epinephrine, norepinephrine and dopamine. Calculations for the samples were carried out by linear regression. The regression coefficients for each catecholamine varied from day to day within the following range: Norepinephrine = 0.994 to 0.999, epinephrine = 0.982 to 0.999 and dopamine = 0.938 to 0.996.

2.6 Data Analysis

For comparison among groups, data were analyzed using a one-way analysis of variance (ANOVA). The level of significance was $p<0.05$ for all comparisons. If the ANOVA showed significant differences, an appropriate post hoc test, Duncan's Multiple Range Test (Duncan, 1955) was performed to determine which individual treatment groups were significantly different.

For serum ALT data, all statistics were done on log transformed values because the log transformed data for this parameter are normally distributed, and raw values are not normally distributed. Statistical analyses of glutathione and catecholamine data were performed using raw (untransformed) data.

Statistics were computed using Systat stastical package (Systat Inc., Evanston, IL) for IBM compatible personal computers.

RESULTS

3.1 Assessment of Iodobenzene-Induced Hepatotoxicity

Since there are only a few studies reported in the scientific literature that examine the toxic effects of iodobenzene, the first experiments were designed to give basic information concerning the toxic effects of iodobenzene on the liver. Temporal changes and dose response differences were derived for both hepatotoxicity, as determined by serum alanine aminotransferase activity and hepatic glutathione concentrations. In order to derive as much data as possible from these experiments, all premature deaths were recorded along with the dose and time of death. Thus, lethality data were gathered without additional justification and expense of lethality studies.

3.1.A Temporal Changes in Serum ALT Activity after Iodobenzene Administration.

In order to determine the time of maximum response of serum ALT activity, the temporal pattern of serum ALT activity was determined following iodobenzene treatment. To accomplish this, iodobenzene was administered in a dose of 0.4 ml/kg to a total of 32 animals. This dose was chosen because preliminary experiments had indicated that doses above 0.4 ml/kg resulted in less than 50% survival. The animals were sacrificed at 24, 48, 72 or 96 hours post-treatment and the results are shown in Figure 3.1. Some animals died before 72 hours had elapsed (lethality ranged from 11-22%/day) leaving a total of 22 animals. The serum ALT activity was significantly elevated above control values at 24 hours post-treatment. At 48, 72 and 96 hours post-treatment serum ALT activity remained significantly elevated in treated groups when compared to controls. Although there was no significant difference among the treated groups, there was a decreasing trend in serum ALT concentrations as time progressed. The arithmetic mean for the control group was 24 ± 2 (Mean ± SEM). Thus, the maximum serum ALT activity occurred approximately 24 hours after treatment with iodobenzene.

3.1.B Dose Response of Serum ALT Activity after Iodobenzene Administration.

Iodobenzene was administered in varying doses to B6C3F1 male mice. The results are shown in Figure 3.2a. The animals were sacrificed at 24 hours post-treatment. All animals in the highest dose group (1.0 ml/kg) died before the 24 hour test period elapsed, thus no data on serum ALT activity was obtained for that group. In the 0.5 ml/kg iodobenzene group, only 3 animals survived. However, serum ALT activity was elevated to such an extent in those 3 animals that significant differences were obtained. There was no significant differences between

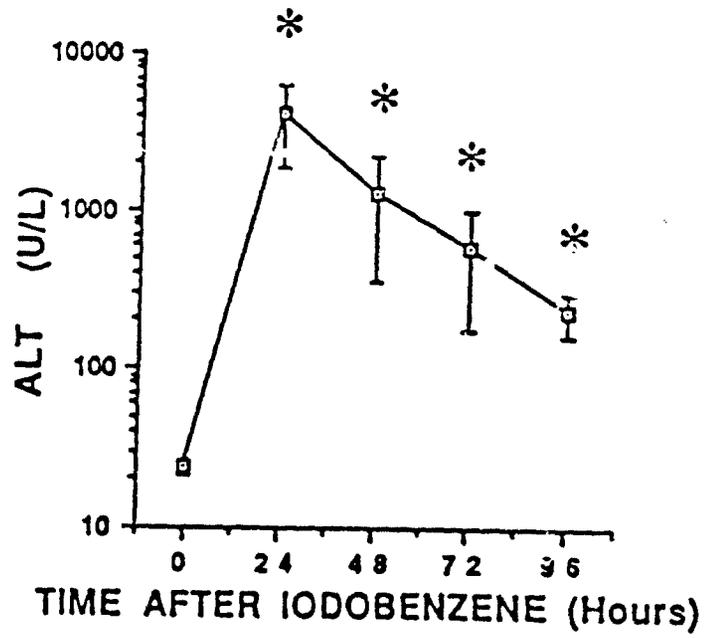


Figure 3.1. Temporal Pattern of Serum Alanine Aminotransferase Activity (ALT) after Treatment with Iodobenzene.

Iodobenzene was administered by intraperitoneal (ip) injection at a dose of 0.40 ml/kg. Animals were sacrificed at 24, 48, 72 or 96 hours post-treatment. Serum ALT activity is reported in Units (U)/liter (L). Asterisks signify a significant difference from the control group. Each point represents the arithmetic mean ± SEM of at least 7 animals/group and is graphed on a log scale. Significant differences were determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test.

the 2 lowest doses (0.01 and 0.1 ml/kg) and the control group (vehicle only). The 0.25 and 0.5 ml/kg iodobenzene treatment groups were significantly elevated above controls but there was no difference in the extent of elevations between these two groups.

Since the dose response curve was very steep between the 0.1 and the 0.25 ml/kg dose in Figure 3.2a, an additional experiment was completed to determine if smaller increment doses would result in a more graded response. The experiment was repeated using the following doses of iodobenzene: 0.1, 0.15, 0.20 and 0.25 ml/kg. There were at least 6 animals per group. The results are shown in Figure 3.2b. One animal in the 0.25 ml/kg group died by the 24 hour time period. Even with these small increments there was a very sharp increase in serum ALT activity between the 0.15 and the 0.20 ml/kg doses. The 2 smaller doses (0.10 and 0.15 ml/kg) were not significantly different from the control group. The two larger doses (0.20 and 0.25 ml/kg) were significantly different from the control group as well as from the two lower doses. Therefore, with dose increases of only 0.05 ml/kg difference, the dose response was steep suggesting a dose threshold rather than a more typical graded dose response curve.

3.1.C Lethality Following Iodobenzene Administration.

To further assess the toxicity of iodobenzene, the percentage of deaths following administration of various doses of iodobenzene were recorded. As shown in Table 3.1, the dose resulting in approximately fifty percent lethality (LD_{50}) 24 hours after treatment was 0.5 ml/kg.

3.1.D Temporal Changes In Hepatic Glutathione Concentrations Following Iodobenzene Treatment.

In order to determine the time of maximum glutathione depletion and to determine whether hepatic glutatione concentrations would return to normal following a hepatotoxic dose, a time course of hepatic glutathione was completed and the results are shown in Figure 3.3. B6C3F1 male mice received one ip injection of 0.4 ml/kg iodobenzene and were sacrificed at 2, 4, 8 or 24 hours post-injection. Iodobenzene administration resulted in the depletion of hepatic glutathione by 2 hours post-treatment and remained at that low concentration for at least 8 hours post-treatment. After 2, 4 and 8 hours post-injection, hepatic glutathione concentrations in animals that had received iodobenzene were only 31%, 19% and 16%, respectively, of concentrations in animals receiving corn oil vehicle. Although the maximum depletion of glutathione appeared to be at eight hours post-iodobenzene, there was not a statistical difference in the three early time periods. The 2, 4 and 8 hour iodobenzene-treated groups were all significantly different from their time matched controls. Twenty-four hours after receiving iodobenzene, hepatic glutathione concentrations were not different from control

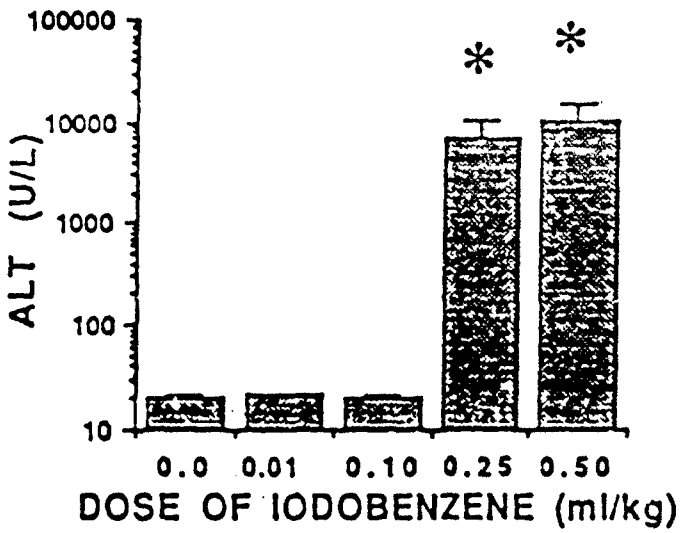


Figure 3.2a. Dose Response of Serum Alanine Aminotransferase Activity after Iodobenzene Administration (0.01 - 1.0 ml/kg).

Iodobenzene was administered ip in the following doses: 0.01, 0.10, 0.25, 0.50 and 1.00 ml/kg. All groups had 8 animals per group at time zero, however by 24 hours post-treatment, 5 animals had died in the 0.50 ml/kg group and all animals had died in the 1.0 ml/kg group. This left 3 animals in the 0.50 ml/kg dose group and 8 animals in all other groups. Asterisks signify a significant difference from the control group.

Each point represents the arithmetic mean ± SEM. The y axis is shown on a log scale. Significant differences were determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test.

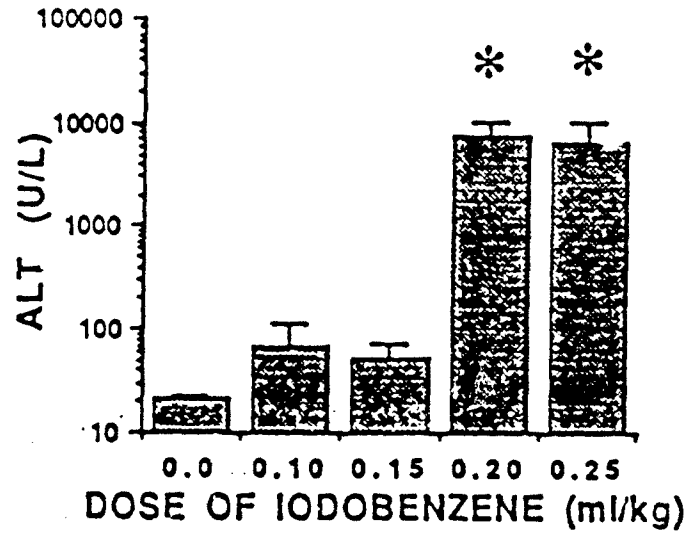


Figure 3.2b. Dose Response of Serum Alanine Aminotransferase Activity after Iodobenzene Administration (0.10 - 0.25 ml/kg).

Iodobenzene was administered ip in the following doses: 0.10, 0.15, 0.20 or 0.25 ml/kg. Corn oil vehicle was administered to the 0 ml/kg group and all groups were sacrificed 24 hours post-treatment. One animal in the 0.25 ml/kg group died by the time of sacrifice but all groups had at least 6 animals per group. Asterisks indicate a significant difference from the control group. Each point represents the mean ± SEM of serum ALT activity expressed in U/L. Significant differences were determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test.

TABLE 3.1

Percent Lethality After Iodobenzene Administration

Dose	% Lethality
0 ml/kg	0 (8)
0.25 ml/kg	6 (16)
0.50 ml/kg	52 (23)
1.00 ml/kg	78 (23)
1.50 ml/kg	100 (7)

Percent lethality determined 24 hours after iodobenzene administration. The total number of animals receiving treatment are shown in parenthesis.

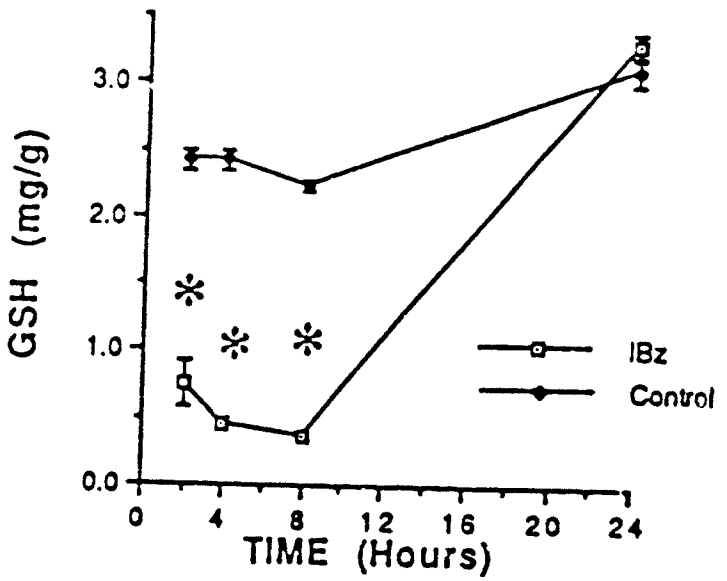


Figure 3.3. Temporal Changes in Hepatic Glutathione Concentrations after Treatment with Iodobenzene (IBz).

Iodobenzene was administered ip at a dose of 0.40 ml/kg in corn oil vehicle to B6C3F1 male mice. Control animals received corn oil. Animals were sacrificed at 2, 4, 8 or 24 hours post-treatment. Results are expressed as milligrams (mg) of hepatic glutathione (GSH) per gram (g) of liver tissue and represent the mean ± SEM of at least 7 animals. Asterisks indicate significant differences from control groups. Significant differences were determined by analysis of variance followed by a Duncan's Multiple Range Test at $p<0.05$.concentrations.

Absolute values for control groups were 2.42 ± 0.08 (mean ± SEM) mg/g liver for the 2- and 4-hour time points, 2.23 ± 0.05 mg/g liver tissue for the 8-hour time point and 3.11 ± 0.10 mg/g liver tissue for the 24-hour time point. Hepatic glutathione concentrations from treatment groups at 2, 4 and 8 hours post-injection were not significantly different from each other but were different from the 24 hour treatment group. Serum alanine aminotransferase (ALT) activities were determined for the 24 hour time point to confirm that this dose of iodobenzene resulted in hepatotoxicity. All treated animals showed increases in serum ALT at least 17-times greater than control. The treated mean was 3860 U/L while the control mean was 25 U/L (Data not shown). Thus, at a dose of iodobenzene shown to be hepatotoxic, hepatic glutathione concentrations were significantly depleted from 2 to 8 hours post-treatment but had recovered fully by 24 hours post-treatment.

3.1.E Effect of Iodobenzene Dose on Hepatic Glutathione Concentrations.

To determine the minimal dose of iodobenzene needed to affect hepatic glutathione concentrations as well as the dose needed to deplete glutathione maximally, varying doses of iodobenzene (0.01, 0.1, 0.25 and 0.5 ml/kg) were administered ip to mice. Based upon the previous time course studies, a 3-hour time interval after iodobenzene administration was selected as representing a useful time for assessing maximal glutathione depletion. The results are shown in Figure 3.4. Both 0.25 ml/kg and 0.5 ml/kg doses lowered glutathione concentrations to 10-12% of control values, while 0.1 ml/kg iodobenzene showed an intermediate response of 61% of control concentrations. The low dose group (0.01 ml/kg) was not significantly different from the control group but was significantly different from all other treatment groups. The 0.1 ml/kg dose group was significantly different from all other treatment groups. The 0.25 and 0.5 ml/kg dose groups were not significantly different from each other but were different from 0.01 ml/kg, 0.1 ml/kg and control groups. The actual hepatic glutathione concentrations for the control group was 2.24 ± 0.04 mg/g of liver tissue. To summarize, 0.01 ml/kg of iodobenzene did not affect hepatic glutathione concentrations, 0.1 ml/kg depleted glutathione to 61% of normal values and 0.25 and 0.5 ml/kg doses depleted glutathione concentrations maximally at three hours post-treatment.

