THESIS

This is to certify that the

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MECHANISM OF CHLOROFORM-INDUCED NEPHROTOXICITY

presented by

Jacqueline Hagan Smith

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of the requirements for

Ph.D. degree in Pharmacology/Toxicology
and
Environmental Toxicology

Major professor

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MECHANISM OF CHLOROFORM-INDUCED NEPHROTOXICITY

By

Jacqueline Hagan Smith

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ABSTRACT

Mechanism of Chloroform-Induced Nephrotoxicity

by

Jacqueline Hagan Smith

Chloroform ($CHCl_3$) is hepatotoxic and nephrotoxic in most species; in mice only males are susceptible to nephrotoxicity. Hepatotoxicity appears to result from hepatic cytochrome P-450-mediated metabolism of $CHCl_3$ to a reactive intermediate, probably phosgene. The purposes of this investigation were two-fold: (1) To test the hypothesis that the kidney metabolizes $CHCl_3$; and (2) To characterize the mechanism of $CHCl_3$-induced nephrotoxicity.

Assessment of $CHCl_3$ toxicity in male and female ICR mice suggested that nephrotoxicity occurred independent of hepatotoxicity. Nephrotoxicity could be detected 2 hr after $CHCl_3$ administration as decreased accumulation of p-aminohippurate (PAH) and tetraethylammonium (TEA) by renal cortical slices from male mice. $CHCl_3$ was not nephrotoxic to females whereas hepatotoxicity was similar in both sexes. Partial hepatectomy (50-70%) did not alter $CHCl_3$ nephrotoxicity. Susceptibility to $CHCl_3$ nephrotoxicity was increased in males and females treated with testosterone as were renal mixed function oxidase activities. Both enzyme activities and susceptibility to toxicity were decreased in castrated males.

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Renal cortical slices incubated with $CHCl_3$ *in vitro* indicated metabolism was related to toxicity. $CHCl_3$ decreased the ability of slices from male, not female, mice to accumulate PAH and TEA within a similar time and dose as observed to produce nephrotoxicity *in vivo*. Deuterated-$CHCl_3$ was less nephrotoxic to slices than $CHCl_3$. $^{14}CHCl_3$ was metabolized to $^{14}CO_2$, covalently bound radioactivity and aqueous soluble metabolites in greater amounts by male than female renal slices. Metabolism and toxicity were reduced when incubations were conducted under an atmosphere of carbon monoxide.

$^{14}CHCl_3$ was metabolized by male renal cortical microsomes in the presence of NADPH; carbon monoxide inhibited metabolism. $CHCl_3$ produced a type I binding spectrum. Incubation of glutathione with microsomes and $^{14}CHCl_3$ increased the amount of aqueous soluble metabolites, suggesting the formation of a reactive intermediate, such as phosgene.

These investigations support the hypothesis that renal cytochrome P-450 metabolizes $CHCl_3$ to a nephrotoxic intermediate.

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My deepest thanks go to my mentor, Dr. Jerry B. Hook, for his intellectual guidance, psychological support and high standards of performance throughout my graduate studies at Michigan State University. I also would like to thank the members of my dissertation committee, Drs. Theodore M. Brody, Robert A. Roth, W. Emmett Braselton, and Rory Connolly, for their suggestions and assistance in the preparation of this thesis. I wish to acknowledge and thank Drs. Keizo Maita and Stuart D. Sleight for their collaboration in the histopathological aspects of this work. The enthusiastic technical assistance of Ms. Laura Everett, Ms. Gay deShone, Mr. Allen Reynolds, Ms. Julie Eldredge and Ms. Virginia Adler is gratefully acknowledged. Special thanks are due to Diane Hummel for her patience and skillful preparation of this thesis.

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INTRODUCTION

Chloroform ($CHCl_3$) was introduced as a general anesthetic by Simpson in 1847. Compared to diethyl ether, $CHCl_3$ was less irritating and nonflammable and, thus, became the major anesthetic used during the next several decades (Pohl, 1979; Davidson et al., 1982). The major disadvantage of $CHCl_3$ use was the potential for hepatic, renal and cardiac toxicity. Thus, the use of $CHCl_3$ as a general anesthetic declined gradually as alternative, less toxic anesthetics were developed. In 1912, the Committee on Anesthesia of the American Medical Association condemned $CHCl_3$ for use in major surgery (Pohl, 1979).

$CHCl_3$ was used as a preservative and a flavor enhancer in pharmaceutical products such as cough medicines, mouth washes and toothpastes. In 1976, these uses were banned by the Food and Drug Administration following a study by the National Cancer Institute indicating that $CHCl_3$ was a renal and hepatic carcinogen in rodent bioassays (U.S. Food and Drug Administration, 1976; Reuber, 1979). $CHCl_3$ may be found in residual amounts in some drug products from its use as a processing solvent in manufacture or as a by-product in chemical synthesis (IARC, 1979).

Currently, $CHCl_3$ is a compound of toxicological concern due to its presence in municipal water supplies. $CHCl_3$ is known to be

produced environmentally in the chlorination of water where chlorine, added as a disinfectant, interacts with small organic molecules in the water (Bellar et al., 1974; Rook, 1974; Bunn et al., 1975; Deinzer et al., 1978). $CHCl_3$ also is used extensively in industry as a solvent and chemical intermediate.

Experimentally, $CHCl_3$ is a relatively potent toxicant. The acute oral LD_{50} of $CHCl_3$ for adult rats and mice ranged from 0.8 to 1.3 ml/kg (Klaassen and Plaa, 1967; Kimura et al., 1971; Torkelson et al., 1976; Winslow and Gerstner, 1978). Administration of $CHCl_3$ by ingestion, inhalation, injection or percutaneous absorption produced hepatic and renal damage in all species studied, including man (Davidson et al., 1982). Other toxic effects of $CHCl_3$ included eye irritation, cardiovascular effects and depression of the respiratory and central nervous systems (Torkelson et al., 1976; Winslow and Gerstner, 1978).

A. Susceptibility of the Kidney as a Target Organ for Chemicals Requiring Metabolic Activation

Xenobiotics are eliminated primarily by the kidney, lung and/or liver. Consequently, these organs are frequently targets for toxicity produced by a variety of chemicals. The role of metabolic activation of xenobiotics in the liver in relation to the occurrence of hepatotoxicity has been studied extensively in the past 15 years with such model hepatotoxicants as acetaminophen, bromobenzene, carbon tetrachloride and chloroform. In contrast to the liver, very little information is available on biochemical mechanisms of metabolic activation within the kidney as a prerequisite for the manifestation of nephrotoxicity. Fortunately, the extensive information on $CHCl_3$-induced

hepatotoxicity provides a background for an evaluation of the mechanisms of metabolism and nephrotoxicity of this compound.

The kidney, and particularly the proximal tubular cells, may be much more susceptible than other organs to the toxic effects of a variety of chemicals for a number of reasons. The kidneys comprise only 0.4% of the body weight in most mammals, but receive 20% of the cardiac output (Maher, 1976). This high blood flow dictates that large quantities of xenobiotics in the systemic circulation will be delivered to the kidneys, especially to the renal cortex, which receives over 90% of the renal blood flow (Maher, 1976). Furthermore, the ability of the kidney to concentrate tubular fluid may enhance toxicity due to increased xenobiotic concentrations.

Additionally, specialized functions of the proximal tubular cells may enhance toxicity in several ways which may contribute to high intracellular concentrations of potentially toxic xenobiotics. For example, solutes may be reabsorbed by passive or active mechanisms by the tubular cells. Additionally, many organic compounds are secreted into the tubular lumen by organic acid or base transport mechanisms, hence passing through or accumulating within the proximal tubular cells, exposing those cells to very high concentrations. These secretory processes can transport protein-bound xenobiotics as well as those in free solution.