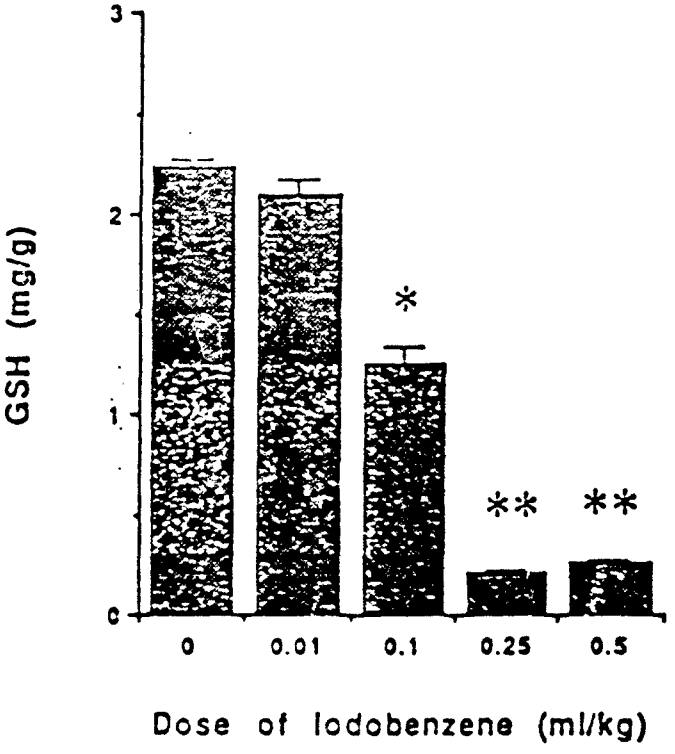


Figure 3.4. Dose Response Relationships for Hepatic Glutathione Concentrations after Treatment with Iodobenzene.

Hepatic glutathione concentrations were determined after ip injection of iodobenzene at the following doses: 0.01, 0.10, 0.25, 0.50 ml/kg. Iodobenzene was diluted with and administered in a corn oil vehicle to B6C3F1 male mice. Control animals received an ip injection of corn oil. Animals were sacrificed 3 hours post-treatment and all groups had 8 animals per group. A single asterisk indicates a significant difference from the control group. Two asterisks signifies a significant difference from the control group as well as the .01 and 0.1 ml/kg treated groups. Each point represents the mean ± SEM. Significant differences were determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test.

3.2 Effect of Phentolamine Co-Treatment on Iodobenzene- Induced Toxicities.

Previous studies in our laboratory had shown that phentolamine co-treatment prevented the hepatotoxicity induced by bromobenzene treatment. The next series of experiments were initiated to determine whether or not phentolamine was capable of blocking the hepatotoxicity induced by iodobenzene administration in a similar manner as that seen with bromobenzene.

3.2.A Treatment Protocol Needed for Phentolamine Protection Against Iodobenzene-Induced Hepatotoxicity.

Preliminary experiments were conducted to test the ability of phentolamine to antagonize iodobenzene-induced hepatotoxicity. The doses and phentolamine treatment regimen were selected based on previous observations with bromobenzene. Additionally, it was a concern that multiple injections could produce stress in the mice that would affect the outcome of the experiment or that the injected volume alone might affect the absorption and/or distribution of iodobenzene. Thus, two groups were included in the first study to compare the hepatotoxicity resulting from treatment with iodobenzene alone or with five additional injections of saline as a positive control. Phentolamine was also administered as a co-treatment with iodobenzene in the same manner as described above for the saline controls. The results of this study are shown in Figure 3.5. By 24 hours post-treatment, 1 animal had died in the saline plus iodobenzene treated group, 3 animals had died in the iodobenzene alone treated group and no animals had died in the phentolamine plus iodobenzene treated group or in the corn oil control group. There was a significant difference in serum ALT activities between the group that received phentolamine plus iodobenzene and the other treated groups. There was no significant difference between the corn oil control group and the phentolamine plus iodobenzene treated groups. Thus, the injections themselves had no effect on the hepatotoxicity or the protection from hepatotoxicity as demonstrated by iodobenzene alone or phentolamine plus iodobenzene treated groups, respectively. This experiment indicated that phentolamine was protective against iodobenzene-induced hepatotoxicity if given in the same dosage and treatments as previously described with bromobenzene (Kerger et al., 1988a). However, the number of injections needed to protect against bromobenzene toxicity was five. If possible, it was desirable to use fewer injections to reduce the stress on the animals and to reduce the chance of nonspecific effects on the outcome of the experiment. Therefore another preliminary experiment was completed to determine if five injections of phentolamine were needed for protection against iodobenzene-induced hepatotoxicity. Phentolamine was administered ip to four groups of animals. One group received the previously described treatment of five injections of phentolamine begining 15 minutes before the iodobenzene injection and one injection of phentolamine at 90 minute intervals thereafter for 4 more injections. Since the critical time of phentolamine administration to protect against bromobenzene toxicity had been determined to be about 3 hours post-treatment (Kerger *et al.*, 1988a), the other three groups received

injections beginning at 3 hours post-treatment of iodobenzene. They received additional injections at 90 minute intervals since phentolamine had been shown to be significantly metabolized by that time period (Kerger *et al.*, 1988a). The results of this experiment are shown in Table 3.2. Although this was not intended to be a definitive study, the preliminary data indicated that attempts to reduce the number of injections of phentolamine did not result in the protection against hepatotoxicity as well as five injections of phentolamine. Thus, in all the following studies using phentolamine as an antagonist against hepatotoxicity, this same treatment regimen was used and will be referred to as the phentolamine co-treatments. All these studies utilized the same phentolamine treatment protocol as had been used with bromobenzene and confirmed in this study to be effective.

3.2.B Temporal Changes in Serum Alanine Aminotransferase Activity after Co-Treatment with Iodobenzene and Phentolamine.

To investigate the effects of phentolamine on iodobenzene-induced hepatotoxicity and to ascertain that phentolamine was preventing and not simply delaying hepatotoxicity, the temporal changes in serum ALT activity were determined following co-treatments of phentolamine and iodobenzene. The experimental groups received phentolamine (10 mg/kg) co-treatments and a single injection of iodobenzene (0.4 ml/kg). Concurrent positive control groups received one injection of iodobenzene (0.4 ml/kg) at time zero. One control group received corn oil for the vehicle control. Animals were sacrificed at 12, 24, 48, or 72 hours after iodobenzene treatment and serum ALT activity determined. Nine animals in the group that received iodobenzene alone died before the 72 hour expermental period had elapsed while none died in the group that received phentolamine plus iodobenzene. Each group contained at least 6 animals per group at the end of the experiment. The results of this experiment are shown in Figure 3.6. The groups that received iodobenzene had serum ALT activity significantly elevated above control groups at 24, 48 and 72 hours post-treatment, however the groups that received co-treatments of phentolamine and iodobenzene were not significantly different from control at any time point tested. The iodobenzene treated animals showed a maximum of serum ALT activity at 24 hours post-treatment. The phentolamine co-treated groups and the 12 hour iodobenzene treated group were significantly different from the groups that received iodobenzene alone at 24, 48 and 72 hours.

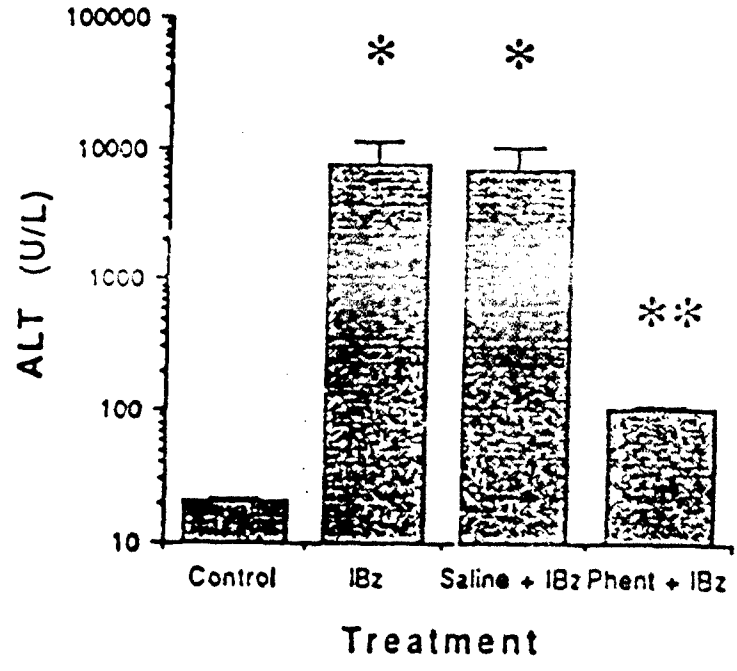


Figure 3.5. Comparison of Five Injections of Saline or Phentolamine in Protecting Against Iodobenzene-Induced Hepatotoxicity.

Four groups composed of 8 animals per group were administered one of the following treatments: corn oil alone, iodobenzene (0.50 ml/kg) alone, phentolamine (10 ml/kg) and iodobenzene, or saline and iodobenzene. Phentolamine or saline was administered as a 15 minute pretreatment to the iodobenzene which was followed by 4 more injections at 90 minute intervals. By 24 hours, 1 animal had died in the saline and iodobenzene treated group and 3 had died in the iodobenzene alone group, but none had died in the phentolamine and iodobenzene treated group or in the corn oil control group. A single asterisk signifies a significant difference from the control group and a double asterisk signifies a significant difference from the other treated groups. Each point represents the mean ± SEM. Significant differences were determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test for multiple comparisons.

TABLE 3.2

Number of Phentolamine Injections Needed to Protect Against Iodobenzene-Induced Hepatotoxicity

Number of Phentolamine Injections	Time After Iodobenzene for 1st Injection	ALT U/L (mean ± SEM)
0	—	6094 ± 2688
1	3 HR	4114 ± 1448
2	3 HR	3747 ± 2386
3	3 HR	2852 ± 1736
5	-15 min.	343 ± 147
Corn Oil Controls		25 ± 2

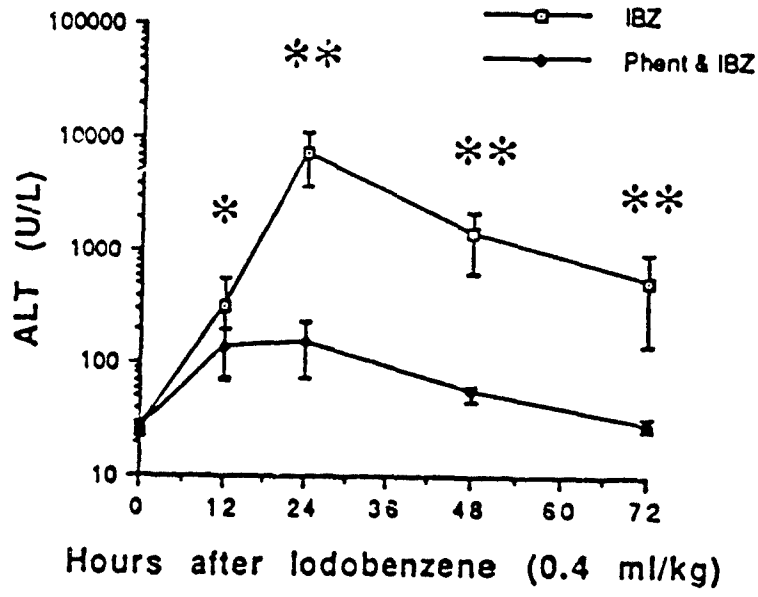


Figure 3.6. Temporal Changes in Serum Alanine Aminotransferase Activity after Co-Treatments of Phentolamine and Iodobenzene.

Iodobenzene (IBz; 0.40 ml/kg) was administered at time zero to B6C3F1 male mice. Groups co-treated with phentolamine (Phent & IBz) received an ip injection of phentolamine (10 mg/kg) 15 minutes prior to the iodobenzene injection and then 4 more injections at 90 minute intervals thereafter. Animals were sacrificed at 12, 24, 48 or 72 hours after iodobenzene treatment. A single asterisk indicates a significant difference from the control group and double asterisks indicate a significant difference from the time-matched phentolamine co-treated group as well as from control. Each point represents the mean ± SEM of serum ALT activity from at least 6 animals. Serum ALT activity is expressed as U/L and is shown on a log scale. Significant differences were determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test for multiple comparisons.

3.2.C Temporal Changes in Hepatic Glutathione after Co-Treatments with Iodobenzene and Phentolamine.

As shown in the previous series of experiments, phentolamine had been successful in blocking the hepatotoxicity resulting from iodobenzene administration. Thus the next series of experiments were designed to determine if phentolamine might also be capable of blocking the decrease in hepatic glutathione that also accompanies iodobenzene intoxication. Iodobenzene (0.4 ml/kg) or iodobenzene plus the phentolamine (10 mg/kg) co-treatments were administered to the mice. The animals received the usual phentolamine co-treatment regimen unless the time course required sacrifice before the regimen was completed. Thus, the 2 hour group received a total of 2 injections of phentolamine, the 4 hour group received a total of 3 injections of phentolamine, and the 8 and 24 hour groups both received the usual 5 injections of phentolamine. The results of this experiment are shown in Figure 3.7a. Hepatic glutathione concentrations were decreased significantly below controls at 2, 4 and 8 hours after treatments in both iodobenzene alone and phentolamine plus iodobenzene treated groups. At 24 hours post-treatment there was no difference in glutathione concentrations for the group receiving iodobenzene and the controls, but the phentolamine plus iodobenzene treated group was significantly less than controls. When groups receiving iodobenzene alone were compared to groups receiving iodobenzene plus phentolamine, the phentolamine co-treated animals had a significantly higher concentration of glutathione than did the iodobenzene alone group at 8 hours post-iodobenzene administration. However, by 24 hours the group receiving iodobenzene alone had glutathione concentrations significantly elevated above phentolamine co-treated groups. Serum was collected and ALT activity was determined from the samples at the 24 hour time point to make certain that this treatment did indeed result in hepatotoxicity to the animals. The results of that assay are shown in Figure 3.7b. As in the previous experiments, iodobenzene administration resulted in a significant increase in serum ALT activity and phentolamine co-treatment significantly diminished the toxicity confirming that iodobenzene administration induced hepatotoxicity in these animals and that phentolamine co-treatment significantly diminished this toxicity.

3.2.D Protection from Lethality by Co-Administration of Phentolamine with Iodobenzene.

As shown in Table 3.1, iodobenzene administration resulted in some lethality when the doses exceeded 0.25 ml/kg. To demonstrate whether phentolamine (10 mg/kg) protected against this lethality, iodobenzene (IBz; 0.53 ml/kg) alone or iodobenzene plus phentolamine (Phent & IBz) was administered to animals and animals were checked for 24 hours. The results of this experiment are shown in Figure 3.8. A dramatic difference was seen in the number of animals that survived with the phentolamine co-treatments. Of the animals receiving iodobenzene alone, only 14.3% survived until 24 hours post-treatment. All of the animals in

the iodobenzene plus phentolamine co-treated and control groups survived until 24 hours post-treatment.

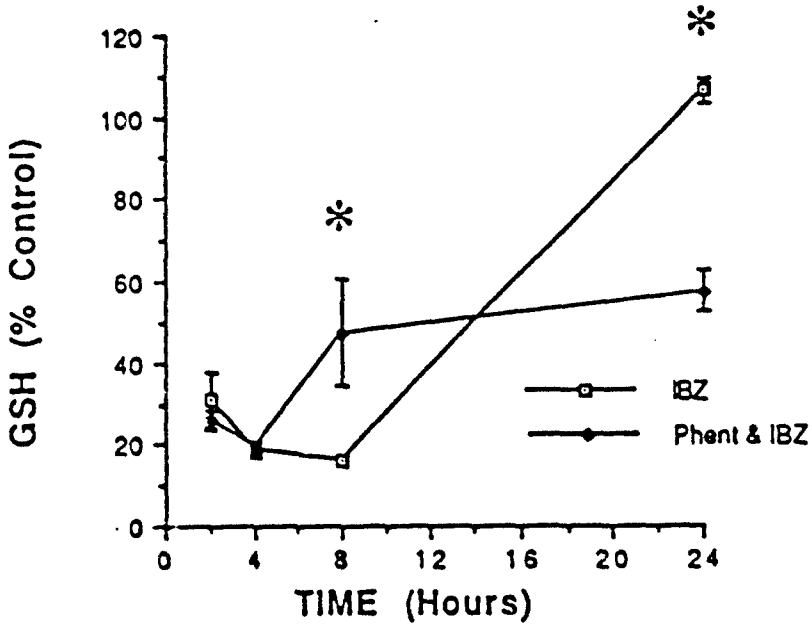


Figure 3.7a. Temporal Changes in Hepatic Glutathione after Treatment with Iodobenzene and Phentolamine.

Iodobenzene (IBz; 0.40 ml/kg) was administered at time zero to B6C3F1 male mice. Groups co-treated with phentolamine (Phent & IBz) received an ip injection of phentolamine (10 mg/kg) 15 minutes prior to the iodobenzene injection followed by a maximum of 4 more injections at 90 minute intervals. The animals were sacrificed at 2, 4, 8 or 24 hours after the iodobenzene treatment. Therefore, the animals co-treated with phentolamine and sacrificed at 2 hours, received a total of 2 phentolamine injections; at 4 hours, they received a total of 3 injections of phentolamine; and at 8 and 24 hours, they received a total of 5 injections of phentolamine. Each group contained at least 7 animals per group. Asterisks signify a significant difference from the iodobenzene-alone time matched positive control group. Each point represents the mean ± SEM and is expressed as percentage of control concentrations. Significant differences were determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test for multiple comparisons.

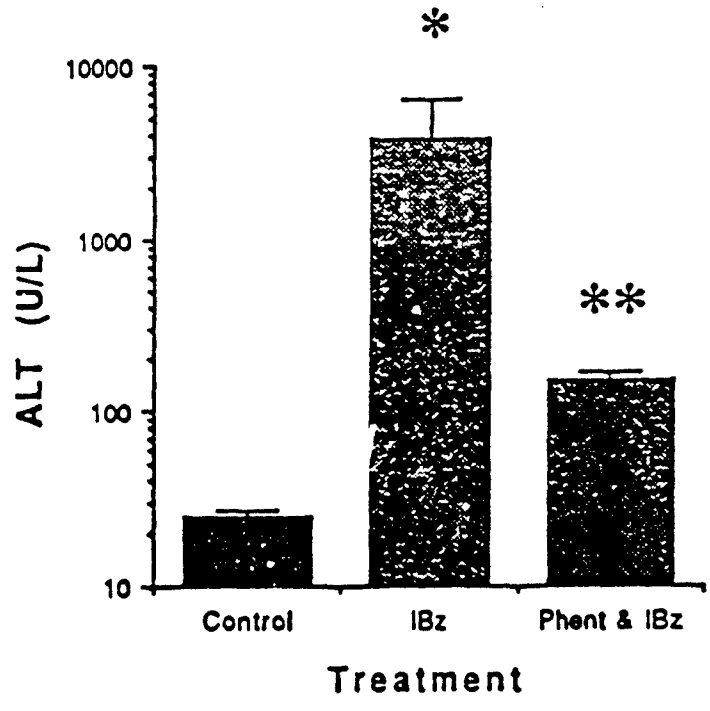


Figure 3.7b. Serum Alanine Aminotransferase Activity for the 24 Hour Time Point from Figure 3.7a.

When animals from the 24 hour time point were sacrificed, serum was collected and assessed for hepatotoxicity by measuring serum ALT activity. Serum ALT activity is expressed in U/L and is shown on a log scale. A single asterisk signifies a significant difference from the control group. Two asterisks signify a signifcant difference from a treatment group. Each point represents the mean ± SEM of at least 7 determinations. Significant differences were determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test for multiple comparisons between treatment groups.

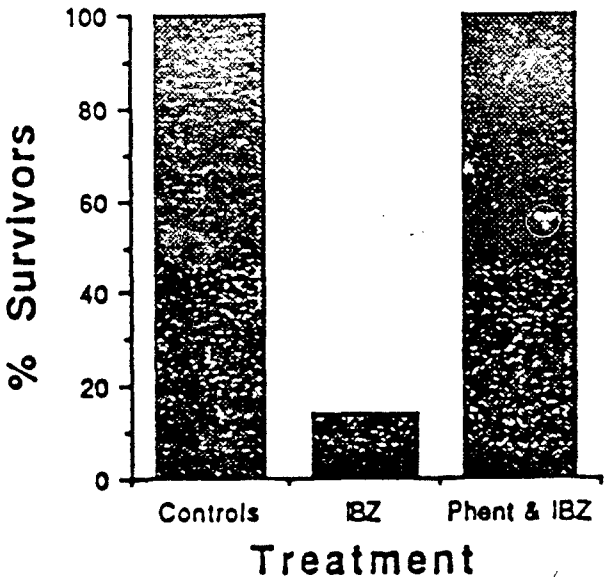


Figure 3.8. Percent Lethality after Treatment with Iodobenzene or Iodobenzene Plus Phentolamine Co-Treatments.

Iodobenzene (IBz; 0.53 ml/kg) or phentolamine (10 mg/kg) plus iodobenzene (Phent & IBz) were administered to B6C3F1 male mice. There were 7 animals per group. Groups co-treated with phentolamine received an ip injection of phentolamine 15 minutes prior to the iodobenzene injection and followed by 4 more injections at 90 minute intervals. None of the iodobenzene and phentolamine treated animals had died by 24 hours post-treatment.

3.3 Effects of Iodobenzene Administration on Serum Catecholamine Concentrations.

To determine whether iodobenzene administration results in the release of catecholamines into the blood stream as had been shown for bromobenzene (Kerger *et al.*, 1988a), iodobenzene (0.53 ml/kg) was administered to five groups of mice and the temporal changes in serum catecholamine concentrations were determined. The animals were sacrificed at 0, 0.5, 1.0, 2.0 or 4.0 hours after treatment. The results of this experiment are shown in Figure 3.9a and the data are expressed as ng catecholamines/ml serum. To determine the catecholamine concentrations, serum concentrations of norepinephrine, epinephrine and dopamine were determined from the same sample and were summed together. No significant elevations in serum catecholamine concentrations were seen before 2 hours post-treatment. However, serum catecholamines were significantly elevated above controls at 2 hours post-treatment. A positive control, bromobenzene (0.5 ml/kg), was administered and epinephrine determined 4 hours post-treatment since previous studies in our laboratory had shown that at this dosage and time bromobenzene treatment resulted in an increase in serum epinephrine (Kerger *et al.*, 1988a). Norepinephrine concentrations were 114% and epinephrine concentrations were 128% of control for the bromobenzene treated animals (data not shown). To ascertain that these doses of iodobenzene and bromobenzene were capable of inducing hepatotoxicity, 3 groups of animals were injected in the same experiment as described in Figure 3.9a with iodobenzene (0.53 ml/kg), bromobenzene (0.50 ml/kg) or corn oil and were sacrificed 24 hours later. Serum ALT was determined for these animals and the results are shown in Figure 3.9b. Serum ALT was significantly elevated above controls in either halobenzene-treated group. Thus, at a hepatotoxic dose of iodobenzene, serum catecholamines were not significantly increased before 2 hours post-treatment, therefore, the remaining experiments were designed to examine only the 2, 4 and 8 hour time points.

3.3.A Comparison of Serum Catecholamine and Glutathione Concentrations after Iodobenzene Administration.

One postulated mechanism by which exposure to halobenzenes might result in hepatotoxicity is by the halobenzene-induced release of catecholamines into the bloodstream which may further deplete glutathione, perhaps below a threshold concentration, and damage the hepatocyte. To test for this mechanism, two doses of iodobenzene were chosen for their different effects on hepatic glutathione and serum ALT activity. The low dose (0.1 ml/kg iodobenzene) depletes glutathione to approximately 60% of control, whereas 0.25 ml/kg iodobenzene depletes

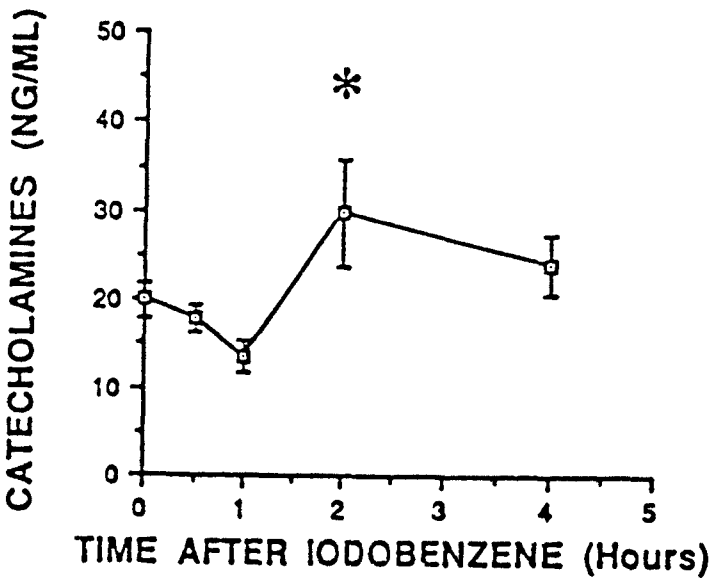


Figure 3.9a. Temporal Changes in Serum Catecholamine Concentrations after Administration of Iodobenzene.

Iodobenzene (IBz; 0.53 ml/kg) was administered ip in a corn oil vehicle and animals were sacrificed at 0, 0.5, 1.0, 2.0 or 4.0 hours after treatment. Results are expressed as ng catecholamines/ml of plasma. Each group contained 6 animals per group and all three catecholamines were determined and summed together for each animal. The asterisk signifies a significant difference from the control group. Each point represents the mean ± SEM from at least 6 animals per time period. Significant differences were determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test.

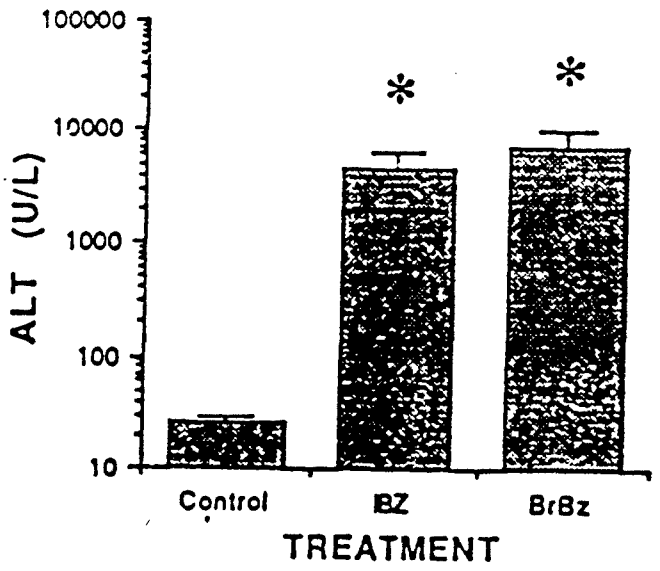


Figure 3.9b. Serum Alanine Aminotransferase Activity after Administration of Bromobenzene or Iodobenzene.

Bromobenzene (BrBz; 0.50 ml/kg), iodobenzene (IBz; 0.53 ml/kg) or corn oil vehicle (control) was administered ip to B6C3F1 male mice. These animals were injected on the same day as animals in Figure 3.9a. All animals were sacrificed 24 hours post-treatment. Hepatotoxicity was evaluated by elevations in serum ALT activity. Serum ALT activity is expressed as U/L and is shown on a log scale. Asterisks signify a significant difference from the control group. Each point represents the mean ± SEM. Significant differences were determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test.

glutathione to below 20% of control as shown in Figure 3.4. No increase in serum ALT activity is seen after administration of 0.1 ml/kg iodobenzene, but administration of 0.25 ml/kg iodobenzene results in a significant increase in serum ALT activity as shown in Figure 3.2a and 3.2b. Serum was collected and evaluated for norepinephrine, epinephrine and dopamine. Data are expressed as the sum of the three catecholamines. Since no significant changes in catecholamines occur before 2 hours post-treatment (as shown in Figure 3.9a), the times examined in the following experiments were 2, 4 and 8 hours. The 8 hour time point was added to examine the long-term effects of iodobenzene on catecholamine concentrations.

3.3.B Comparison of Hepatotoxic Versus Non-Hepatotoxic Doses of Iodobenzene on Serum Catecholamine Concentrations.

Animals were dosed with either 0.1 ml/kg or 0.25 ml/kg iodobenzene or corn oil vehicle and were sacrificed at 2, 4 or 8 hours post-treatment. Serum norepinephrine, epinephrine and dopamine concentrations were determined, data were summed for each sample and the results are shown in Figure 3.10. Two hours after treatment with either dose of iodobenzene, serum catecholamine concentrations were depressed to approximately one-half of control concentration. There was no significant difference between control and the 0.1 ml/kg group at either 4 or 8 hours post-treatment. The high dose group (0.25 ml/kg iodobenzene) was not significantly different from controls at 4 hours or 8 hours post-treatment.

3.3.C Comparison of Hepatotoxic Versus Non-Hepatotoxic Doses of Iodobenzene on Hepatic Glutathione Concentrations.

Hepatic glutathione concentrations were determined at 2, 4 and 8 hours after treatment with either 0.0 ml/kg, 0.10 ml/kg or 0.25 ml/kg iodobenzene. Liver tissue was removed from the mice treated in the above experiments. It was essential to show that the doses used for the catecholamine studies did indeed affect glutathione in the expected manner. The results are shown in Figure 3.11 and are expressed as percentage of control. These results confirm the previous findings (shown in Figure 3.4) that hepatic glutathione concentrations are depressed maximally by 2 to 4 hours post-treatment to 73% and 26% of control at 0.1 and 0.25 ml/kg iodobenzene, respectively. Thus, these doses were effective in depleting hepatic glutathione at the desired levels. At both dosages glutathione concentrations had recovered to normal concentrations by 8 hours post-treatment.

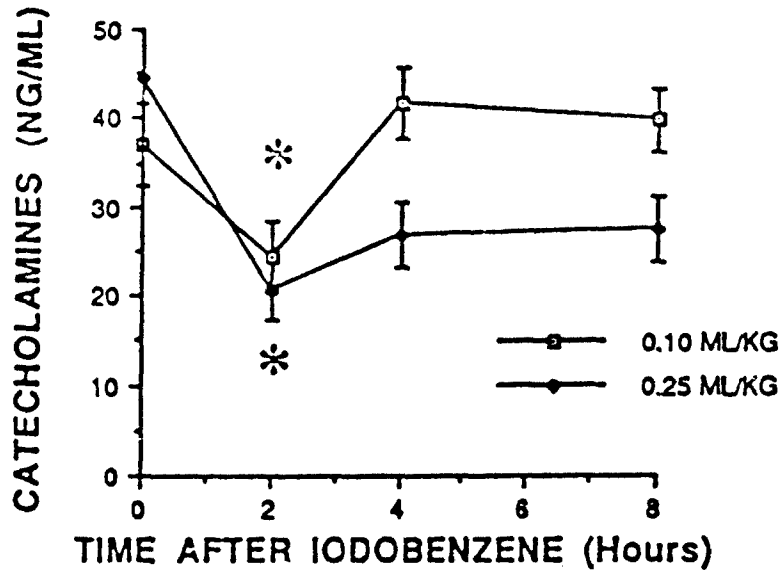


Figure 3.10. Comparisons of the Temporal Changes in Serum Catecholamine Concentrations after Treatment with a Hepatotoxic or a Non-Hepatotoxic Dose of Iodobenzene.

Iodobenzene was administered ip to B6C3F1 male mice at the following doses: 0.10 or 0.25 ml/kg and were sacrificed at 2, 4 or 8 hours post-treatment. There were 6 animals per group. Results are expressed as ng catecholamines/ml of plasma. Each point represents the mean ± SEM of 6 individual samples. An asterisk indicates a significant difference from control concentration. Significant differences were determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test.

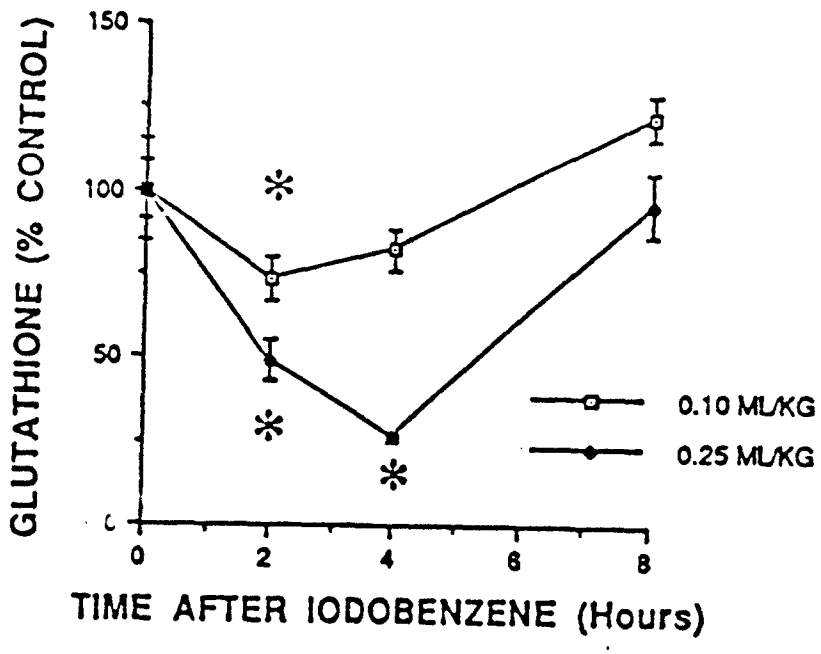


Figure 3.11. Comparison of Hepatic Glutathione Concentrations after Treatment with a Hepatotoxic or a Non-Hepatotoxic Dose of Iodobenzene.

Iodobenzene was administered ip to B6C3F1 male mice at the following doses: 0.00, 0.10 or 0.25 ml/kg. Animals were sacrificed at 2, 4 or 8 hours post-treatment. These samples were taken from the same animals used in Figure 3.10. There were 6 animals per group. Results are expressed as percent of control concentrations. Each point represents the mean ± SEM of 6 individual samples. An asterisk signifies a significant difference from control concentration. Significant differences were determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test.