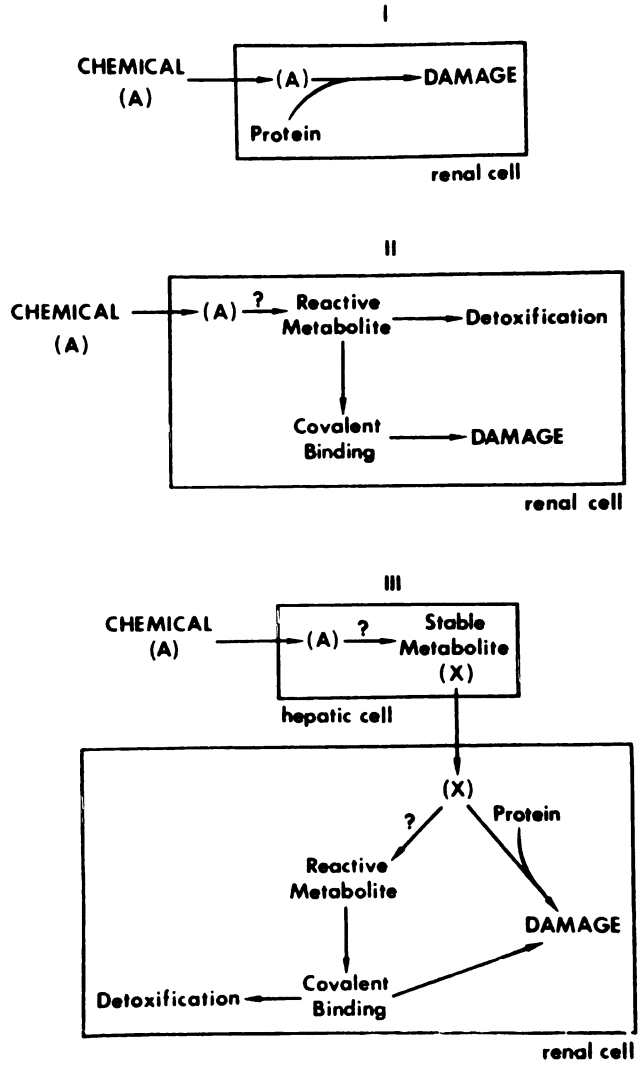
Anatomically, the renal cortex contains glomeruli, proximal and distal tubules, is richly vascular, and receives the majority of renal blood flow. Functionally, the renal cortex contains many active

transport processes, is highly aerobic, is metabolically very active and is exquisitely sensitive to oxygen deprivation.

Once in the kidney, a chemical may act directly or indirectly to produce a toxic response. In general, three independent mechanisms can be envisioned for chemicals that result in renal damage (Figure 1). First, a chemical may enter renal cells and interfere directly with an essential metabolic or functional process resulting in cellular damage (Scheme I). Second, a chemical may be metabolized in the kidney to a highly reactive intermediate that may covalently bind to protein or initiate lipid peroxidation resulting in cellular damage (Scheme II). Finally, a chemical may be metabolized by extrarenal enzymes to a stable metabolite that may enter the systemic circulation (Scheme III). In the kidney, this metabolite may result in toxicity in a manner similar to Scheme I or II.

Drug metabolizing enzymes, including cytochrome P-450 dependent mixed function oxidases, are present in the kidney, as well as in other extrahepatic organs, though the specific activities of enzymes present are typically much less than found in the liver (Litterst et al., 1975, 1977; Fry et al., 1978). Due to the heterogeneity of cell types in this organ, homogenates of whole kidney or kidney cortex contain diluted concentrations of these enzymes. Thus, use of kidney homogenates may dramatically underestimate the metabolic activity of certain regions of the kidney. The subcellular locations and actions of renal drug metabolizing enzymes are generally analogous to those described for the liver and other extrahepatic tissues. In contrast to the liver, there are regional differences in the relative amounts

Figure 1. Schematic representation of mechanisms of toxic injury induced by xenobiotics.


I
CHEMICAL
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Protein
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Detoxification
Covalent
Binding
renal cell

Figure 1

of certain enzymes due to the greater cellular heterogeneity of the kidney. Renal mixed function oxidases are not uniformly distributed within the kidney, but exhibit a cortico-papillary gradient with activity being highest in the cortex (Dees et al., 1982; Rush et al., 1983).

Very little information is available on the direct metabolic activation of nephrotoxicants by renal cytochrome P-450. However, due to the similarities of the renal mixed function oxidases to those in the liver the potential for activation of chemicals by this mechanism in the kidney, as has been shown in the liver, must be considered. Furthermore, while cytochrome P-450-type reactions have been most extensively investigated in the kidney to date, there are other possible biochemical mechanisms of intrarenal metabolic activation.

Considering the unstable nature of putative reactive intermediates, it seems likely that the site of toxicity within a tissue would be in close proximity to the site of activation, which is consistent with in situ metabolism of nephrotoxicants within the kidney. This may be reflected by the discrete regions of renal damage produced by a variety of nephrotoxic agents believed to require some type of metabolic activation.

Thus, the kidney is a target organ for chemicals requiring metabolic activation. The extent of in situ metabolism and the susceptibility to toxicity may be greatly exaggerated by the achievement of much greater concentrations of a toxic chemical within certain regions of the nephron. Additionally, it is quite possible for complex interactions of these various mechanisms to produce a nephrotoxic response that might not be seen with any of the events occurring individually.

B. Relationship Between $CHCl_3$ Metabolism and Toxicity in the Liver

The concept that metabolism of $CHCl_3$ was required for toxicity and the molecular basis for the toxicity of $CHCl_3$ has been a subject of investigation for many years. In 1883, Zeller observed an increase in chloride ion excretion after $CHCl_3$ administration to dogs (Zeller, 1883). Phosgene ($COCl_2$) was reported to be a component of $CHCl_3$ in 1894 (Hofmeister and Lenz, 1894). As early as 1912, it was known that $CHCl_3$ could be oxidized to phosgene and hydrochloric acid in the presence of sunlight at room temperature (Baskerville and Hamor, 1912). Furthermore, phosgene was known to form carbon dioxide and hydrochloric acid in the presence of water. The net chemical reaction was expressed as:

$$CHCl_3 + O \rightleftharpoons COCl_2 + HCl$$
$$COCl_2 + H_2O \rightleftharpoons CO_2 + 2HCl \qquad \text{(Graham, 1915)}$$

Therefore, it was reasoned that since one molecule of $CHCl_3$ could produce 3 molecules of hydrochloric acid and since the liver was known to be a metabolically active organ, the most favored mechanism proposed for $CHCl_3$ toxicity was the formation of hydrochloric acid resulting in tissue necrosis (Graham, 1915). The earlier hypothesis of Müller (1911) that phosgene might be the agent responsible for the tissue necrosis produced by $CHCl_3$ was discounted until recently.

Research on the mechanism of $CHCl_3$ toxicity during the next 60 years concentrated primarily on documenting the metabolism of $CHCl_3$ to hydrochloric acid (Lucas, 1928; Heppel and Porterfield, 1948; Bray et al., 1952) and to carbon dioxide (Paul and Rubinstein, 1963; Van Dyke

et al., 1964; Fry et al., 1972; Brown et al., 1974b; Lavigne and Marchand, 1974). However, as research in the area of chemical carcinogenesis indicated the role of metabolism to reactive electrophilic metabolites and covalent binding to tissue macromolecules was related to tissue damage (Miller and Miller, 1947; 1966), emphasis on the mechanism of toxicity of drugs and chemicals also was shifted toward the concept of reactive intermediates (Gillette et al., 1974; Gillette, 1974).

Evidence had accumulated that a reactive intermediate was formed during $CHCl_3$ metabolism. Whole body autoradiography experiments in mice 2 hr after inhalation of $^{14}CHCl_3$ indicated the presence of nonvolatile radioactivity in the liver and duodenum, suggesting the presence of a compound other than $^{14}CHCl_3$ or $^{14}CO_2$ (Cohen and Hood, 1969). Autoradiograms of liver and kidney of male mice killed 30 hr after $^{14}CHCl_3$ administration revealed localized accumulation of radioactivity in necrotic centrilobular hepatocytes and in necrotic proximal convoluted tubular cells, further suggesting a relationship between covalent binding and necrosis (Ilett et al., 1973). A similar correlation between covalently bound radioactivity and the degree and site of hepatotoxicity had been reported for other model hepatoxicants believed to require metabolic activation, such as acetaminophen (Mitchell et al., 1973; Jollow et al., 1973) and bromobenzene (Reid et al., 1971; Brodie et al., 1971). Evidence that radioactivity associated with necrotic tissue observed in vivo occurred as a result of covalent binding of a reactive metabolite(s), and not merely the incorporation of a $CHCl_3$ metabolite such as $^{14}CO_2$ into normal cellular

macromolecules, was provided by experiments with hepatic microsomes. Covalent binding to protein and lipid was observed after incubation of $^{14}CHCl_3$ with liver microsomes prepared from rats (Reynolds and Yee, 1967; Brown et al., 1974a; Uehleke and Werner, 1975; Sipes et al., 1977), mice (Ilett et al., 1973; Uehleke and Werner, 1975) or rabbits (Uehleke and Werner, 1975). In a microsomal reaction, the enzymes necessary for incorporation of CO_2 into cellular macromolecules, such as lipids or proteins, are not available. This would indicate that a metabolite of $CHCl_3$ could be bound covalently to tissue macromolecules in vitro.