3.4 Assessment of Chlorobenzene-Induced Hepatotoxicity

The next series of experiments was designed to give basic information concerning the toxic effects of chlorobenzene on the liver. Temporal changes and dose response data were derived for both hepatotoxicity, as determined by serum alanine aminotransferase activity, and hepatic glutathione concentrations.

3.4.A Temporal Changes in Serum Alanine Aminotransferase Activity after Chlorobenzene Treatment.

To determine the time of maximum response of serum ALT activity after chlorobenzene treatment temporal changes in serum ALT activity were examined. Chlorobenzene was administered by ip injection at a dose of 0.48 ml/kg, which represented a molar dose equivalent to that used in the previous bromobenzene studies (Kerger *et al.*, 1988a). Chlorobenzene was administered in a corn oil vehicle. Animals were sacrificed at 24, 48, 72 or 96 hours post-treatment. Results of this experiment are shown in Figure 3.12. By 24 hours post-treatment serum ALT activity in the treated group was significantly increased above controls. At 48 hours post-treatment, serum ALT activity remained at the same high level as that seen in the 24 hour group. By 72 and 96 hours post-treatment, the chlorobenzene treated animals had serum ALT activities that were not significantly different from controls. At 48 hours, 2 animals had died from the treated group, but no additional animals died by 72 or 96 hours post-treatment indicating that the animals were recovering from the initial toxic insult.

Serum ALT response to chlorobenzene was also tested at a higher dose (1.0 ml/kg). Chlorobenzene was injected ip in a corn oil vehicle and the animals were sacrificed at 12, 24, 48 or 72 hours post-treatment. The maximum activity was 3116 ± 1439 U/L at 24 hours post-treatment and serum ALT did not decline significantly by 72 hours post-treatment (Data not shown). Two of the chlorobenzene treated animals had died by 72 hours post-treatment. Therefore, serum ALT activity can be increased by increasing the dose of chlorobenzene, but the time course changes since serum ALT activity does not return to normal by 72 hours post-treatment.

3.4.B Dose Response of Serum Alanine Aminotransferase Activity after Chlorobenzene Treatment.

Since the temporal changes in serum ALT activity after chlorobenzene treatment had indicated that serum ALT activity was maximal at 48 hours post-treatment, the dose response was examined at that time period. Chlorobenzene was administered ip at 0.01, 0.10, 0.25, 0.50 or 1.00 ml/kg doses. Animals were sacrificed 48 hours post-treatment and the results are presented in Figure 3.13. The maximum value for serum ALT activity was 3845 ± 1649

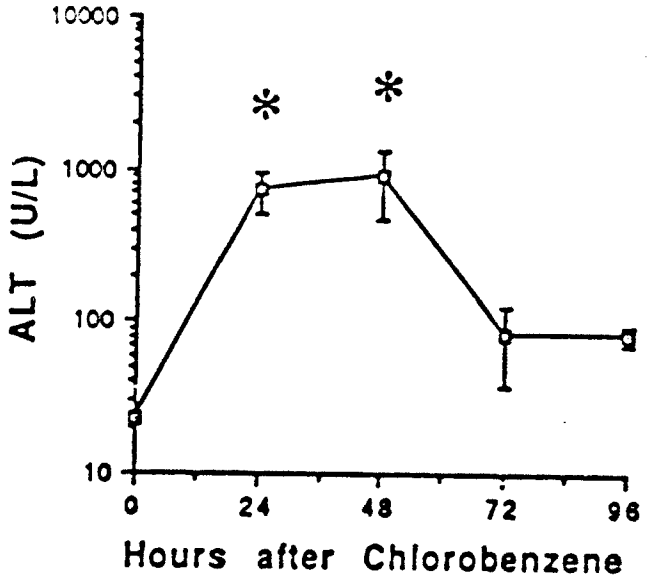


Figure 3.12. Temporal Changes in Serum Alanine Aminotransferase Activity After Treatment with Chlorobenzene.

Chlorobenzene (0.48 ml/kg) was administered ip to B6C3F1 male mice and animals were sacrificed at 24, 48, 72, or 96 hours post-treatment. Each group contained at least 7 animals. Serum ALT activity is expressed in U/L. The results are means ± SEM and are shown on a log scale. Asterisks indicate significant differences from the control group. Significant differences were determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test.

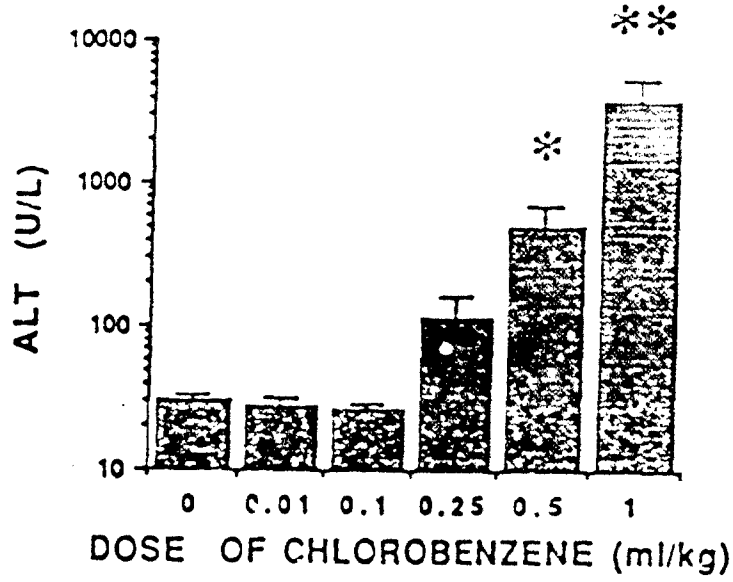


Figure 3.13. Dose Response of Serum Alanine Aminotransferase Activity after Treatment with Chlorobenzene.

B6C3F1 male mice were injected ip with chlorobenzene at doses of 0, 0.01, 0.1, 0.25, 0.5 or 1.0 ml/kg and animals were sacrificed 48 hours after treatment. Corn oil was used as the diluent and as vehicle for chlorobenzene. Each group had at least 9 animals per group at end of the experiment. Two animals died in the 0.1 ml/kg group and one animal died in each of the following groups: 0.25, 0.5 and 1.0 ml/kg. The results are expressed as mean ± SEM. Serum ALT is expressed in U/L and is shown on a log scale. A single asterisk indicates a significant difference from control treatment and double asterisks indicate significant differences from control and other treated groups. Significant differences were determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test.

U/L from the 1.0 ml/kg dose group. The 1.0 ml/kg treated group was significantly different from the control group as well as all other treatment groups. The only other group that was signifcantly different from the control group was the 0.50 ml/kg dose group which showed an intermediate response between the control and the 1.0 ml/kg dose group. Although the 0.25 ml/kg dose group appeared to have serum ALT activities greater than controls, it was not significant at $p<0.05$ level probably due to the variability within the group.

3.4.C Lethality after Administration of Chlorobenzene.

As seen in Table 3.3, chlorobenzene administered in high doses appears to be extremely toxic. However, at doses below 1.0 ml/kg, animals did not die from the intoxication. At doses above 1.0 ml/kg, all animals died before 24 hours had elapsed.

3.4.D Temporal Changes in Hepatic Glutathione Concentrations Following Chlorobenzene Treatment.

In order to determine the time of maximum glutathione depletion and to determine whether hepatic glutatione concentrations would return to normal following chlorobenzene treatment, temporal changes in hepatic glutathione concentrations were determined. Chlorobenzene (0.48 ml/kg) was injected ip to B6C3F1 male mice and animals were sacrificed at 2, 4, 8 or 24 hours after receiving chlorobenzene. The results of this experiment are shown in Figure 3.14. Hepatic glutathione concentrations are expressed as mean ± SEM in mg of glutathione/g of liver tissue. By 2 hours post-treatment, hepatic glutathione was 28% of control concentrations. Hepatic glutathione concentrations were depressed to a maximum of 10% of control concentrations at 4 hours after treatment with chlorobenzene. At 8 hours post-treatment, glutathione levels had recovered to 39% of control and finally, by 24 hours post-treatment, glutathione concentrations from treated animals had recovered back to the normal concentrations found in control animals. The treated groups at 2, 4 and 8 hours were all significantly different from control or the 24 hour treated group. The treated group at the 24 hour time point was not significantly different from control. Thus, chlorobenzene depleted hepatic glutathione concentrations maximally at four hours post-treatment.

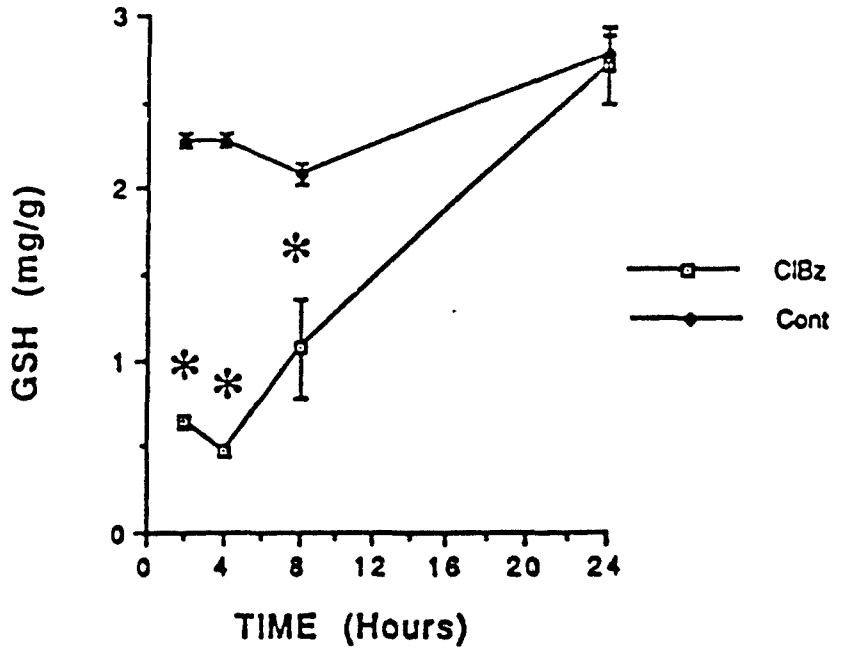


Figure 3.14. Changes in the Temporal Patterns of Hepatic Glutathione Concentrations after Treatment with Chlorobenzene.

Chlorobenzene (ClBz) at a dose of 0.48 ml/kg or corn oil vehicle (control) was injected ip to B6C3F1 male mice. The mice were sacrificed at 2, 4, 8 or 24 hours post-treatment. Each group contained 8 animals/group. Results are expressed as mg hepatic glutathione/g of liver tissue and represents the mean ± SEM of 8 animals. Serum ALT was determined on the 24 hour time point to confirm hepatotoxicity. Asterisks indicate significant differences from control concentrations. Significant differences were determined by analysis of variance followed by a Duncan's Multiple Range Test at $p<0.05$.

3.4.E Dose Response of Hepatic Glutathione Concentrations Following Chlorobenzene Treatment.

To further describe the effect of chlorobenzene on hepatic glutathione concentrations, a dose response of glutathione following chlorobenzene administration was completed. Results of this experiment are shown in Figure 3.15. Although it appears that the 0.01 ml/kg dose resulted in a slight increase of hepatic glutathione concentrations, it was not significantly different from control at $p<0.05$ level of significance. However, all other doses were significantly different from control concentrations and their administration resulted in hepatic glutathione concentrations that were 77%, 22%, 24% and 29% percent of control for 0.10, 0.25, 0.50 and 1.0 ml/kg, respectively. Additionally, the groups receiving 0.01 ml/kg and 0.10 ml/kg doses were significantly different from all other treatment groups. The 3 higher doses (0.25, 0.5 and 1.0 ml/kg) were not significantly different from each other. For chlorobenzene, a minimum dose of 0.25 ml/kg was capable of depleting glutathione to the same extent as the higher doses by 3 hours post-treatment.

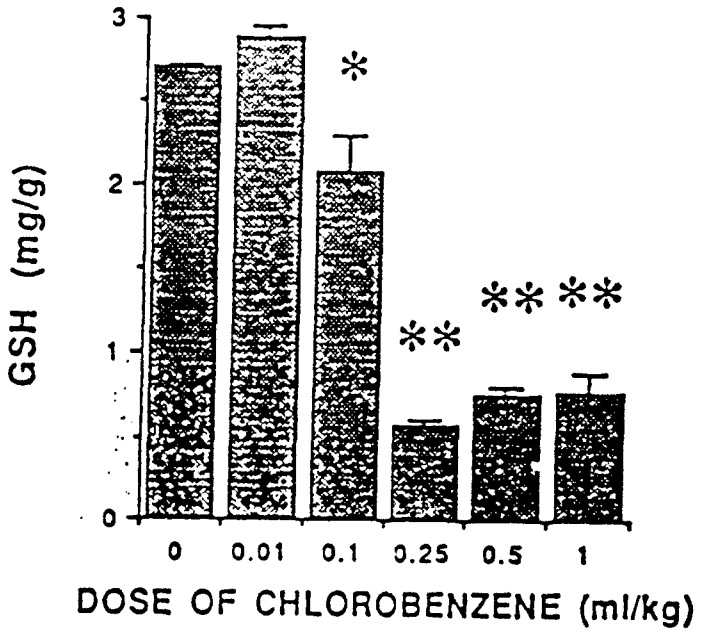


Figure 3.15. Dose Response of Hepatic Glutathione Concentrations After Treatment with Chlorobenzene.

Chlorobenzene was injected ip at doses of 0.01, 0.1, 0.25, 0.5 or 1.0 ml/kg to B6C3F1 male mice and mice were sacrificed 3 hours post-injection. Corn oil was used as the vehicle for administration. Each group contained at least 8 animals. The results are expressed as mean ± SEM. A single asterisk indicates a significant difference from the control group and double asterisk indicates a significant difference from the .01 and .1 ml/kg treated groups as determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test. Control animals had an average hepatic glutathione concentration (GSH) of 2.69 ± 0.08 mg/g of liver tissue.

3.5 Effect of Phentolamine Co-Treatments on Chlorobenzene-Induced Toxicities.

Although chlorobenzene intoxication resulted in hepatotoxicity to a lesser magnitude than either iodobenzene or bromobenzene, it was desirable to know whether phentolamine would protect against this hepatotoxicity. If so, this would suggest that all three compounds worked by a similar mechanism that resulted in hepatotoxicity, albeit to a different extent. If phentolamine did not protect against chlorobenzene hepatotoxicity, then idodbenzene and bromobenzene might be working by different, or perhaps additional, mechanisms to exert their toxicity. To determine this, the following series of studies were completed examining the effects of phentolamine co-treatments on chlorobenzene toxicities.

3.5.A Phentolamine Protection of Chlorobenzene-Induced Glutathione Depletion.

To determine if phentolamine could protect against chlorobenzene-induced toxicity similar to that seen with iodobenzene, chlorobenzene was administered in a dose (0.48 ml/kg) known to deplete glutathione. Experimental groups also received phentolamine (10 mg/kg) co-treatments. This experiment was designed to test whether phentolamine would block chlorobenzene-induced hepatic glutathione depletion. The temporal pattern of changes in glutathione were examined to ascertain that any effect seen was due to antagonism rather than a delay of toxicity. Chlorobenzene or chlorobenzene plus phentolamine co-treatments were administered to the animals. The phentolamine treatment regimen was the same as that previously described in the iodobenzene experiments. Thus, the number of phentolamine injections received by the groups depended upon the time of sacrifice. The group sacrificed 2 hours after chlorobenzene treatment received a total of 2 injections of phentolamine, the 4 hour group received a total of 3 injections of phentolamine, the 8 and 24 hour groups both received the usual 5 injections of phentolamine. The results of this experiment are shown in Figure 3.16a. Both groups, chlorobenzene alone and chlorobenzene plus phentolamine co-treatments, had hepatic glutathione concentrations decreased significantly below controls at 2, 4 and 8 hours after treatments. At 24 hours post-treatment, glutathione concentrations for the chlorobenzene treated group as well as the chlorobenzene plus phentolamine co-treated group had returned to control levels. The only time point where glutathione concentrations were significantly different between the phentolamine co-treated and the chlorobenzene alone treated groups was at 4 hours post-treatment. All other time points were not significantly different from each other.