The metabolism of $CHCl_3$ by hepatic microsomes suggested that $CHCl_3$ was metabolized by a cytochrome P-450-dependent mechanism. In vitro activation of $CHCl_3$ by liver appeared to be catalyzed by a phenobarbital-inducible form of cytochrome P-450 and required oxygen (Ilett et al., 1973; Uehleke and Werner, 1975; Sipes et al., 1977; Pohl et al., 1980), required NADPH (Rubinstein and Kanics, 1964; Ilett et al., 1973; Sipes et al., 1977), was inhibited by carbon monoxide (Ilett et al., 1973; Sipes et al., 1977), and was inhibited by the cytochrome P-450 inhibitor SKF 525-A (Sipes et al., 1977).

Further evidence for the role of cytochrome P-450 was the observation that there were parallel alterations in the hepatotoxicity and metabolism of $CHCl_3$ in rats and mice pretreated with inducers and inhibitors of hepatic microsomal enzymes. Inducing agents which increased $CHCl_3$-induced hepatotoxicity included phenobarbital (Scholler, 1970; Ilett et al., 1973; Brown et al., 1974a; Lavigne and Marchand, 1974; Gopinath and Ford, 1975; Uehleke and Werner, 1975; Docks

and Krishna, 1976; Cascorbi et al., 1976; Pohl and Krishna, 1977; Sipes et al., 1977; Pohl et al., 1980; McMartin et al., 1981), ketones and ketogenic chemicals (Hewitt et al., 1980a,b), DDT (Gopinath and Ford, 1975), ethanol (Klaassen and Plaa, 1966), polybrominated biphenyls (Kluwe and Hook, 1978) and phenylbutazone (Gopinath and Ford, 1975). Not all hepatic microsomal inducers increased hepatotoxicity; inactive agents included 3-methylcholanthrene (Kluwe et al., 1978; Pohl et al., 1980), polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin (Kluwe et al., 1978). Hepatotoxicity was decreased when animals were pretreated with inhibitors of cytochrome P-450 such as SKF 525-A (Gopinath and Ford, 1975), piperonyl butoxide (Ilett et al., 1973; Kluwe et al., 1978) or carbon disulfide (Watrous and Plaa, 1972; Gopinath and Ford, 1975). These data were consistent with the in vitro observations that a phenobarbital-inducible form of cytochrome P-450 mediated the metabolism of $CHCl_3$.

Additional evidence that $CHCl_3$ hepatotoxicity was associated with the formation of a reactive metabolite was the observation that hepatic glutathione concentrations were decreased in phenobarbital pretreated rats that were subsequently treated with $CHCl_3$ (Brown et al., 1974a; Docks and Krishna, 1976). Glutathione, a nucleophilic tripeptide containing cysteine, was believed to play a role in protecting the liver from electrophilic metabolites of other hepatotoxicants such as bromobenzene (Reid et al., 1971) and acetaminophen (Mitchell et al., 1973).

In 1977, two laboratories independently reported the identification of $COCl_2$ as a hepatic metabolite of $CHCl_3$ (Mansuy et al., 1977;

Pohl et al., 1977). In these studies, $^{14}CHCl_3$ was incubated with cysteine, a nucleophilic trapping agent, in the presence of liver microsomes prepared from phenobarbital pretreated rats. The metabolite identified from the incubation was 2-oxothiazolidine-4-carboxylic acid (OTZ). An earlier report had indicated that phosgene reacted spontaneously with cysteine to form OTZ (Kaneko et al., 1964). Pohl and coworkers have investigated extensively the hepatic metabolism of $CHCl_3$ and the role of phosgene in $CHCl_3$-mediated toxicity. Phosgene has been trapped as a metabolite of $CHCl_3$ in vivo with cysteine (Pohl et al., 1979) and in vivo and in vitro with glutathione to form diglutathionyl dithiocarbonate (Pohl et al., 1981). Figure 2 illustrates the metabolic pathway of $CHCl_3$ proposed by Pohl and coworkers on the basis of these experiments (Pohl, 1979; Pohl et al., 1981).

The chemical properties of phosgene make this compound a likely candidate for a role in the toxicity produced by $CHCl_3$. Phosgene is extremely electrophilic and reacts readily with various nucleophiles; the half-life of phosgene in water at 37°C was calculated to be less than 0.04 sec (Nash and Pattle, 1971). For example, phosgene reacts with water to produce CO_2 and HCl (Babad and Zeiler, 1973), a reaction which would be consistent with the metabolism of $CHCl_3$ in vitro and in vivo to these products. Phosgene was predicted to react rapidly with various endogenous nucleophiles such as thiols, hydroxyls, and amino groups in proteins and lipids (Nash and Pattle, 1971; Babad and Zeiler, 1973; Pohl, 1979), a reaction that would be consistent with the metabolism of $CHCl_3$ in vitro and in vivo to covalently bound radioactivity. The conclusion is supported further by the observed

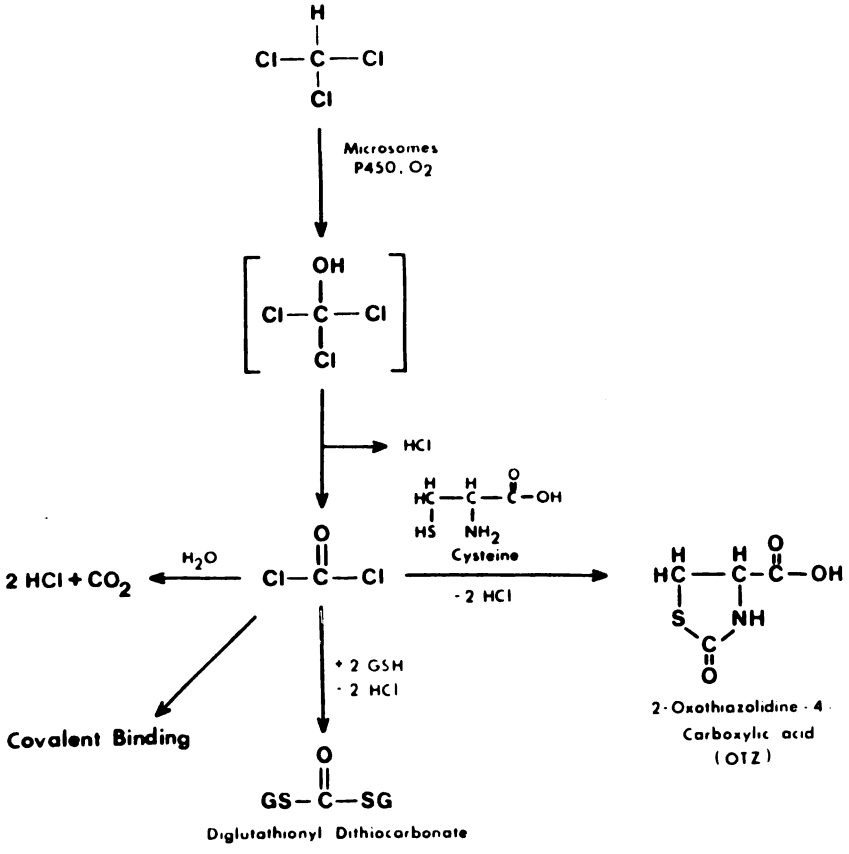


Figure 2. Hepatic metabolism and activation of chloroform.

interactions of phosgene with protein *in vitro* (Cessi *et al.*, 1966) and *in vivo* (Reynolds, 1967), with glutathione *in vitro* (Pohl *et al.*, 1981) and with cysteine *in vitro* (Kaneko *et al.*, 1964). The interaction of phosgene with glutathione probably was responsible for the $CHCl_3$-mediated depletion of hepatic glutathione *in vivo* (Johnson, 1965; Brown *et al.*, 1974a; Docks and Krishna, 1976).

Pohl and coworkers have reviewed several possible metabolic pathways for the metabolism of $CHCl_3$ (Pohl, 1979; Pohl *et al.*, 1980). These include metabolism of $CHCl_3$ by (1) oxidative dechlorination, (2) hydrogen abstraction, (3) reductive dechlorination, and (4) hydrogen ion abstraction. These pathways are illustrated in Figures 3 and 4 and are discussed below.