Serum ALT activity was determined in the animals for the 24 hour time point to make certain that this treatment did indeed result in hepatotoxicity to the animals. The results of that assay are shown in Figure 3.16b. In this experiment, chlorobenzene administration resulted in

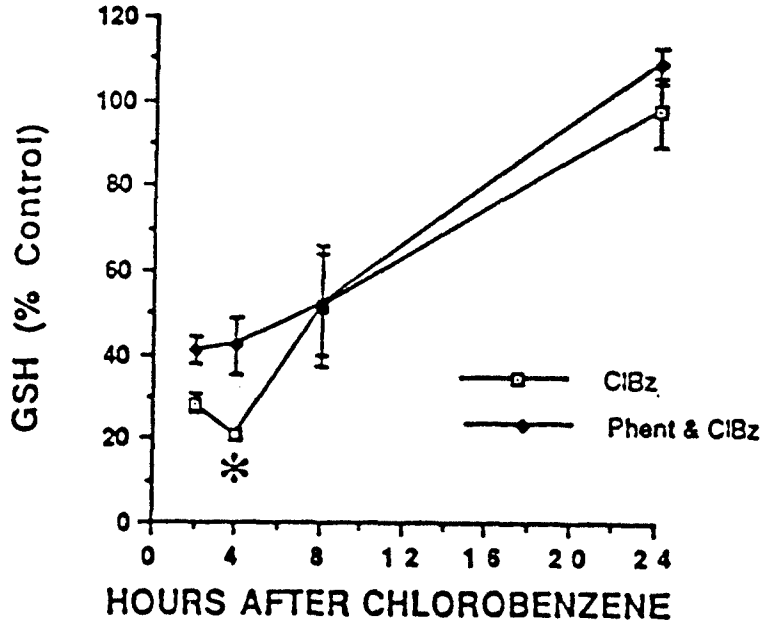


Figure 3.16a. Temporal Changes in Hepatic Glutathione Concentrations after Chlorobenzene and Phentolamine Co-Treatments.

Animals received chlorobenzene (ClBz; 0.48 ml/kg) alone or with phentolamine (10 mg/kg) co-treatment (Phent & ClBz). Groups receiving the phentolamine co-treatment received an injection 15 min before receiving chlorobenzene followed by a maximum of 4 more injections of phentolamine at 90 min intervals. Animals were then sacrificed at 2, 4, 8 or 24 hrs after the chlorobenzene injection. As in the previous experiments using phentolamine co-treatments, each animal in the 2 hr group received a total of 2 injections of phentolamine, in the 4 hr group each animal received a total of 3 injections of phentolamine and in the 8 hr and 24 hr groups each animal received a total of 5 injections of phentolamine. There were at least 7 animals per group.

Hepatic glutathione concentrations were determined in all animals. Results are expressed as percentage of control. Average concentrations for control groups were: 2.28 ± 0.03 mg/g for 2 and 4 hr controls; 2.08 ± 0.06 mg/g for 8 hr controls; and 2.77 ± 0.10 for 24 hr controls (mean ± SEM). Asterisks indicate significant differences between chlorobenzene alone treated groups and the time-matched phentolamine co-treated group. Significant differences were determined by an analysis of variance at $p<0.05$ followed by Duncan's Multiple Range Test.

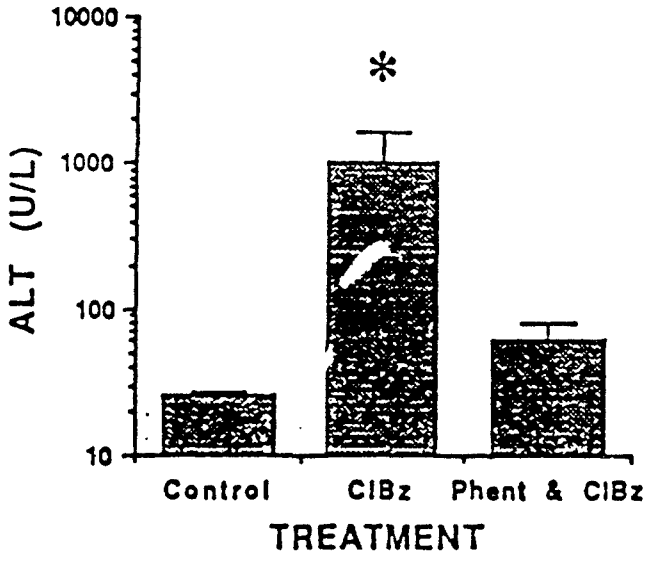


Figure 3.15b. Serum Alanine Aminotransferase Activities after Co-Treatments with Chlorobenzene and Phentolamine from Figure 3.16a.

Serum ALT activities were determined on the animals sacrificed at the 24 hour time point from Figure 3.16a in order to verify the chlorobenzene-induced hepatotoxicity and to describe the extent of the hepatotoxicity. An asterisk indicates significant differences between treatment and control groups. Significant differences were determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test.

an increase in serum ALT activity that was significantly different from control. Phentolamine co-treatment significantly diminished the toxicity and the phentolamine co-treated group was not significantly different from control, but was significantly different from the chlorobenzene group.

3.5.B Temporal Changes in Serum Alanine Aminotransferase Activity Following Co-Treatments of Phentolamine and Chlorobenzene.

Phentolamine was tested in this experiment as an antagonist against chlorobenzene-induced hepatotoxicity. The temporal changes in serum ALT were examined to ascertain that phentolamine was preventing rather than simply delaying hepatotoxicity. The experimental groups were co-treated with phentolamine (10 mg/kg) in the same treatment regimen as described in the previous experiments.

Concurrent positive control groups received one injection of chlorobenzene (0.48 ml/kg) at time zero. One control group received corn oil for the vehicle control. Animals were sacrificed at 12, 24, 48, or 72 hours after chlorobenzene treatment. None of the animals died before the 72 hour time period had elapsed. The results of this experiment are shown in Figure 3.17. The chlorobenzene treated groups were significantly different from their time matched phentolamine co-treated groups at 48 and 72 hours post-treatment. The chlorobenzene treated groups were significantly elevated above control groups at 24, 48 and 72 hours post-treatment, however the chlorobenzene plus phentolamine co-treated groups were not significantly different from control at any time except the 12 hour time point. The chlorobenzene treated animals showed a peak in serum ALT activity at 48 hours post-treatment (429 ± 173 U/L), while the phentolamine co-treated groups did not exceed 100 U/L at any time period examined.

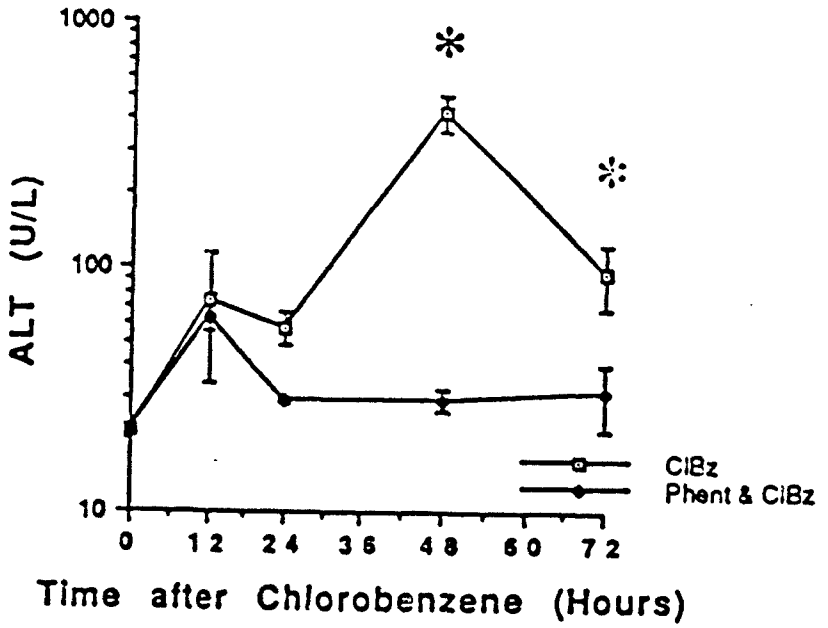


Figure 3.17. The Effect of Phentolamine Co-Treatments on the Temporal Pattern of Serum Alanine Aminotransferase Activity after Treatment with Chlorobenzene.

Animals received an ip injection of chlorobenzene (ClBz; 0.48 ml/kg) alone or with co-treatments of phentolamine (10 mg/kg). For co-treatments (Phent & ClBz), animals received an ip injection of phentolamine 15 minutes before receiving chlorobenzene. Following the injection of chlorobenzene, animals received 4 more injections of phentolamine at 90 minute intervals. Control animals received an injection of corn oil. Animals were sacrificed at 12, 24, 48 or 72 hours post-treatment of chlorobenzene. Serum ALT concentrations are expressed as mean ± SEM. There were at least 7 animals per group. Asterisks indicate significant differences from time-matched phentolamine co-treated groups. Significant differences were determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test.

3.6 Effect of Chlorobenzene Administration on Serum Catecholamine Concentrations.

Previous experiments had shown that these two doses were effective in depleting hepatic glutathione (Figure 3.15) and that the 1.0 ml/kg dose was effective in increasing serum ALT while the 0.25 ml/kg dose was not (Figure 3.13). Therefore, to determine the effect of chlorobenzene administration on serum catecholamine concentrations, two doses of chlorobenzene (0.25 or 1.0 ml/kg) were administered by ip injection. The results are described in the following sections.

3.6.A Temporal Patterns of Serum Catecholamine Concentrations after Treatment with a Hepatotoxic or a Non-Hepatotoxic Dose of Chlorobenzene.

A single injection of chlorobenzene (0.25 or 1.0 ml/kg) was administered to the animals and animals were sacrificed at 2, 4 or 8 hours later. The results of this experiment are shown in Figure 3.18 and are expressed as ng catecholamines/ml serum. At no time point (2, 4 or 8 hours) were the catecholamine concentrations for the low dose group (0.25 ml/kg) significantly different from the control group. At 4 hours post-treatment, the high dose group (1.0 ml/kg) was depressed below control concentrations. This was significantly different from both the control group and the low dose group. However, by 8 hours post-treatment the high dose group had recovered back to the normal range of concentrations. Therefore, at the 2 doses chosen for this experiment, the only significant change was seen at the four hour time point for the high dose group.

3.6.B Effects of a Non-Hepatotoxic Versus a Hepatotoxic Dose of Chlorobenzene on Hepatic Glutathione Concentrations.

To establish that the 2 doses of chlorobenzene chosen (0.25 and 1.0 ml/kg) did indeed depress hepatic glutathione in the expected manner, hepatic glutathione concentrations were determined on the animals used in the catecholamine studies. The results of this study are presented in Figure 3.19. Hepatic glutathione concentrations are expressed as percentage of control concentrations. The average control concentrations in mg of hepatic glutathione/g of liver tissue were 2.75 ± 0.08 for the 2 and 4 hour time points and 1.92 ± 0.15 for the 8 hour time point. By 2 hours post-treatment, hepatic glutathione concentrations in both the low (0.25 ml/kg) and high (1.0 ml/kg) doses were depleted to 28% and 32% of control concentrations, respectively. These concentrations did not change significantly by 4 hours post-treatment (34% and 22%, respectively). However, by 8 hours post-treatment the low dose group had hepatic glutathione concentrations that had fully recovered to normal levels

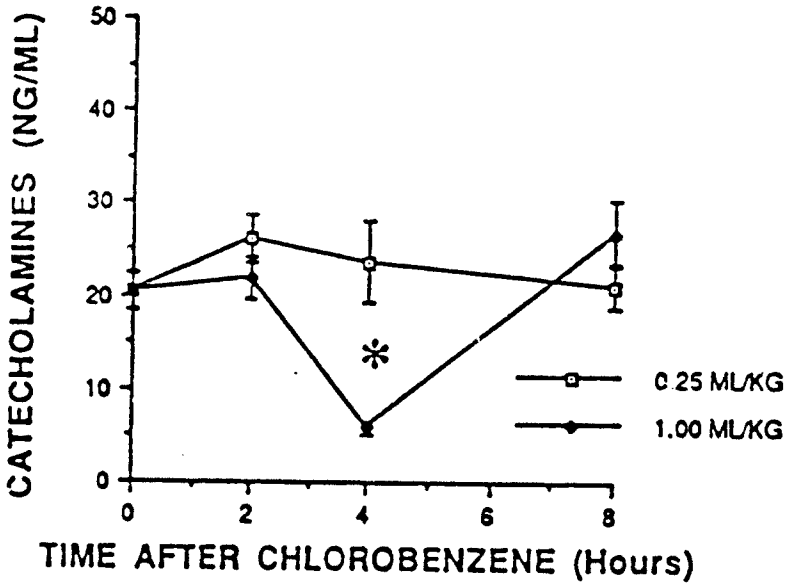


Figure 3.18. Temporal Patterns of Serum Catecholamine Concentrations after Treatment with a Hepatotoxic or a Non-Hepatotoxic Dose of Chlorobenzene.

Chlorobenzene was injected ip at 0.25 or 1.0 ml/kg doses and animals were sacrificed at 2, 4 or 8 hours post-treatment. There were 6 animals per group. Results are expressed as ng catecholamines/ml plasma. Total catecholamine concentrations were calculated by summing the norepinephrine, epinephrine and dopamine concentrations for each sample. The results are expressed as the mean ± SEM for 6 samples at each time point. The asterisk indicates a significant difference from control and from the 4 hour low dose (0.25 ml/kg) treatment group. Significant differences were determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test for multiple comparisons.

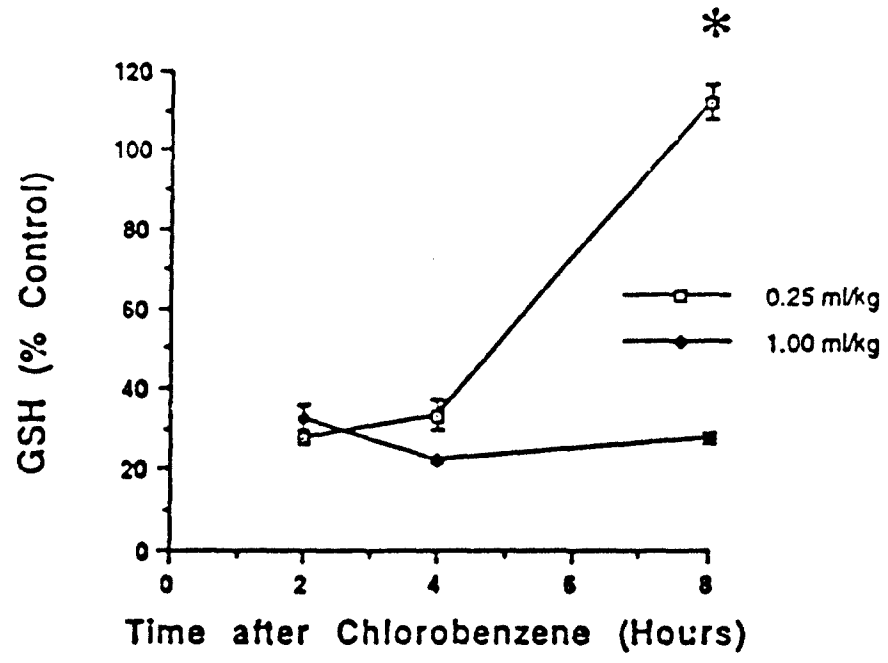


Figure 3.19. Temporal Patterns of Hepatic Glutathione after Treatment with a Hepatotoxic or a Non-Hepatotoxic Dose of Chlorobenzene.

Chlorobenzene was injected ip at 0.25 or 1.0 ml/kg doses and animals were sacrificed at 2, 4 or 8 hours post-treatment. There were 6 animals per group. Livers were removed from the animals used in the catecholamine study and glutathione concentrations were determined. Results are expressed as percentage of control. The average control concentrations for the 2 and 4 hour time points were 2.75 ± 0.08 mg/g of liver tissue (Mean ± SEM) and 1.93 ± 0.15 mg/g of liver tissue. The asterisk indicates a significant difference from the 1.0 ml/kg group. Significant differences were determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test for multiple comparisons.

(112%). This was in contrast to the high dose group which had glutathione concentrations that remained significantly depressed to the same low level (28%) as in the 2 and 4 hour time points. Thus, exposure to a high dose (1.0 ml/kg) of chlorobenzene resulted in hepatic glutathione concentrations that were significantly depressed at all three time points examined. Low dose (0.25 ml/kg) exposure to chlorobenzene resulted in a depletion of hepatic glutathione at the 2 and 4 hours time points, but by 8 hours post-treatment hepatic glutathione concentrations had recovered to normal.

3.7 Comparisons of the Toxicity Resulting from Exposure to Chlorobenzene, Bromobenzene or Iodobenzene.

In order to assess the effects of these three halobenzenes and to look for possible correlations between their structure and toxicity, the following series of experiments were conducted.

3.7.A Comparisons of Hepatotoxicity Resulting from Exposure to Molar Equivalent Doses (4.8 mM) of Chlorobenzene, Bromobenzene or Iodobenzene.