1. Oxidative Dechlorination

The most likely pathway for $CHCl_3$ metabolism is an oxidative dechlorination in which an activated oxygen would be inserted directly across the C-H bond of $CHCl_3$ to form trichloromethanol (Cl_3C-OH). The reaction is depicted in Figures 2-4. Trichloromethanol has not been identified directly as a metabolite; it is believed to be very unstable such that it would rapidly and spontaneously dehydrochlorinate to phosgene ($COCl_2$) (Pohl, 1979). The trihalomethanol derivative, trifluoromethanol (F_3C-OH), has been synthesized and observed to dehydrofluorinate spontaneously to carbonyl fluoride (COF_2) at temperatures above -20°C (Seppelt, 1977; Klöter and Seppelt, 1979). The potential role of phosgene in mediating the toxicity of $CHCl_3$ has been discussed previously.

CHLOROFORM METABOLISM

1. Oxidative Dechlorination

$$CHCl_3 \longrightarrow Cl_3COH$$

2. Hydrogen Abstraction

$$CHCl_3 \longrightarrow Cl_3\dot{C} + \dot{H}$$

3. Reductive Dechlorination

$$CHCl_3 \longrightarrow Cl_2\dot{C}H + \dot{Cl}$$

4. Hydrogen Ion Abstraction

$$CHCl_3 \longrightarrow Cl_3\ddot{C}^- + H^+$$

Figure 3. Possible mechanisms for metabolic activation of chloroform.

Figure 4. Possible metabolic pathways for metabolism of chloroform by (1) oxidative dechlorination, (2) hydrogen abstraction, (3) reductive dechlorination, and (4) hydrogen ion abstraction.

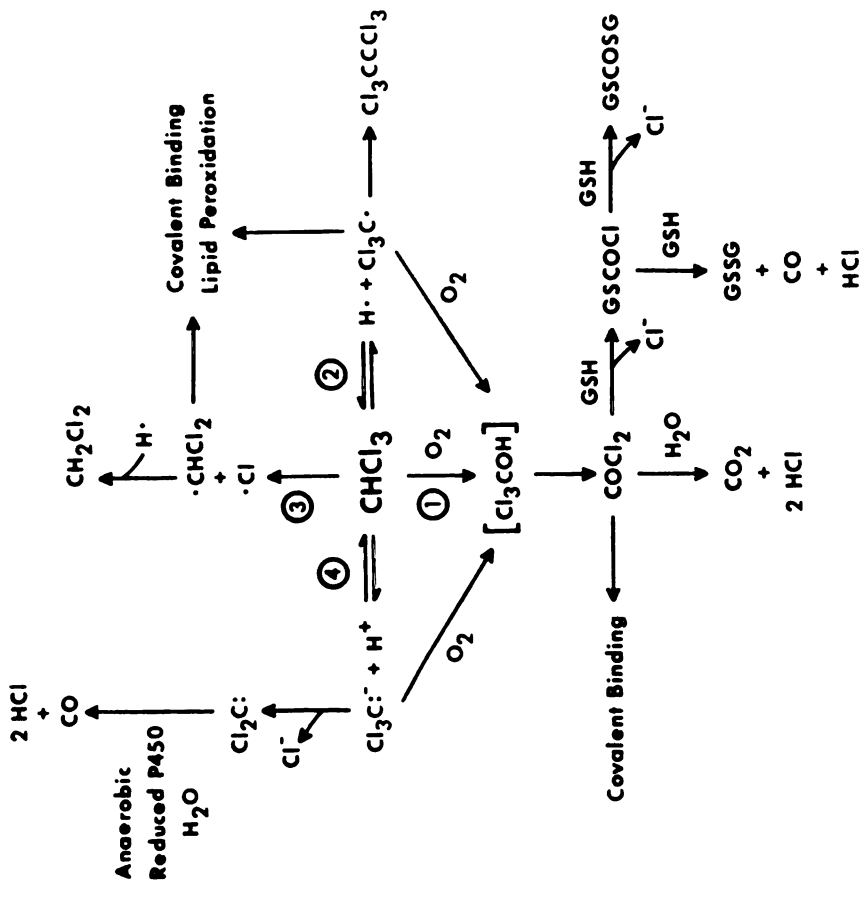

2 HCl
+
CO
Anaerobic
Reduced P450
H_2O
Cl_2C:
Cl^-
$Cl_3C:^- + H^+$
O_2
$[Cl_3COH]$
$CHCl_3$
O_2
CH_2Cl_2
$H^\cdot$
$\cdot CHCl_2$
+
$\cdot Cl$
Covalent Binding
Lipid Peroxidation
$H^\cdot + Cl_3C^\cdot$
Cl_3CCCl_3
O_2
Covalent Binding
$COCl_2$
H_2O
CO_2
+
2 HCl
GSH
Cl^-
GSCOCl
GSH
Cl^-
GSCOSG
GSH
GSSG
+
CO
+
HCl

Figure 4

Carbon monoxide has been detected as a very minor metabolite of $CHCl_3$, though the structural analogs iodoform (CHI_3) and bromoform ($CHBr_3$) yield greater amounts of CO *in vitro* (Ahmed *et al.*, 1977) and *in vivo* (Stevens and Anders, 1981). From these data, Anders and coworkers have proposed that sulfhydryl compounds, primarily glutathione (GSH), may mediate the conversion of phosgene to CO (Figure 4) (Ahmed *et al.*, 1980). This could occur, for example, via the attack of glutathione on phosgene to yield glutathione-S-formyl chloride (GSCOCl), and subsequent attack by a second glutathione on the sulfur of the first to result in formation of carbon monoxide (CO) and oxidized glutathione (GSSG) (Stevens and Anders, 1979, 1980; Ahmed *et al.*, 1980). This pathway has been developed primarily using bromoform as a model (Ahmed *et al.*, 1977; Anders *et al.*, 1978; Stevens and Anders, 1979). In these studies, it was observed that CO produced in the presence of $^{18}O_2$ was enriched in $C^{18}O$ while no $C^{18}O$ was seen when $H_2{}^{18}O$ was used in the incubation mixtures (Stevens and Anders, 1979). These data indicate that the oxygen atom in CO was derived from molecular oxygen, and thus are consistent with the oxidative dechlorination pathway.

Evidence that molecular oxygen is the source of oxygen in the chloroform metabolite was provided by incubation of rat liver microsomes with $CHCl_3$ in the presence of cysteine under an atmosphere of $^{18}O_2$ (Pohl *et al.*, 1977). In these incubations, ^{18}O was incorporated into the 2-oxo position of 2-oxothiazolidine-4-carboxylic acid. It was estimated that nearly 100% of 2-oxo oxygen was derived

from molecular oxygen based on the isotope purity of $^{18}O_2$ used in these experiments (Pohl et al., 1977).

Evidence for the direct involvement of cytochrome P-450 as the enzyme catalyzing the metabolism of $CHCl_3$ to phosgene in vitro was provided by the observations that molecular oxygen was the source of oxygen in phosgene (Pohl et al., 1977); that production of phosgene was inhibited by SKF 525-A and carbon monoxide (Pohl and Krishna, 1978) and was stimulated by phenobarbital pretreatment of rats (Pohl et al., 1981).

The importance of the metabolism of the C-H bond in the metabolic activation and toxicity of $CHCl_3$ has been demonstrated in experiments using deuterium-labelled $CHCl_3$ ($CDCl_3$). The C-D bond has a higher chemical energy than the C-H bond; thus, metabolism and toxicity should be reduced if cleavage of the bond is a requisite step in $CHCl_3$ metabolism and toxicity. The formation of 2-oxothiazolidine-4-carboxylic acid from $CDCl_3$ was 50% of that formed from an equimolar concentration of $CHCl_3$ (Pohl and Krishna, 1978). This indicated that less $COCl_2$ was formed from $CDCl_3$ than from $CHCl_3$. Likewise, $CDCl_3$ was significantly less hepatotoxic than $CHCl_3$ in rats 24 hr after administration (Pohl and Krishna, 1978). These results indicated that cleavage of the C-H bond was a requisite step in the hepatotoxicity of $CHCl_3$ and that $COCl_2$ probably was produced in vivo in the liver. Subsequent experiments have revealed there is less in vivo formation of phosgene from $CDCl_3$ than from $CHCl_3$ (measured as formation of 2-oxothiazolidine-4-carboxylic acid in the livers of rats pretreated with cysteine and phenobarbital) (Pohl et al., 1979). Likewise, less

diglutathionyl dithiocarbonate was formed from $CDCl_3$ than from $CHCl_3$ in rat liver microsomes incubated in the presence of glutathione (Pohl et al., 1981).

2. Hydrogen Abstraction

Another possible pathway for $CHCl_3$ metabolism involves the abstraction of a hydrogen atom resulting in the formation of a trichloromethyl radical ($Cl_3C\cdot$, Figure 3) (Pohl et al., 1980). Trichloromethyl radical is a reactive metabolite produced during the reductive metabolism of carbon tetrachloride (CCl_4), another extensively studied model hepatotoxicant (Butler, 1961; Reynolds, 1967, 1977; Recknagel and Glende, 1973; Recknagel et al., 1977). Formation of this intermediate should produce a pattern of metabolites and characteristics of toxicity similar to those observed for CCl_4. The characteristics of hepatotoxicity produced by CCl_4 are markedly different from those produced by $CHCl_3$; these differences will be discussed below.

The trichloromethyl radical has several fates, which have been summarized in a recent review by Hanzlik (1981). Both the trichloromethyl radical and chlorine radical could bind irreversibly to protein and lipid (Butler, 1961; Reynolds, 1967, 1977) or could abstract hydrogen atoms from lipids or other potential sources of hydrogen atoms to form $CHCl_3$ (Butler, 1961; Paul and Rubinstein, 1963; Rubinstein and Kanics, 1964; Uehleke et al., 1973; Shah et al., 1979; Kubic and Anders, 1981). Additionally, the trichloromethyl radical could react with molecular oxygen to form phosgene (Shah et al., 1979; Kubic and Anders, 1980); or it could react with another

trichloromethyl radical to form hexachloroethane (Fowler, 1969; Uehleke et al., 1973). The reactions of the trichloromethyl radical are depicted in Figure 4. An additional reactive metabolite which may be formed is dichloromethyl carbene (Cl_2C:), which would result from the further reductive metabolism via chloride elimination of the trichloromethyl radical (Wolf et al., 1977). The metabolic fate of dichloromethyl carbene (Cl_2C:) will be discussed with (4) Hydrogen Ion Abstraction.

3. Reductive Dechlorination

The reductive dechlorination of $CHCl_3$ would produce a dichloromethyl radical ($Cl_2HC\cdot$) which would undergo reactions similar to the trichloromethyl radical (Figure 3) (Pohl et al., 1980). Thus, as illustrated in Figure 4, this radical intermediate could bind irreversibly to tissue macromolecules, or abstract hydrogen atoms from lipids or other potential sources of hydrogen atoms to form dichloromethane (CH_2Cl_2). The toxicity produced by this intermediate should be similar to that produced by other free radical intermediates, such as trichloromethyl radical.

4. Hydrogen Ion Abstraction

The final alternative pathway of $CHCl_3$ metabolism involves the abstraction of a hydrogen ion to produce trichloromethyl carbanion ($Cl_3C:^-$) (Figure 4) (Pohl, 1979; Pohl et al., 1980). This intermediate could be hydroxylated to produce phosgene ($COCl_2$), or could abstract a hydrogen ion (H^+) to form $CHCl_3$, or could spontaneously eliminate a chloride ion (Cl^-) to form the reactive electrophile

dichloromethyl carbene (Cl_2C:). Dichloromethyl carbene may bind irreversibly to tissue macromolecules or may be further metabolized, under anaerobic conditions in the presence of water, to form carbon monoxide (CO) and HCl (Wolf et al., 1977; Ahmed et al., 1977; Anders et al., 1978; Ahr et al., 1980; deGroot and Haas, 1981). Although there is evidence for a small amount of carbon monoxide formation during $CHCl_3$ metabolism, this appears to occur under aerobic conditions with the incorporation of molecular oxygen as discussed above (Stevens and Anders, 1979).

Thus, extensive studies of hepatic metabolism of $CHCl_3$ and its trihalomethane analogs indicate that oxidative dechlorination is the primary metabolic pathway. There is little evidence to support the formation of $CHCl_3$ metabolites that would be produced by the alternative metabolic pathways of hydrogen abstraction, reductive dechlorination, or hydrogen ion abstraction.

The main evidence against most of these alternative pathways comes from the differences observed between $CHCl_3$ and CCl_4 metabolites and manifestations of toxicity. For example, the pattern of ^{14}C incorporation into constituents of liver in vivo and liver microsomes in vitro is markedly different for CCl_4 and $CHCl_3$. A larger portion of $^{14}CHCl_3$ is bound to protein than with $^{14}CCl_4$ (Reynolds and Yee, 1967; Uehleke and Werner, 1975). Likewise, CCl_4 produces extensive lipid peroxidation in livers of untreated rats (Recknagel et al., 1977) whereas $CHCl_3$ does not (Klaassen and Plaa, 1969; Reynolds, 1972; Brown et al., 1974a) except when hepatic mixed function oxidases have been induced (Brown et al., 1974a). CCl_4 will rapidly decrease

hepatic cytochrome P-450 concentrations (Recknagel and Glende, 1973) whereas $CHCl_3$ does not in non-induced rats (Brown et al., 1974a). Additionally, $CHCl_3$ depletes hepatic glutathione concentrations whereas CCl_4 does not (Johnson, 1965; Brown et al., 1974a; Docks and Krishna, 1976).

Furthermore, covalent binding of radiolabelled CCl_4 indicates incorporation of both ^{14}C and ^{36}Cl (Reynolds, 1967) suggesting the generation of free radicals. Negligible amounts of ^{3}H or ^{36}Cl incorporation have been detected after incubation of $CHCl_3$ with rat liver microsomes under various incubation conditions (Pohl et al., 1980). Electrophilic chlorine has been trapped with 2,6-dimethylphenol after incubation of CCl_4, but not $CHCl_3$, with rat liver microsomes (Mico et al., 1982). These data indicate that trichloromethyl radical ($Cl_3C\cdot$), dichloromethyl carbene ($Cl_2C:$), or dichloromethyl radical ($Cl_2HC\cdot$) are not major reactive metabolites of $CHCl_3$ (Pohl et al., 1980).

Dehalogenation, CCl_4 metabolism and irreversible binding are favored when CCl_4 is incubated anaerobically with rat liver microsomes whereas the metabolism and binding of $CHCl_3$ requires oxygen (Uehleke and Werner, 1975; Sipes et al., 1977).

Finally, when $CDCl_3$ was administered to mice, no exchange of the deuterium label to form $CHCl_3$ was observed (Krantz et al., 1967). $CHCl_3$ would be expected to form if the alternate metabolic pathways of hydrogen abstraction, reductive dechlorination, or hydrogen ion abstraction were of significance.

C. Characteristics of $CHCl_3$-Induced Nephrotoxicity

Susceptibility to $CHCl_3$ nephrotoxicity varies between species. The renal lesion induced by $CHCl_3$ was localized primarily to the proximal tubule and was characterized by increased kidney weight, cloudy swelling of tubular epithelium, fatty degeneration, tubular casts and/or marked necrosis of proximal tubular epithelium with a lesser involvement of the distal tubules (Eschenbrenner, 1944; Deringer et al., 1953; Plaa and Larson, 1965; Thompson et al., 1974; Torkelson et al., 1976; Clemens et al., 1979). Renal functional changes include proteinuria and glucosuria, decreased excretion of exogenously administered phenolsulfonphthalein and increased blood urea nitrogen (Plaa and Larson, 1965; Klaassen and Plaa, 1967; Kluwe and Hook, 1978). In vitro accumulation of organic ions by renal cortical slices also was decreased by in vivo $CHCl_3$ administration (Watrous and Plaa, 1972; Kluwe and Hook, 1978; Kluwe, 1981). In mice, $CHCl_3$ did not produce glomerular damage and the renal lesion was sex-dependent; only males were susceptible (Eschenbrenner, 1944; Deringer et al., 1953).

The mechanism of $CHCl_3$-induced nephrotoxicity is unknown but may be the result of 1) direct $CHCl_3$ nephrotoxicity, 2) a hepatic metabolite that is directly nephrotoxic; 3) a hepatic metabolite that is further metabolized by the kidney to a nephrotoxic compound, or 4) the renal metabolism of $CHCl_3$ to a nephrotoxic compound. Most of the experimental evidence supports either the in situ metabolic activation of $CHCl_3$ or a hepatic metabolite of $CHCl_3$ as the mechanism for nephrotoxicity. For example, autoradiography studies indicated that the location and distribution of radioactivity to the kidney after

administration of $^{14}CHCl_3$ was proportional to the extent of nephrotoxicity (Ilett et al., 1973; Taylor et al., 1974). Furthermore, the radioactivity was localized to the necrotic proximal tubular cells and centrilobular hepatocytes, the regions of the highest mixed function oxidase activity in these organs (Ilett et al., 1973). Since $CHCl_3$ does not bind readily to macromolecules without metabolism, renal metabolism of $CHCl_3$ to some reactive intermediate is probably required to elicit nephrotoxicity. $CHCl_3$ also produced a dose-dependent reduction in renal glutathione concentrations in male ICR mice similar to that found in the liver which may result from an electrophilic intermediate of $CHCl_3$. Glutathione depletion alone did not produce nephrotoxicity. However, depletors of glutathione content such as diethyl maleate potentiated the extent of nephrotoxicity induced by $CHCl_3$ (Kluwe and Hook, 1981). In the liver, cleavage of the C-H bond in $CHCl_3$ was a rate-limiting step in $CHCl_3$ metabolism as $CDCl_3$ was less hepatotoxic than $CHCl_3$ (Pohl and Krishna, 1978; Pohl et al., 1979). Likewise, the nephrotoxicity in mice was less in animals receiving $CDCl_3$ than in those receiving an equal dose of $CHCl_3$ (Ahmadizadeh et al., 1981).