Molar equivalent doses of chlorobenzene (0.48 ml/kg), bromobenzene (0.50 ml/kg) or iodobenzene (0.53 ml/kg) were administered by ip injection in a corn oil vehicle. Serum ALT activities were determined as a measure of hepatotoxicity at the time of maximum response (24 hours post-treatment for iodobenzene and bromobenzene and 48 hours post-treatment for chlorobenzene). Results of this experiment are presented in Figure 3.20. Both bromobenzene and iodobenzene treated groups had serum ALT activities that were increased significantly above control concentrations by 24 hours post-treatment. By 48 hours post-treatment the majority of the animals in the bromobenzene and iodobenzene groups had died; thus samples could not be collected. Chlorobenzene treated animals did not show a significant increase above controls at 24 hours. However, they were significantly elevated above controls at 48 hours post-treatment. Thus, among the three halobenzenes, bromobenzene and iodobenzene appear to be more hepatotoxic at an earlier time period than chlorobenzene, and this toxicity results in death for the majority of the animals by 48 hours.

3.7.B Comparisons of the Effects of Molar Equivalent Doses of Halobenzenes on Hepatic Glutathione Concentrations.

Molar equivalent doses of chlorobenzene (0.48 ml/kg), bromobenzene (0.50 ml/kg) or iodobenzene (0.53 ml/kg) were administered by ip injection in a corn oil vehicle. Animals were sacrificed 12 hours after treatment in the same experiment as described for Figure 3.20. The results are presented in Figure 3.21 and are expressed as mg glutathione/g liver tissue. Bromobenzene and iodobenzene treated groups had glutathione concentrations that remained significantly depressed at 12 hours after treatment. In chlorobenzene treated animals, glutathione concentrations had recoved to control concentrations by 12 hours post-treatment. Since previous time course experiments have shown that all three of these halobenzenes deplete

glutathione maximally by 4 hours post-treatment, it appears that there are no significant differences in the ability of the halobenzenes to deplete hepatic glutathione in the early hours after treatment but that the time to recovery for glutathione is much longer for the more hepatotoxic halobenzenes, bromobenzene and iodobenzene.

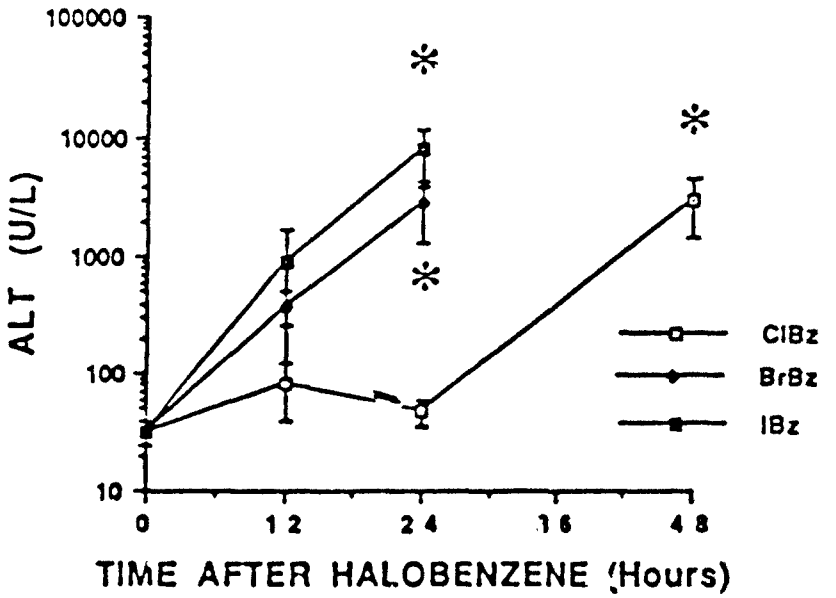


Figure 3.20. Comparison of Hepatotoxicity after Molar Equivalent Doses (4.8 mM) of Chlorobenzene, Bromobenzene and Iodobenzene.

Molar equivalent doses (4.8 mM) of chlorobenzene (0.48 ml/kg), bromobenzene (0.50 ml/kg) or iodobenzene (0.53 ml/kg) were administered to male B6C3F1 mice. Halobenzenes were administered as an ip injection in a corn oil vehicle. Control animals received an injection of corn oil alone. Animals were sacrificed at 12, 24 or 48 hours after treatment and serum analyzed for ALT. Serum ALT activity is expressed as mean ± SEM and is shown on a log scale. All groups had at least 6 animals/group. Significant differences were determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test. An asterisk indicates a significant difference from control.

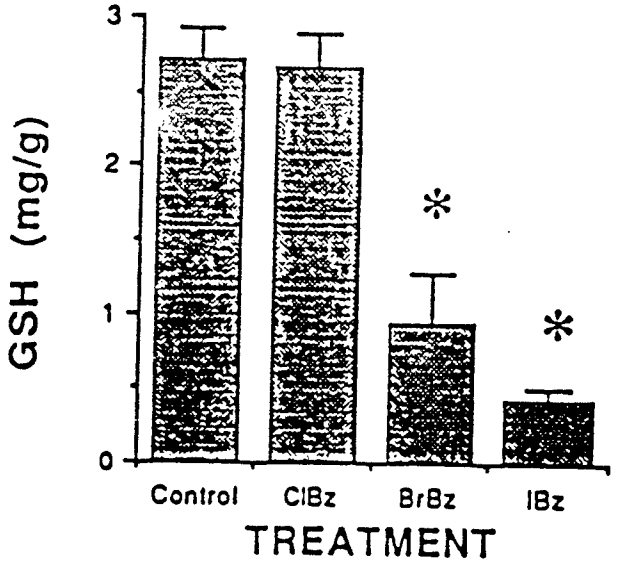


Figure 3.21. Comparisons of Hepatic Glutathione Concentrations after Treatment with Molar Equivalent Doses of Chlorobenzene, Bromobenzene or Iodobenzene.

Chlorobenzene (0.48 ml/kg), bromobenzene (0.50 ml/kg) or iodobenzene (0.53 ml/kg) were administered at molar equivalent doses (4.8 mM) to B6C3F1 male mice. Animals were sacrificed at 12 hours post-treatment and hepatic glutathione concentrations were determined. The halobenzenes were administered as an ip injection in corn oil vehicle. The results are expressed as mg GSH/g of liver tissue. All groups had at least 7 animals per group. An asterisk indicates a significant difference from control concentrations. Significant differences were determined by an analysis of variance at $p<0.05$ followed by a Duncan's Multiple Range Test for multiple comparisons.

3.7.C Comparisons of Hepatic Glutathione Concentrations Versus Serum Alanine Aminotransferase Activity after Treatment with Molar Equivalent Doses of Halobenzenes.

In an attempt to directly compare an individual's hepatic glutathione concentration with the serum ALT activity, molar equivalent doses (4.8 mM) of chlorobenzene (0.48 ml/kg), bromobenzene (0.50 ml/kg) or iodobenzene (0.53 ml/kg) were administered to B6C3F1 male mice. This method of directly comparing glutathione and serum ALT activity has been reported by Casini et al. (1988). Animals were sacrificed 12 hours post-treatment and both hepatic glutathione concentrations and serum alanine aminotransferase activities were determined for each animal and plotted as log values. The results of the three halobenzenes are combined and presented in Figure 3.22. Since both hepatic glutathione and serum ALT determinations were completed on individual samples, each individual sample is plotted and the results are shown on a histogram. There appears to be a trend to the data indicating that as glutathione concentrations decrease, serum ALT increases.

3.7.D Comparisons of Catecholamines after Hepatotoxic Doses of Chlorobenzene, Bromobenzene or Iodobenzene.

Known hepatotoxic doses of chlorobenzene (1.0 ml/kg), bromobenzene (0.50 ml/kg) or iodobenzene (0.25 and 0.53 ml/kg) were administered ip in a corn oil vehicle. Animals were sacrificed 4 hours post-treatment and serum catecholamine concentrations were determined. The results are presented in Figure 3.23 and are shown as percentage of control. The low dose of iodobenzene (0.25 ml/kg) and chlorobenzene (1.0 ml/kg) either significantly decreased or had no effect on serum concentrations of norepinephrine or epinephrine at 4 hours post-treatment. In contrast to this, the high dose of iodobenzene (0.53 ml/kg) and the molar equivalent dose of bromobenzene (0.50 ml/kg) both resulted in elevations of serum norepinephrine and epinephrine at 4 hours post-treatment.

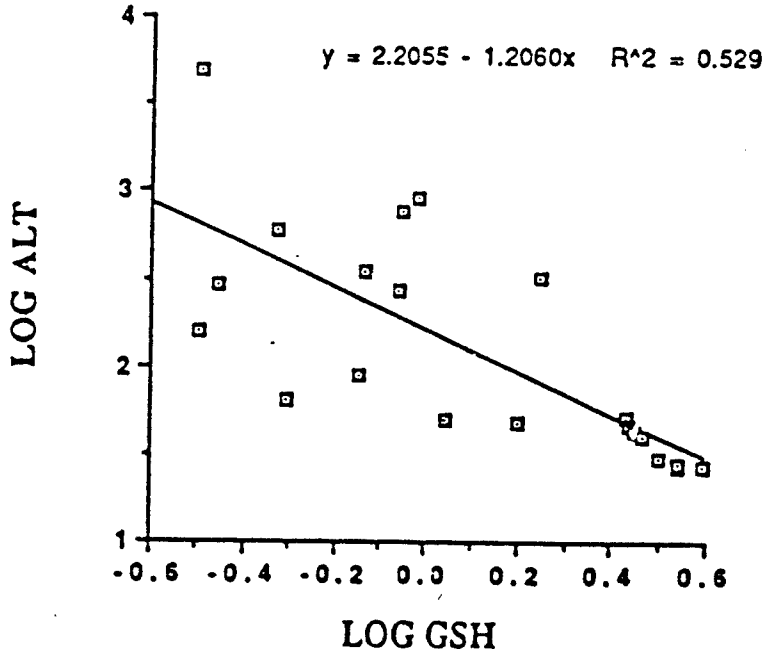


Figure 3.22. Comparisons of Hepatic Glutathione Concentrations Versus Serum Alanine Aminotransferase Activity 12 Hours after Treatment with a Halobenzene.

Chlorobenzene (0.48 ml/kg), bromobenzene (0.50 ml/kg) or iodobenzene (0.53 ml/kg) were administered ip at molar equivalent doses (4.8 mM) and animals were sacrificed 12 hours post-treatment. Hepatic glutathione concentrations and serum ALT activities were determined for each animal. Each point on the histogram represents the log of the hepatic glutathione concentration versus the log serum ALT activity for one animal. Each group contained at least 6 animals per group.

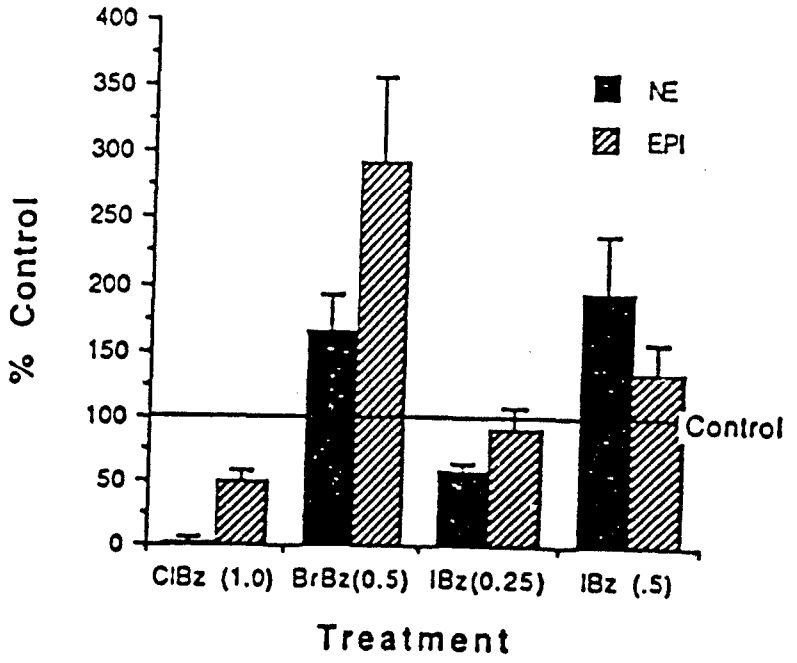


Figure 3.23. Comparisons of Catecholamine Concentrations 4 Hours after Treatment with Chlorobenzene, Bromobenzene or Iodobenzene.

Chlorobenzene (1.0 ml/kg), bromobenzene (0.5 ml/kg) or iodobenzene (0.25 or 0.53 ml/kg) were administered ip in corn oil vehicle and animals were sacrificed 4 hours post-treatment. Results are expressed as percentage of conirol. Each group contained at least 6 animals per group.

3.7.E Comparisons of Serum Alanine Aminotransferase Activity Response after Molar Equivalent Doses of Halobenzene and Phentolamine Co-Treatments.

In a final comparison of phentolamine's ability to block halobenzene-induced hepatotoxicity, molar equilavent doses of chlorobenzene, bromobenzene or iodobenzene were administered to male B6C3F1 mice. The halobenzene was administered alone as a positive control or with phentolamine co-treatments. The experiment was terminated at the time of maximum response for each halobenzene. The results are shown in Figure 3.24. Serum ALT is shown on a log scale and each point represents the mean ± SEM. Phentolamine was equally effective in preventing the halobenzene-induced increase in serum ALT acitivity for chlorobenzene and iodobenzene as it was for bromobenzene. For each halobenzene, the halobenzene administered alone was significantly higher than the halobenzene plus phentolamine co-treated groups. Likewise, none of the groups receiving phentolamine co-treatments were significantly differently from controls.

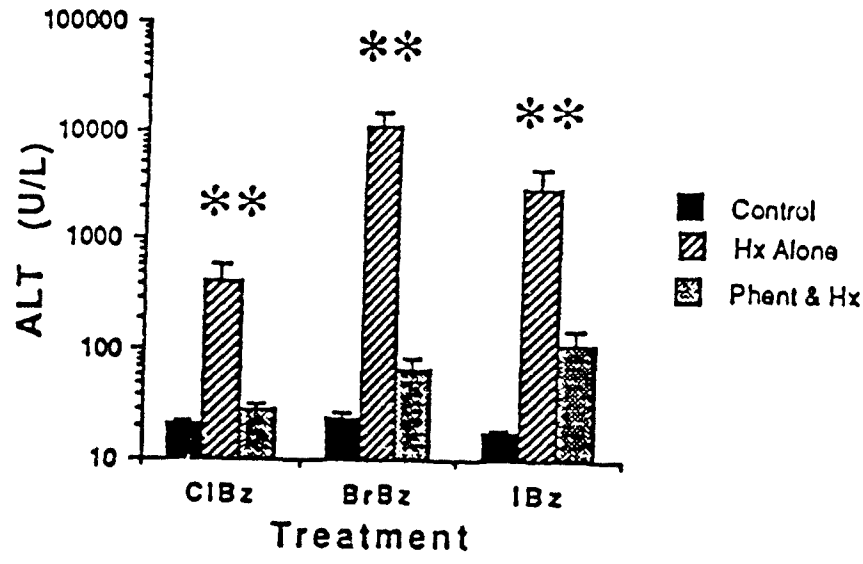


Figure 3.24 Comparisons of Serum Alanine Aminotransferase Activity Response After Molar Equivalent Doses of Halobenzenes Plus Phentolamine Co-Treatments.

Molar equivalent doses (4.8 mM) of chlorobenzene, bromobenzene or iodobenzene were administered to male B6C3F1 mice. Groups co-treated with phentolamine received an injection of phentolamine (10 mg/kg) 15 minutes prior to the halobenzene injection and then four more injections at 90 minute intervals thereafter. The animals were sacrificed at the time of maximum serum ALT response (ClBz = 48 hrs; BrBz and IBz = 24 hrs). A double asterisk indicates a significant difference from the control and its matched phentolamine co-treated group. Each point represents the mean ± SEM and serum ALT is plotted on a log scale. Significant differences were determined by an analysis of variance at $p < 0.05$ followed by a Duncan's Multiple Range Test.

DISCUSSION

The hepatotoxic effects of halogenated hydrocarbons and the mechanisms by which they induce these effects have been an area of active research for many years (Brody and Calvert, 1960; Calvert and Brody, 1960; Larson and Plaa, 1963, 1965; Schwetz and Plaa, 1969; Jollow *et al.*, 1974; Casini et al., 1982, 1985; Kerger *et al.*, 1988a). Thus far, bromobenzene has been the subject of most published research on halobenzenes (Jollow *et al.*, 1974; Casini *et al.*, 1982; 1985; Kerger *et al.*, 1988a), and much less is known about the related compounds, chlorobenzene and iodobenzene (Casini *et al.*, 1982, 1985). While examining bromobenzene hepatotoxicity, Kerger *et al.* (1988a) described a phenomenom by which the hepatotoxicity and lethality resulting from bromobenzene intoxication was significantly reduced by the alpha-adrenergic antagonist, phentolamine. The purpose of this study was to systematically describe the toxic effects of iodobenzene and chlorobenzene on the liver, to test for antagonism of these effects by phentolamine and whether the antagonism might correlate with changes in catecholamines, and to compare the hepatotoxicity of the halobenzendescribe possible structure activity relationships among the halobenzenes.