Thus, it appears that metabolism of $CHCl_3$ is a requisite step in $CHCl_3$ nephrotoxicity. However, whether this metabolism occurs in the kidney or liver is unknown. Several experimental approaches utilizing different sexes and strains of mice, as well as dose-response relationships, indicated that the hepatic and renal toxicity of $CHCl_3$ were independent events. Eschenbrenner (1944) originally observed renal necrosis in the absence of liver necrosis following low concentrations

of $CHCl_3$ in male mice. Watrous and Plaa (1972) determined that the minimum subcutaneous dose of $CHCl_3$ effective in producing nephrotoxicity in mice was 0.005 ml/kg with a near maximal effect at 0.025 ml/kg, as determined by the decreased ability of renal cortical slices to accumulate p-aminohippurate (PAH). These doses produced little or no hepatotoxicity. Functional studies also have detected renal toxicity in ICR mice at doses of $CHCl_3$ showing no evidence of hepatotoxicity (Kluwe and Hook, 1978).

In contrast, to other animal species, in mice only males were susceptible to $CHCl_3$-induced nephrotoxicity, whereas hepatotoxicity occurred in both sexes (Eschenbrenner, 1944; Eschenbrenner and Miller, 1945; Shubik and Ritchie, 1953; Hewitt, 1956; Culliford and Hewitt, 1957; Klaassen and Plaa, 1967; Ilett et al., 1973; Taylor et al., 1974; Clemens et al., 1979). This sex difference in $CHCl_3$ nephrotoxicity in mice appeared to be an effect of testosterone. Immature or castrated male mice were not susceptible to $CHCl_3$ nephrotoxicity (Eschenbrenner and Miller, 1945; Deringer et al., 1953; Culliford and Hewitt, 1957); female mice or castrated male mice treated with testosterone were susceptible to $CHCl_3$ nephrotoxicity (Eschenbrenner and Miller, 1945; Culliford and Hewitt, 1957; Taylor et al., 1974). The lack of a testosterone effect on $CHCl_3$ nephrotoxicity in female mice treated concomitantly with testosterone and the antiandrogen flutamide, or in androgen unresponsive Tfm/Y mice, suggested that an androgen receptor may mediate the effect of testosterone on $CHCl_3$ nephrotoxicity (Clemens et al., 1979). In fact, it has been suggested that the dramatic strain differences in mice with respect to the degree of

$CHCl_3$ nephrotoxicity may by related to strain differences in androgen production (Hill et al., 1975; Clemens et al., 1979). Alteration of sex-hormone status had no effect on $CHCl_3$ hepatotoxicity (Ilett et al., 1973; Taylor et al., 1974).

Testosterone induces dramatic morphological and biochemical alterations in the proximal tubules of mouse kidney. These changes include renal hypertrophy (Selye, 1939), enhanced RNA and protein synthesis (Kochakian et al., 1963; Koths et al., 1972), larger and more extensively developed organelles such as mitochondria, lysosomes, smooth and rough endoplasmic reticulum, and Golgi apparatus (Koenig et al., 1980) and increased activity of many renal enzymes. The effect of testosterone on renal mixed function oxidases has not been quantified. The inability of female mouse kidney to activate dimethylnitrosamine to a mutagen in contrast to male mice suggests there may be sex-related differences in mouse renal cytochrome P-450 concentrations (Weekes and Brusick, 1975). Morphologically, male mice have a greater amount of smooth endoplasmic reticulum than female mice (Koenig et al., 1980), also suggesting there may be sex-related differences in renal cytochrome P-450 concentrations. Therefore, sex-related differences in the susceptibility to $CHCl_3$-induced nephrotoxicity may be explained by alterations in the ability to generate a nephrotoxic metabolite within the kidney.

Further evidence for intrarenal metabolic activation of $CHCl_3$ comes from studies employing inducers and inhibitors of drug metabolizing enzymes (Table 1). However, these results are complicated by the simultaneous induction of hepatic as well as extrahepatic enzymes,

TABLE 1

Effects of Inducers and Inhibitors on Mouse Renal and Hepatic Mixed Function Oxidase (MFO) Activity and the Effect on $CHCl_3$ Toxicity

	Hepatic MFO Activities	Hepato-toxicity	Renal MFO Activities	Nephro-toxicity	Reference
Phenobarbital	+	+	0	0	Kluwe et al., 1978
3-Methylcholanthrene	+	0	+	-	Kluwe et al., 1978
2,3,7,8-Tetrachlorodibenzo-p-dioxin	+	-	+	-	Kluwe et al., 1978
Polychlorinated biphenyls	+	0	+	-	Kluwe et al., 1978
Polybrominated biphenyls	+	-	+	+	Kluwe and Hook, 1978
Methyl-n-butyl ketone	+	+	0	+	Hewitt et al., 1980b; Branch-flower and Pohl, 1981
Piperonyl butoxide	-	-	-	-	Kluwe and Hook, 1981
SKF 525-A	-	+	-	+	Kluwe and Hook, 1981
Diethyl maleate	-	+	-	+	Kluwe and Hook, 1981

+ = increase - = decrease 0 = no change

induction of different forms of cytochrome P-450, and effects on the distribution of unmetabolized $CHCl_3$ due to potential alterations in blood flow and hepatic metabolism. Phenobarbital enhanced hepatotoxicity, hepatic covalent binding and the metabolism of $CHCl_3$ (Scholler, 1970; Ilett et al., 1973; Brown et al., 1974a; Lavigne and Marchand, 1974; Uehleke and Werner, 1975; Gopinath and Ford, 1975; Docks and Krishna, 1976; Sipes et al., 1977; Pohl and Krishna, 1978; Pohl et al., 1980). On the other hand, phenobarbital did not affect renal mixed function oxidase activity nor the nephrotoxic effects of $CHCl_3$ in mice or rats (Ilett et al., 1973; Kluwe et al., 1978; Clemens et al., 1979; McMartin et al., 1981). The effects of cytochrome P-448-specific and mixed-type inducers are not as clear (Table 1). For example, 3-methylcholanthrene and polychlorinated biphenyls increased mixed function oxidases in both organs, but reduced $CHCl_3$ nephrotoxicity despite little or no effect on hepatotoxicity (McMartin et al., 1981). On the other hand, polybrominated biphenyls increased hepatic and renal mixed function oxidases and increased $CHCl_3$ toxicity in both organs (Kluwe and Hook, 1978). Clearly, the renal toxicity did not parallel the alterations in hepatotoxicity. This has been a strong argument in favor of the in situ formation of a nephrotoxic $CHCl_3$ metabolite. If the liver was the source of a directly nephrotoxic $CHCl_3$ metabolite, such as phosgene, or of a stable metabolite that was further metabolized to a reactive metabolite in the kidney, then alterations in hepatic toxicity and metabolism should produce parallel alterations in renal toxicity and metabolism (Kluwe and Hook, 1978, 1980). Inhibitors of drug metabolism also modulate nephrotoxicity;

$CHCl_3$ nephrotoxicity (and hepatotoxicity) in mice could be reduced by pretreatment with piperonyl butoxide but not by SKF 525-A (Table 1) (Kluwe and Hook, 1981).

Methyl n-butyl ketone (MBK) effectively potentiated $CHCl_3$ hepatotoxicity and nephrotoxicity in rats (Hewitt et al., 1980b). MBK potentiated the hepatotoxicity produced by $CHCl_3$ in rats by decreasing hepatic glutathione and increasing the oxidative metabolism of $CHCl_3$ to phosgene, the latter possibly due to the inductive effect of MBK on hepatic cytochrome P-450 (Hewitt et al., 1980b; Branchflower and Pohl, 1981). In these studies the nephrotoxicity of $CHCl_3$ was also increased by MBK; however, no effect on renal cytochrome P-450 or on glutathione concentrations could be detected. Branchflower and Pohl (1981) have suggested that MBK may alter the different isozymes of renal cytochrome P-450 without altering the total content and/or produce local depletion of glutathione which was not relfected in total renal glutathione content.

Evidence for the direct renal metabolism of $CHCl_3$ must come from in vitro or isolated organ experiments, since the effects of hepatic metabolism render evaluation of in vivo data difficult. Ilett et al. (1973) assessed the in vitro and in vivo covalent binding of $CHCl_3$ to renal and hepatic microsomal protein as an indication of renal $CHCl_3$ metabolism. In contrast to in vivo covalent binding, where covalent binding of $^{14}CHCl_3$ to male mouse kidney was equal to liver, very little covalent binding in vitro to male mouse renal microsomal protein was detected. Covalent binding to microsomal protein in vitro was almost 13 times greater in male mouse liver than kidney; the

magnitude of difference in covalent binding in male mouse verses female mouse kidneys was 3 *in vitro* in contrast to 10 *in vivo*. Ilett and coworkers suggested this may indicate that the kidney does not metabolize $CHCl_3$ but rather, further transforms hepatic $CHCl_3$ metabolites. There are few reports of *in vitro* metabolism of $CHCl_3$ by the kidney. In one investigation, $^{14}CHCl_3$ was metabolized to $^{14}CO_2$ *in vitro* by rat kidney slices, however, at a much reduced rate compared to liver slices (Paul and Rubinstein, 1963).

In contrast to the liver, omission of an NADPH-generating system only slightly decreased the amount of covalent binding of $CHCl_3$ to renal microsomal protein while incubation under nitrogen or carbon monoxide had no effect (Ilett *et al.*, 1973). Hence, the covalent binding to kidney microsomal protein may not be dependent on a cytochrome P-450 system or, alternatively, the incubation conditions were not optimal for renal microsomal metabolism of $CHCl_3$.

It is possible that a relatively stable liver metabolite may reach the kidney and be further metabolized to a nephrotoxic compound in the kidney. One could envision the phosgene conjugates, diglutathionyl dithiocarbonate or 2-oxothiazolidine-4-carboxylic acid, as likely compounds in this role. Indeed, excretion of diglutathionyl dithiocarbonate into the bile of rats after $CHCl_3$ has been demonstrated (Pohl *et al.*, 1981). There are data to suggest that glutathione and/or cysteine conjugates of certain xenobiotics are nephrotoxic to the proximal tubules where they are subsequently bioactivated to a toxic metabolite by a renal enzyme, C-S lyase (Bhattacharya and Schultz, 1967). It has been postulated that a cysteine conjugate of

trichloroethylene, dichlorovinylcysteine, may produce nephrotoxicity via this mechanism (Anderson and Schultz, 1965). The cleavage of dichlorovinylcysteine by C-S lyase yielded pyruvate, ammonia and chloride in a 1:1:2 ratio and another unknown fragment containing carbon from the vinyl group and sulfur. This unknown fragment was demonstrated to combine with proteins, glutathione and probably with the C-S lyase enzyme resulting in enzymatic inactivation (Anderson and Schultz, 1965). The nephrotoxicants chlorotrifluoroethylene and hexachlorobutadiene also have been suggested to be activated metabolically within the kidney by C-S lyase following conjugation with glutathione (Bonhaus and Gandolfi, 1981).

D. Purpose

The primary purpose of this investigation was to test the hypothesis that direct renal metabolism of $CHCl_3$ is required for $CHCl_3$-induced nephrotoxicity. In the past, circumstantial evidence has been used to support the contention that intrarenal metabolism is responsible for the nephrotoxic response to $CHCl_3$. This investigation was designed as a mechanistic approach to evaluate the role of renal metabolism in the generation of nephrotoxic metabolites. The mouse was chosen as the model species in order to utilize the dramatic sex and strain differences in susceptibility to $CHCl_3$-induced nephrotoxicity.

There are several possible mechanisms of $CHCl_3$-induced nephrotoxicity. (1) $CHCl_3$ may be directly toxic to the kidney, possibly due to a solvent effect on the cell membrane. (2) $CHCl_3$ may be metabolized

directly by the kidney to a nephrotoxic metabolite, possibly by a cytochrome P-450 mediated mechanism similar to that in the liver. (3) The kidney may not metabolize $CHCl_3$ directly, but possibly metabolize a hepatic $CHCl_3$ metabolite to a nephrotoxic intermediate. The most likely compounds would be the phosgene conjugates, 2-oxothiazolidine-4-carboxylic acid and diglutathionyl dithiocarbonate. (4) A metabolite of $CHCl_3$ formed in the liver, such as phosgene or one of the phosgene conjugates, may be directly nephrotoxic. Cells in the proximal tubules may be more susceptible to toxicity caused by a circulating hepatic metabolite because they are exposed to a high proportion of cardiac output, are very active metabolically, and can be exposed to higher concentrations of certain drugs and chemicals than other cells due to the active transport processes and concentrating mechanisms that occur in the kidney.

The specific objectives of this investigation were:

(1) To define better the relationship between hepatic and renal toxicity of $CHCl_3$ *in vivo*.

(2) To evaluate the role of the liver and of hepatic metabolites of $CHCl_3$ in the manifestation of $CHCl_3$-induced nephrotoxicity.

(3) To determine the effect of sex hormone status and strain differences on mouse renal and hepatic mixed function oxidases in relation to the susceptibility to $CHCl_3$ nephrotoxicity.

(4) To determine whether $CHCl_3$ could be metabolized directly by the kidney, and if so, whether the mechanism was similar to that occurring in the liver.

METHODS

A. Animals

Adult male and female ICR mice (30 g, Harlan Farms, Haslett, MI) and adult male C57Bl/6 and DBA/2 mice (20 g, Charles River, Portage, MI) were maintained on a 12 hr light-dark cycle (0800 to 2000 light) in a temperature and humidity controlled room. Animals were allowed free access to food (Wayne Lab Blox, Continental Grain Co., Chicago, IL) and water.

B. Assessment of Renal and Hepatic Toxicity

1. Serum Analysis

Mice were killed by cervical dislocation and decapitated for blood collection. Blood was allowed to clot for 2 hr at room temperature and then centrifuged. Serum was collected and refrigerated until assayed. Serum blood urea nitrogen (BUN) concentration and serum glutamic-pyruvic transaminase (SGPT) activities were determined with Sigma reagents (Sigma Technical Bulletin Nos. 640 and 505, respectively, Sigma Chemical Co., St. Louis, MO). BUN concentration was expressed as mg/dl serum. SGPT activity was expressed as Sigma-Frankel (SF) units/ml where one SF unit of SGPT will form 4.82×10^{-4} μmol glutamate/min in phosphate buffer, pH 7.5 and 25°C.

2. Determination of Renal and Hepatic Non-Protein Sulfhydryl Content

Livers and kidneys were quickly removed and weighed. Samples of liver and renal cortex dissected from one kidney were immediately homogenized in 20 volumes of ice-cold 6% trichloroacetic acid and centrifuged. The concentration of non-protein sulfhydryls in the supernatant fraction was measured as an index of reduced glutathione according to the method of Ellman (1959) with slight modifications as described by Kuo and Hook (1982).

3. Histology

Thin slices of liver and kidney were fixed in pH 7.4 phosphate-buffered formalin (10%), embedded in paraffin blocks, sectioned, mounted on glass slides, and stained with hematoxylin and eosin for examination by light microscopy.

4. Renal Cortical Slice Accumulation of Organic Ions

Kidneys were placed in ice-cold 0.9% NaCl briefly until thin renal cortical slices could be prepared for determination of p-aminohippurate (PAH) and tetraethylammonium (TEA) accumulation. Thin renal cortical slices (0.3 to 0.5 mm) were prepared free-hand and incubated in 4.0 ml of phosphate-buffered medium containing 96.7 mM NaCl, 7.4 mM sodium phosphate buffer, 40 mM KCl and 0.74 mM $CaCl_2$ at pH 7.4 (Cross and Taggart, 1950) supplemented with 10 mM sodium lactate and also containing 7.4×10^{-5}M PAH and 1×10^{-5}M [1-^{14}C]TEA, specific activity 2.0 mCi/mmole (New England Nuclear, Boston, MA). After incubation for 90 min at 25°C under 100% O_2 in a Dubnoff metabolic shaker, slices were removed from the medium, blotted and weighed. The slices

were homogenized in 10 ml of 3% trichloroacetic acid. A 2 ml aliquot of the incubation medium was treated similarly. After centrifugation, 0.4 ml of 1.2 N HCl was added to 2 ml of the supernatant or to PAH standards. Each test tube was capped with a marble and subjected to hydrolysis in a boiling water bath (100°C) for 1 hr since mice rapidly acetylate PAH (Carpenter and Mudge, 1980). The hydrolyzed samples were then assayed for PAH by the method of Smith et al. (1945). To quantify [^{14}C]TEA, 1 ml of slice or medium supernatant was added to 10 ml of ACS counting scintillant (Amersham Corp., Arlington Heights, IL) and radioactivity was determined in a Searle Delta 300 liquid scintillation spectrophotometer. PAH or TEA accumulation was expressed as the S/M ratio, where the concentration of PAH or TEA per gram of tissue was divided by the concentration of PAH or TEA per ml of medium.