4.1 Chlorobenzene- and Iodobenzene-Induced Changes in Hepatotoxicity.

Hepatotoxicity can be evaluated by several methods including the examination of enzyme levels in the serum. According to Zimmerman (1978), serum enzymology is appropriate for determining possible hepatotoxicity of previously untested agents, for comparing the toxicity of known agents, for detecting the onset of liver injury, and for studying the potentiation or inhibition of toxicity resulting from the administration of combinations of drugs or from various physiologic manipulations. Since this dissertation study compared the extent and onset of hepatotoxicity from three halobenzenes as well as the inhibition of toxicity by an alpha-adrenoreceptor antagonist, serum enzymology was an appropriate test to describe these parameters. Serum alanine aminotransferase (serum ALT) activity was selected as the enzyme of choice because it is relatively specific to the liver and is accurately measured.

Changes in the temporal patterns of serum ALT activity after treatment with iodobenzene or chlorobenzene show somewhat different profiles. While serum ALT activity after iodobenzene treatment is maximal at 24 hours post-treatment, it remains significantly elevated above control values for at least 48 more hours. In comparison with a slightly higher dose of chlorobenzene (0.48 ml/kg vs 0.40 ml/kg), iodobenzene shows a much higher activity of serum ALT (4096 ± 2188 U/L; mean ± SEM) than does chlorobenzene (903 ± 442 U/L). Although it appears that serum ALT activity was maximal at 24 hours for iodobenzene and 48 hours for chlorobenzene, there was not a significant difference between the 24 and 48 hour time point for chlorobenzene. Iodobenzene treated groups had serum ALT activities that remained significantly elevated above controls through 72 hours, while chlorobenzene treated groups had serum ALT activities that were not significantly different from control by 72 hours

post-treatment.

Using serum ALT activity as an index of hepatotoxicity, an interesting difference in the response to varying doses of chlorobenzene and iodobenzene emerged. For iodobenzene, there was no significant increase in serum ALT activities until a dose of 0.20 ml/kg was administered. At that dose there was a sharp increase in serum ALT activities (Figures 3.2a and 3.2b) suggesting a dose threshold response. This was in contrast to chlorobenzene, which showed a more graded response to increasing doses (Figure 3.13), suggesting that the mechanism by which iodobenzene and chlorobenzene result in hepatotoxicity may be somewhat different. Previous studies with bromobenzene (Kerger *et al.*, 1988a) showed an intermediate response between these two extremes. Like the other two halobenzenes, bromobenzene administered at 0.10 ml/kg had no effect on serum ALT activities, however, at 0.25 ml/kg bromobenzene administration increased serum ALT activity to approximately 1000 U/L. At the 0.50 ml/kg dose, bromobenzene showed a maximum response similar to iodobenzene, with increases in serum ALT activity in the range of 10,000 U/L (Kerger *et al.*, 1988a). This indicates that like chlorobenzene, bromobenzene showed a linear dose response curve, but like iodobenzene, the maximum response is approximate 10-fold greater than with chlorobenzene. It is interesting to note that the three structurally similar compounds, iodobenzene, bromobenzene and chlorobenzene showed these different dose response relationships.

To summarize their effects on serum ALT activity, chlorobenzene and bromobenzene showed a similar linear relationship with dose although bromobenzene elevated serum ALT activities approximately 10 times greater than chlorobenzene. Iodobenzene, which showed a dose threshold effect, had a similar toxicity to bromobenzene.

4.2 Chlorobenzene- and Iodobenzene-Induced Changes in Hepatic Glutathione Concentrations.

Previous studies have presented evidence that chlorobenzene and iodobenzene are metabolized in a manner similar to bromobenzene. Early work by Jollow *et al.* (1974) demonstrated that bromobenzene is metabolized by hepatic cytochrome P-450 to 3,4-bromobenzene oxide (an epoxide metabolite), conjugated to glutathione and excreted from the body. They showed that a significant decrease in hepatic glutathione (by either the amount of the epoxides generated or by chemical pre-treatment prior to bromobenzene administration) resulted in an increase in covalent binding of the epoxide to cellular proteins and increased hepatotoxicity. Jollow *et al.* (1974) concluded that a dose-threshold exists for bromobenzene-induced hepatic necrosis beyond which detoxification of bromobenzene decreases and hepatic necrosis increases. In an *in vitro* system using either mouse or human liver microsomes, chlorobenzene is metabolized to the same extent and to the same phenol metabolites as bromobenzene (Kerger *et al.*, 1988b). Iodobenzene has been shown to rapidly deplete glutathione (Casini *et al.*, 1985). However temporal changes and dose response studies

describing and comparing the effects of chlorobenzene and iodobenzene on hepatic glutathione have not been published.

This study shows that the dose response of hepatic glutathione after either iodobenzene or chlorobenzene is similar (Figures 3.4 and 3.15). Hepatic glutathione is depleted to 10% of control values for iodobenzene and to 22% of control values for the same dose (0.25 ml/kg) of chlorobenzene. An intermediate dose of either halobenzene (0.10 ml/kg) results in a similar response from hepatic glutatnione, 61% and 77% of control, for iodobenzene and chlorobenzene, respectively, while a ten-fold lower dose (0.01 ml/kg) does not result in any significant change in hepatic glutathione concentrations. Increasing the doses of either halobenzene does not result in any significant change in hepatic glutathione concentrations but does increase lethality in some instances. Thus, the smallest dose of iodobenzene and chlorobenzene that was maximally effective in depleting hepatic glutathione at 3 hours post-treatment was 0.25 ml/kg for both compounds. These data compare favorably with that previously found for bromobenzene, where a dose of 0.25 ml/kg was found to deplete hepatic glutathione concentrations to near maximal at 4 hours post-treatment (Kerger *et al.*, 1988a).

To obtain the desired hepatotoxic effects, 3 different doses of iodobenzene were utilized to examine temporal changes in hepatotoxicity during these studies. These 3 doses show different profiles in the time-dependent changes seen in hepatic glutathione. Low dose iodobenzene (0.10 ml/kg) treatment resulted in glutathione depletion to 73% of control values by 2 hours post-treatment, after which hepatic glutathione concentrations begin to recover towards control concentrations. Control concentrations were attained by 8 hours post-treatment (Figure 3.11). Administration of the intermediate dose of iodobenzene (0.25 ml/kg) resulted in a decrease in hepatic glutathione by 2 hours, but the maximum decrease is seen between 3 and 4 hours post-treatment (Figures 3.4 and 3.11). However, as seen with the low dose of iodobenzene, hepatic glutathione concentrations have returned to normal values by 8 hours post-treatment. This is in contrast to the higher dose of iodobenzene (0.4 ml/kg) which depletes glutathione significantly by 2 hours and remains maximally depleted for up to 8 hours post-treatment (Figure 3.3). The chlorobenzene dosages used for these studies were somewhat higher than iodobenzene since chlorobenzene is less acutely toxic than iodobenzene (Figure 3.20). However, a similar pattern of altered hepatic glutathione emerges. Administration of the low dose of chlorobenzene (0.25 ml/kg) depletes hepatic glutathione maximally between 2 and 4 hours post-treatment (Figure 3.19). However, as seen with the lower dose of iodobenzene, hepatic glutathione concentrations have returned to normal concentrations by 8 hours post-treatment. Treatment with chlorobenzene at 0.48 ml/kg, depletes glutathione maximally by 4 hours and, although glutathione concentrations have not attained control values by 8 hours post-treatment, the trend appears to be in that direction (Figure 3.14).

This is in contrast to the high dose (1.0 ml/kg) group which shows depletion of hepatic glutathione near maximal by 2 hours post-treatment and which does not significantly change by

8 hours post-treatment. Thus, it appears that the major difference between iodobenzene and chlorobenzene in their effects on hepatic glutathione concentrations is not so much the extent of the glutathione depletion, but rather how long hepatic glutathione remains at this low concentration. However, Jollow *et al.* (1974) have suggested that bromobenzene toxicity is a function of depleting glutathione below a certain threshold concentration (10-20% of control), beyond which the organism is no longer able to metabolize the toxic compounds to non-toxic metabolites. Since iodobenzene and chlorobenzene are similar in structure to bromobenzene and the metabolism of these compounds has been suggested to be similar to bromobenzene (Casini *et al.*, 1982; Casini *et al.*, 1985; Kerger *et al.*, 1988c), it was suspected that they would show similar patterns of glutathione depletion. Administration of equivalent doses of iodobenzene and chlorobenzene resulted in depletion of hepatic glutathione to the same extent (approximately 10% of control concentrations) but dissimilar degrees of hepatotoxicity, suggesting that the mechanism of action for hepatotoxicity may not be linked solely to the extent of depletion of hepatic glutathione. Temporal changes in hepatic glutathione concentrations after bromobenzene administration show results similar to those found with iodobenzene and chlorobenzene. Kerger *et al.* (1988a) demonstrated that hepatic glutathione in mice treated with bromobenzene (0.5 ml/kg) had concentrations that were significantly depleted below controls at 2 hours and that the maximum depletion was seen at approximately 4 hours post-treatment. These concentrations remained at the same low level for over 8 hours post-treatment and were significantly depressed below control concentrations for up to 24 hours post-treatment. Thus chlorobenzene, bromobenzene and iodobenzene are capable of depleting hepatic glutathione to approximately 10-20% of control levels. In hepatotoxic doses these concentrations remain depressed for over 8 hours post-treatment. This study suggests that the length of time that glutathione is depleted maximally may correlate with hepatotoxicity.

It has been proposed that two pools of glutathione exists within the cell (Tateishi *et al.*, 1977; Higashi *et al.*, 1977), a cytoplasmic pool having a half-life of 2 hours and a mitochondrial pool having a half-life of 30 hours (Meredith and Reed, 1982). It is possible that the compounds and doses that are capable of depleting glutathione for extended periods of time are not only depleting the cytoplasmic pool but the mitochondrial pool of glutathione as well. This could prevent the detoxification of metabolites and disrupt oxidative metabolism within the mitochondria.

4.3 Comparison of Lethality after Chlorobenzene or Iodobenzene Treatments.

Iodobenzene appears to be more acutely toxic than chlorobenzene since iodobenzene treatment results in lethality at a much lower dose than chlorobenzene. For iodobenzene, administration of 0.25 ml/kg resulted in 6% of the animals dying while with chlorobenzene no animals died at doses up to 1.0 ml/kg. It took twice as much chlorobenzene (3.0 ml/kg; Table

3.3) to attain the LD_{100} as it did iodobenzene (1.5 ml/kg; Table 3.1). Bromobenzene administration (0.50 ml/kg) resulted in 67% lethality (Kerger *et al.*, 1988a) which is comparable to the 52% seen with an equivalent dose of iodobenzene (Table 3.1). It was somewhat surprising that chlorobenzene was less toxic than bromobenzene since *in vitro* metabolic studies showed that chlorobenzene was metabolized to the same phenolic compounds as bromobenzene (Kerger *et al.*, 1988b). Also, in this same *in vitro* system using mouse liver microsomes, chlorobenzene was shown to have similar Km and Vmax values as bromobenzene leading Kerger and co-workers (1988b) to suggest that chlorobenzene was metabolized by the same cytochrome P450/P448 isozymes that are responsible for the metabolism of bromobenzene. This dissertation study showed that despite the evidence that chlorobenzene, bromobenzene and iodobenzene are metabolized by the same pathway to similar metabolites, chlorobenzene exposure was much less likely to result in death of the experimental animals.

4.4 Phentolamine Protection of Chlorobenzene- and Iodobenzene-Induced Hepatotoxicity in the Liver.

When the α-adrenergic antagonist phentolamine was administered as a co-treatment with chlorobenzene or iodobenzene, it prevented the increase in hepatotoxicity that was seen with the halobenzene alone. Chlorobenzene- and iodobenzene-induced increases in serum ALT activities are essentially eliminated with phentolamine co-treatments. When comparing the time of maximum elevation of serum ALT activities treated with halobenzene alone with its concurrently co-treated phentolamine plus halobenzene group, the serum ALT activities of phentolamine co-treated groups were only 8% of the chlorobenzene treated groups and only 3% of the iodobenzene treated groups. Phentolamine also protected against bromobenzene-induced increases in serum ALT activities. The phentolamine co-treated group was only 0.5% of the bromobenzene treated group at 24 hours post-treatment, but had increased to 21% of the bromobenzene treated groups by 48 hours (Kerger *et al.*, 1988a). This 48 hour increase in serum ALT activity after treatment with phentolamine and bromobenzene was not seen in this study using either chlorobenzene or iodobenzene (Figures 3.17 and 3.6).

Since hepatic glutathione concentrations had been shown to play an important role in bromobenzene-induced hepatotoxicity (Jollow *et al.*, 1974) and since phentolamine had been shown to antagonize a significant portion of the bromobenzene-induced depletion of glutathione (Kerger *et al.*, 1988a), it was important to determine whether or not phentolamine would play a similar role in chlorobenzene- or iodobenzene-induced depletions. Kerger and co-workers (1988a) found that phentolamine antagonized a small but significant amount of the bromobenzene-induced glutathione depletions at 2, 4, and 8 hours post-treatment. In this study, only one time point was significantly different in the phentolamine co-treated groups than the groups treated with iodobenzene alone. Eight hours post-treatment, the phentolamine co-treated group had glutathione concentrations approximately twice as high as the iodobenzene

treated group (Figure 3.7a). In the chlorobenzene treated animals, hepatic glutathione was significantly higher in the phentolamine co-treated groups at 4 hours post-treatment (Figure 3.16a). If a dose threshold exists for halobenzene toxicity, it can not explain the difference in toxicity seen between these two compounds.

4.5 Role of Catecholamines in Halobenzene-Induced Hepatotoxicity.

Since it had been previously shown that bromobenzene treatment resulted in increases in serum epinephrine (Kerger *et al.*, 1988a), catecholamine concentrations were determined in these studies. The early catecholamine studies were designed primarily to assess whether there was a difference in a hepatotoxic versus a non-hepatotoxic dose of halobenzene as seen with bromobenzene. The results of this study (Figures 3.10 and 3.18) showed that at a dose of halobenzene which was mildly hepatotoxic catecholamine concentrations were not increased but were, in fact, decreased between 2 and 4 hours post-treatment. Therefore, the study design was changed to examine a more acutely hepatotoxic dose. Unlike bromobenzene, chlorobenzene did not elevate catecholamines at the hepatotoxic (1.0 ml/kg) dose. At a dose of iodobenzene that depleted glutathione but was only mildly hepatotoxic (0.25 ml/kg), catecholamines were depressed at 2 hours post-treatment and were not significantly elevated above controls at any time period tested. However, when iodobenzene was given at a severly hepatotoxic dose (0.53 ml/kg), there was an elevation of catecholamines at 2 hours post-treatment. These data are similar to those previously published for bromobenzene at a dose of 0.50 ml/kg and repeated in this study (Figure 3.23). While it is surprising that elevation in catecholamines occurs only at the high dose of iodobenzene, this is in agreement with that previously published for bromobenzene (Kerger *et al.*, 1988) and appears to be a high dose phenomenon.

4.6 Possible Mechanisms of Action for Halobenzene-Induced Hepatotoxicity.

There are at least three ways in which a halocarbon could potentiate its own toxicity through an α-adrenoreceptor mechanism that could be blocked by an α-adrenoreceptor antagonist: 1) by stimulating the α-adrenoreceptor directly, or 2) by stimulating the release of endogenous hormones, such as epinephrine or norepinephrine, or 3) by a receptor-independent mechanism. Also, there are at least three ways by which a halocarbon could depress hepatic glutathione concentrations: 1) by direct conjugation to glutathione, resulting in excretion of both molecules, 2) by interfering with the synthesis or release of glutathione, 3) by stimulating the release of endogenous hormones (i.e. epinephrine, norepinephrine, glucagon), which in turn depress endogenous concentrations of glutathione. In a study examining the role of the α-adrenergic receptor in bromobenzene-induced hepatotoxicity, Kerger *et al.* (1988a) found that co-administration of the α-adrenoreceptor antagonist phentolamine attenuated the acute lethality, extensive hepatocellular necrosis and elevations of serum ALT activity produced by bromobenzene. Administration of another

α-adrenoreceptor antagonist specific for α-2-adrenoreceptors, idazoxan, was also effective in reducing the serum ALT activity seen after bromobenzene treatment, suggesting that bromobenzene hepatotoxicity was somehow mediated through the α-2-adrenoreceptor (Kerger *et al.*, 1988a). This was supported by the finding that serum epinephrine concentrations were elevated after bromobenzene treatment indicating that epinephrine might be released endogenously and stimulate the α-2-adrenoreceptor. The results of the present study are consistant with this in that at high doses, bromobenzene and iodobenzene stimulate the release of epinephrine. However, a confounding factor in this study was that chlorobenzene did not stimulate the release of catecholamines but did respond to phentolamine antagonism of hepatotoxicity. A possible explanation for this is that as an α-adrenoreceptor blocking agent, phentolamine causes vasodilation, changes in blood pressure, hypothermia and regional blood flow. Hypothermia has been correlated with protection from hepatotoxicity with carbon tetrachloride (Larson and Plaa, 1963; 1965) and for bromobenzene (Simmons, 1988) although Kerger *et al.* (1989) found that phentolamine was capable of protecting against hepatotoxicity even when the animals were warmed to maintain normal body temperature. Larson and Plaa (1965) demonstrated that a decrease in rectal temperature of 6° was correlated with a reduction in oxygen consumption of about 50%. Hypothermia could be exerting a protective influence by slowing down the rate of metabolism and decreasing the production of the toxic metabolite. In this way, the toxic metabolite might not be produced in sufficient quantities to damage the cell. It is possible that the reduction in oxygen consumption prevents the formation of the epoxide, the punative toxic metabolite. Also, the decrease in body temperature would affect the activity of many enzymes. Maximum activity of most endogenous enzymes is approximately body temperature. A change in temperature of 6°C would probably decrease their efficiency and perhaps destroy the enzyme molecule. In this mechanism, cellular respiration would most likely be compromised and cell death could occur as a result of insufficient energy production. Thus, phentolamine may be protecting the liver by blocking the catecholamine-induced depletions of glutathione and/or by causing hypothermia or other adrenergic-related events which also decreases hepatotoxicity.

One of the major difficulties in trying to correlate decreasing glutathione concentrations with hepatotoxicity has been the time lapse between maximum glutathione response (2-8 hours) and the time until hepatotoxicity can be quantitated (12-48 hours by serum enzymology). Another complicating problem in quantitating hepatotoxicity has been the large variability in response seen among individuals. In an attempt to correlate these two parameters, Casini *et al.* (1985) examined the correlation coefficients when log serum ALT activities were plotted versus log hepatic glutathione concentrations at 12 hours post-treatment. Since chlorobenzene depleted glutathione to the same extent as iodobenzene but for less time and was not as hepatotoxic as iodobenzene, an experiment was completed to examine the direct correlation between log serum ALT activity and log glutathione concentrations. For

this experiment all three halobenzenes were administered since ideally a wide range of responses was needed to calculate a correlation coefficient. In Figure 3.22, the computed correlation coefficient was 0.53. This indicated that there was a weak correlation between the log glutathione concentrations and the log serum ALT activities. The correlation reported by Casini et al. (1985) is higher (0.867). The lower coefficient seen in the present study may be the result of a small number of samples.

Obviously, the ideal protocol would be to measure both glutathione and serum ALT activity at their maximum reponse times within each individual. However, until a method is developed to measure glutathione from a biopsy sample and then to measure serum ALT activity in the same animal at 24 to 48 hours later, this method appears to be the best available. Indeed, with the possible limitation of a large sample size needed to obtain enough data for good correlation coeficients, it may hold promise as a new method for correlating hepatic glutathione concentrations and hepatotoxicity.

Whether or not decreased concentrations in hepatic GSH is a causal factor in hepatotoxicity or is the result of the metabolism of the xenobiotic, the consequences of decreased concentrations could have devastating effects on the cell. As shown previously in Figure 1.2, glutathione has multiple roles within a cell. Since GSH reduces peroxides and free radicals, the inability of the cell to rid itself of these toxic compounds would likely result in the breakdown of membranes due to lipid peroxidation. The breakdown of the mitochondrial membrane would result in the disruption of cellular respiration and eventual cell death due the lack of energy in the form of ATP. The breakdown of the cell membrane would lead to an immediate change in ion concentration (Na^+; Ca^{++}) which would result in changes in the electrochemical gradients, changes in enzyme activities and changes in the transport processes at the cell membrane and within the endoplasmic reticulum. GSH may serve as a co-enzyme within the cell. If so, then those enzymes would become inactive and would quickly disrupt all involved cell processes. Many amino acids are dependent upon GSH for their transport and metabolism. Disruption of adequate amounts of GSH would result in the accumulation of amino acids in some areas, while normal transport mechanisms could not supply amino acids to other areas. Many xenobiotics as well as endogenous compounds (such as leukotrienes, prostaglandins, estrogens and melanins) are dependent upon GSH for their metabolism. Once GSH is conjugated to these compounds they become water soluble and are excreted out of the body. Insufficient concentrations of GSH could result in the build-up of these adducts. Depending on the chemical composition of these compounds, they could covalently bind to macromolecules within the cell (such as DNA, RNA or proteins) and damage the structural integrity of the cell or disrupt the reproductive processes within each cell. In conclusion, whether decreases in GSH is a direct cause or the result of cellular toxicity, insufficient concentrations of GSH within the cell could lead to cell death by one or more of the processes listed.

Individual characteristics of the halobenzene molecule may influence the resulting

toxicity. The electrochemical forces exerted by the different halogens on the benzene ring may influence the reactivity of that particular halobenzene. It is interesting to note that the halobenzenes show increasing toxicity with increasing molecular weight. This may result in differences in chemical reactivity. For example, if chlorobenzene did not form an epoxide as readily as iodobenzene, then it would be expected that chlorobenzene would not be as toxic as iodobenzene. Similarly, if the chlorobenzene epoxide formed a glutathione adduct and was excreted more readily than iodobenzene, it would also be less toxic (assuming the toxic metabolite is the epoxide). Another possible explanation for the different toxicities for these chemically similar compounds would be differential binding and reactivity with enzymes. Again, if the toxic metabolite was formed more rapidly or conjugated and excreted more slowly, it would have a longer time period in which to exert its toxic effects.

4.7 Conclusions

The results of this study has produced several novel findings:

1) Iodobenzene and bromobenzene are more toxic as measured by serum ALT activity and lethality than is chlorobenzene.
2) Chlorobenzene, bromobenzene and iodobenzene are approximately equal in their ability to deplete hepatic glutathione; however, glutathione is depleted longer with iodobenzene and bromobenzene than with chlorobenzene.
3) Iodobenzene or bromobenzene administration appears to result in the release of endogenous catecholamines at high doses but chlorobenzene does not.
4) Phentolamine protects against hepatotoxicity induced by chlorobenzene, bromobenzene or iodobenzene.

REFERENCES

Ali, S. F., J. M. Cranmer, P. T. Goad, W. Slikker, R. D. Harbison and M. F. Cranmer. 1983. Trimethyltin induced changes of neurotransmitter levels and brain receptor binding in the mouse. Neurotoxicology 4(1):29-36.

Bergmeyer, H. U., P. Scheibe, A. W. Wahlefeld. 1978. Optimization of methods for aspartate aminotransferase and alanine aminotransferase. Clin Chem 24:58.

Brodie, B. B., W. D. Reid, A. K. Cho, G. Sipes, G. Krishna and J. R. Gillette. 1971. Possible mechanism of liver necrosis caused by aromatic organic compounds. Proc Nat Acad Sci USA 68(1):160-164.

Brody, T. M. and D. N. Calvert. 1960. Release of catechol amines from the adrenal medulla by CCl_4. Am J Physiol 198(3):682-685.

Calvert, D. N. and T. M. Brody. 1960. Role of the sympathetic nervous system in CCl4 hepatotoxicity. Am J Physiol 198(3):669-676.

Casini, A., M. Giorli, R. J. Hyland, A. Serroni, D. Gilfor and J. L. Farber. 1982. Mechanisms of cell injury in the killing of cultured hepatocytes by bromobenzene. J Biol Chem 257(12):6721-6728.

Casini, A. F., A. Pompella, and M. Comporti. 1985. Liver glutathione depletion induced by bromobenzene, iodobenzene, and diethylmaleate poisoning and its relation to lipid peroxidation and necrosis. Am J Pathol 118:225-237.

Deichmann, W. B. 1981. Halogenated cyclic hydrocarbons in Patty's Industrial Hygiene and Toxicology, G. D. Clayton and F. E. Clayton (eds). Wiley & Sons, New York, NY. pp 3603-3759.

Docks, E. L. and G. Krishna. 1976. The role of glutathione in chloroform- induced hepatotoxicity. Exp Mol Pathol 24:13-22.

Dowty, B., D. Carlisle, and J. Laseter. 1975. New Orleans drinking water sources tested by gas chromatography mass spectrometry: occurrence and origin of aromatics and halogenated aliphatic hydrocarbons. Environ Sci Tech 9:762- 765.

Duncan, D. B. 1955. Multiple range and multiple F tests. Biometrics 11:1-10.

Ellman, G. L. 1959. Tissue sulfhydryl groups. Arch of Biochem Biophy 82:70- 77.

Fouts, J. R. 1964. Drug interactions: Effects of drugs and chemicals on drug metabolism. Gastroenterology 46(4):486-490.

Gross, R., G. Bertrand, G. Ribes and M. M. Loubatieres-Mariani. 1987a. Alpha-2-adrenergic potentioation of adenosine-stimulating effect on glucagon-secretion. Endocrinol 121(2):765-769.

Hallman, H., L.-O. Farnebo, B. Hamberger and G. Jonsson. 1978. A sensitive method for the determination of plasma catecholamines using liquid chromatography with electrochemical detection. Life Sciences 23:1049-1052.

Higashi, T., N. Tateishi, A. Naruse and Y. Sakamoto. 1977. A novel physiological role of liver

glutathione as a reservoir of L-cysteine. J Biochem (Tokyo) 82(1):117-124.

International Agency for Research on Cancer (IARC). 1986. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Some halogenated hydrocarbons and pesticide exposures. Volume 41. International Agency for Research on Cancer, Lyon, France.

James, R. C. and R. D. Harbison. 1982. Hepatic glutathione and hepatotoxicity: The effects of cytochrome P-450 complexing compounds SKF525-A, L-α-acetylmethadol (LAAM), norLAAM, and piperonyl butoxide. Biochem Pharmacol 31:1829-1835.

James, R. C., D. R. Goodman and R. D. Harbison. 1982. Hepatic glutathione and hepatotoxicity changes induced by selected narcotics. J Pharmacol Exp Therap 221:708-714.

James, R. C., S. M. Roberts and R. D. Harbison. 1983. The perturbation of hepatic glutathione by α2-adrenergic agonists. Fundam Appl Toxicol 3:303-308.

James, R. C., M. A. Schiefer, S. M. Roberts and R. D. Harbison. 1987. Antagonism of cocaine-induced hepatotoxicity by the alpha-adrenergic antagonists phentolamine and yohimbine. J Pharmacol Exp Ther 242(2):726-732.

Jollow, D. J., J. R. Mitchell, N. Zampaglione and J. R. Gillette. 1974. Bromobenzene-induced liver necrosis. Protective role of glutathione and evidence for 3,4-bromobenzene oxide as the hepatotoxic metabolite. Pharmacol 11:151-169.

Kerger, B. D., J. Gandy, T. J. Bucci, S. M. Roberts, R. D. Harbison and R. C. James. 1988a. Antagonism of bromobenzene-induced hepatotoxicity by the *a*-adrenergic blocking agents, phentolamine and idazoxan. Toxicol Appl Pharmacol 95:12-23.

Kerger, B. D., S. M. Roberts, J. A. Hinson, J. Gandy, R. D. Harbison and R. C. James. 1988b. Antagonism of bromobenzene-induced hepatotoxicity by phentolamine: evidence for a metabolism-independent intervention. Toxicol Appl Pharmacol 95:24-31.

Kerger, B. D., S. M. Roberts, R. D. Harbison and R. C. James. 1989. Antagonism of bromobenzene-induced hepatotoxicity by the *a*-adrenoreceptor blocking agents phentolamine and idazoxan: Role of hypothermia. Toxicol Appl Pharmacol 97:360-369.

Kluwe, W. M., G. Dill, R. Persing and A. Peters. 1985. Toxic responses to acute, subchronic, and chronic oral administrations of monochlorobenzene to rodents. J Toxicol Environ Health 15:745-767.

Larson, R. E. and G. L. Plaa. 1963. Spinal cord transection and CCl4- Toxicity. Experientia 19:604-606.

Larson, R. E. and G. L. Plaa. 1965. A correlation of the effects of cervical cordotomy, hypothermia, and catecholamines on carbon tetrachloride-induced hepatic necrosis. J Pharmacol Exp Ther 147(1):103:111.

MacDonald, J. R., A. J. Gandolfi and I. G. Sipes. 1982a. Acetone potentiation of 1,1,2-trichloroethane hepatotoxicity. Toxicol Lett 13:57-69.

Mitchell, J. R., H. Hughes, B. H. Lauterburg and C. V. Smith. 1982. Chemical nature of reactive intermediates as determinant of toxicologic responses. Drug Metabolism Rev 13(4):539-553.

Mitchell, J. R. and D. J. Jollow. 1974. Biochemical basis for drug-induced hepatotoxicity. Israel J Med Sci 10(4):312-318.

Mitchell, J. R., D. J. Jollow, and J. R. Gillette. 1974. Relationship between metabolism of foreign compounds and liver injury. Israel J Med Sci 10:339-344.

Mitchell, J. R., D. J. Jollow, W. Z. Potter, J. R. Gillette and B. B. Brodie. 1973. Acetaminophen-Induced hepatic necrosis. IV. Protective role of glutathione. J Pharmacol Exp Ther 187:211-217.

Mitchell, J. R. , S. D. Nelson, S. S. Thorgeirsson, et al. 1976. Metabolic activation: Biochemical basis for many drug-induced liver injuries. Prog Liver Dis 5:259-279.

National Toxicology Program. 1985. NTP technical report on the toxicology and carcinogenisis studies of 1,4-dichlorobenzene (CAS No. 106-46-7) in F344/N rats and B6C3F1 Mice (gavage studies). NIH Publ.86-2575, Department of Health and Human Services, Research Triangle Park, NC.

Patel, D. G. 1983. Lack of glucagon-response to hypoglycemia in long-term experimental diabetic rats. Diabetes 32(1):55-60.

Plaa, G. L. 1986. Toxic responses of the liver, in "Casarett and Doull's Toxicology," C. D. Klaassen, M. O. Amdur, J. Doull (eds), Macmillan Publishing Co., N.Y., N. Y. pp. 286-309.

Reid, W. D., B. Christie, G. Krishna, J. R. Mitchell, J. Moskowitz and B. B. Brodie. 1971. Bromobenzene metabolism and hepatic necrosis. Pharmacology 6:41-55.

Reid, W. D., G. Krishna, J. R. Gillette and B. B. Brodie. 1973. Biochemical mechanism of hepatic necrosis induced by aromatic hydrocarbons. Pharmacology 10:193-214.

Sedlak, J. and R. H. Lindsay. 1968. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem 25:192-205.

Simmons, H. F. 1988. Doctoral Dissertation (UAMS, 1988).

Sipes, I. G. and A. J. Gandolfi. 1986. Biotransformation of toxicants in "Casarett and Doull's Toxicology," C. D. Klaassen, M. O. Amdur, J. Doull (eds), Macmillan Publishing Co., N.Y.

Sipes, I. G., D. A. Wiersma and D. J. Armstrong. 1986. The role of glutathione in the toxicity of xenobiotic compounds: Metabolic activation of 1,2- dibromoethane by glutathione. Adv Exp Med Biol 197:457-467.

Schwetz, B. A. and G. L. Plaa. 1969. Catechloamine potentiation of carbon tetrachloride-induced hepatotoxicity in mice. Toxicol Appl Pharmacol 14:495- 509.

Tateishi, N., T. Higashi, A. Naruse, K. Nakashima, H. Shiozaki and Y. Sakamoto. 1977. Rat liver glutathione: Possible role as a reservoir of cysteine. J Natr 107(1):51-60.

Torkelson, T. R. and V. K. Rowe. 1981. Halogenated aliphatic hydrocarbons containing chlorine, bromine and iodine. In Patty's Industrial Hygiene and Toxicology, G. D. Clayton and F. E. Clayton (eds). Wiley & Sons, New York, NY. pp 3433-3601.

Wroblewski, F. and J. S. LaDue. 1956. Serum glutamic pyruvic transaminase in cardiac and hepatic disease. Proc Soc Exp Biol Med 91:569-571.

Zimmerman, H. J. 1978. Hepatotoxicity. The adverse effects of drugs and other chemicals on the liver. Appleton-Century-Crofts, New York, New York.

Zimmerman, H. J. 1986. Effects of alcohol on other hepatotoxins. Alcohol Clin Exp Res 10(1):3-15.