5. Definitions of Toxicity

Decreases in PAH and TEA accumulation by renal cortical slices and/or increases in BUN were used as indications of nephrotoxicity. Elevation of SGPT was used as an indication of hepatotoxicity. The presence or absence of histopathological lesions in kidney and liver was defined in selected experiments.

Increases of liver weight to body weight ratios and increases of kidney weight to body weight ratios were used as an additional indication of hepatic and renal toxicity, respectively, in certain experiments.

C. Analysis of Components of Renal and Hepatic Drug-Metabolizing Enzyme Systems

1. Preparation of Subcellular Fractions

Kidneys and livers from mice were quickly excised, pooled and placed in ice-cold 0.1 M sodium phosphate buffer pH 7.4 containing, 1.15% KCl. After being weighed, tissues were minced in this same buffer, rinsed 3 times and homogenized in 3 volumes buffer using a Potter-Elvehjem homogenizer with a teflon pestle followed by centrifugation at 9,000 x g for 30 minutes. The resulting supernatant was then centrifuged at 105,000 x g for 60 minutes. The 105,000 x g supernatant was the cytosol fraction. The pellet was resuspended in the same buffer and recentrifuged at 105,000 x g for 60 minutes. In experiments for comparative assessment of renal cortical and hepatic mixed function oxidase activities, the microsomal fraction (pellet) was resuspended in 0.1 M sodium phosphate buffer, pH 7.4 containing 0.25 M sucrose and 5.4 mM EDTA to a final concentration of 15-25 mg protein per ml and were frozen at -70°. In experiments for determination of microsomal metabolism of $^{14}CHCl_3$, microsomes were resuspended in 0.1 M sodium phosphate buffer, pH 7.4, at a concentration of 10 mg protein per ml and were used immediately.

2. Determination of Mixed Function Oxidase Activities In Vitro

Microsomes (1-2 mg/ml for kidney and 0.25-0.50 mg/ml for liver) were suspended in 1 ml of 0.1 M sodium phosphate, pH 7.4, containing 4.5 μmol glucose-6-phosphate, 0.3 μmol NADH, 0.1 μmol NADPH, 163 μmol $MgCl_2$ and 1 unit of glucose-6-phosphate dehydrogenase.

After 5 minutes preincubation at 37°C the reaction was initiated by the addition of substrate. The deethylation of ethoxyresorufin and ethoxycoumarin was measured by the methods of Johnson et al. (1979) and Aitio (1978), respectively; NADPH cytochrome-c-reductase was measured by the method of Pederson et al. (1973). Cytochrome P-450 concentrations were determined from the dithionite-reduced CO difference spectra and cytochrome b5 concentrations were measured from the NADH reduced difference spectra as described by Omura and Sato (1964). Substrate binding studies were conducted as described by Schenkman et al. (1967). Spectral measurements were made on a Beckman dual beam spectrophotometer (Model No. UV 5260). Microsomal protein concentration was determined by the method of Lowry et al. (1951).

3. Chemicals

Ethoxyresorufin and resorufin were purchased from the Pierce Chemical Co. (Rockford, IL). Ethoxycoumarin, umbelliferone, NADPH (tetrasodium salt), NADP (monosodium salt), NADPH (disodium salt), glucose-6-phosphate (monosodium salt), glucose-6-phosphate dehydrogenase (Bakers yeast, type XV), and cytochrome c (horse heart type III), were purchased from the Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest grade commercially available.

D. Assessment of $^{14}CHCl_3$ Metabolism In Vitro

1. Incubation Procedures

Metabolism of $^{14}CHCl_3$ (diluted to a specific activity of ≈0.5 μCi/μmol, Pathfinder Laboratories, St. Louis, MO) by mouse tissue was evaluated by measuring the conversion of $^{14}CHCl_3$ to $^{14}CO_2$,

radioactivity irreversibly associated with the protein fraction (an indication of covalent binding), and aqueous soluble radioactivity. The incubations were conducted in 25 ml Erlenmeyer flasks containing tissue slices or subcellular fractions in a phosphate-buffered medium, pH 7.4. Specific alterations in the composition of the incubation medium are noted in the figure legends. The flasks were oxygenated with 100% O_2 for 5 min, $^{14}CHCl_3$ was added directly to the incubation medium, the flasks were sealed immediately with a sleeve-type rubber septum (Kontes, Vineland, NJ) and were incubated at 37°C in a metabolic shaker (100 cycles/min). Each flask had a removable center well attached to the rubber septum containing a 1 cm^2 piece of filter paper with 20-50 μmoles of NaOH as a CO_2 trap. The incubation was terminated by the injection of 10% trichloroacetic acid (TCA) at a volume equal to the reaction volume with a syringe through the rubber septum. The flasks remained sealed for at least 3 hr at room temperature after incubation was terminated to allow for trapping of $^{14}CO_2$ as sodium carbonate. In some experiments, the flasks were refrigerated overnight. The rubber septa were removed and the amounts of $^{14}CO_2$, covalently bound radioactivity, and aqueous soluble radioactivity measured as described below.

Non-enzymatic metabolism of $^{14}CHCl_3$ was assessed in reaction flasks containing heat-denatured protein (100°C, 5 min) from either liver or kidney and, where indicated, was subtracted to obtain corrected values for enzymatic $^{14}CHCl_3$ metabolism.

2. Determination of $^{14}CO_2$ Production

$^{14}CO_2$ evolution was detected by the method of Pohl et al. (1980). The filter paper CO_2 traps were transferred to 15 ml screw cap tubes containing 1 ml H_2O. Unmetabolized $^{14}CHCl_3$ was removed by extracting the resulting alkaline solutions with 2 ml of diethyl ether until radioactivity could no longer be extracted for three consecutive washes. This usually required seven extractions. A 0.5 ml aliquot of the washed alkaline solution was counted in 20 ml ACS counting scintillant for 10 min each; quench was corrected by internal standardization with [^{14}C]toluene (New England Nuclear, Boston, MA). Data are expressed as nmol $^{14}CO_2$ detected per reaction vessel per incubation time.

3. Determination of Covalently Bound Radioactivity to Proteins

The trichloroacetic acid precipitated protein was transferred from the Erlenmeyer flask to a 15 ml screw-cap tube. When slices were used, the tissue and incubation medium were homogenized prior to transfer. The denatured protein was separated by centrifugation. The aqueous layer was removed and, in some experiments, extracted for determination of aqueous soluble radioactivity (see below). Radioactivity not covalently bound to proteins was removed as described by Ilett et al. (1973). The protein pellets were washed with 5 ml of hot (50°C) methanol:ether (3:1) until radioactivity could no longer be extracted from the pellets for three consecutive washes; this usually required seven extractions. The protein pellets were dissolved in 1 ml of 1 N NaOH and an aliquot taken for determination of protein (Lowry et al., 1951). The covalently bound radioactivity

was determined by counting a 0.5 ml aliquot of the alkaline solution in 10 ml of ACS containing 0.5 ml of 1 N HCl. Samples were counted for 10 min each; quench was corrected by internal standardization with [^{14}C]toluene. Data are expressed as nmol $^{14}CHCl_3$ covalently bound per mg protein, or were extrapolated to nmol $^{14}CHCl_3$ covalently bound per reaction vessel where indicated.

4. Determination of Aqueous Soluble $^{14}CHCl_3$ Metabolites

In some experiments, the aqueous supernatant of the reaction homogenate was extracted to remove unmetabolized $^{14}CHCl_3$ with 4 ml diethyl ether until radioactivity could no longer be extracted from the aqueous phase for three consecutive washes. A 0.5 ml aliquot of the aqueous phase was counted in 10 ml of ACS for 10 min each; quench was corrected by internal standardization with [^{14}C]toluene. Data are expressed as nmoles $^{14}CHCl_3$ converted to aqueous soluble metabolites per reaction vessel. This radioactivity probably represents phosgene conjugated with small molecular weight proteins, cysteine and glutathione.

E. Individual Experiments

1. Effect of Dose and Route of Administration on $CHCl_3$ Toxicity in Male and Female ICR Mice

The effect of dose and route of administration on male and female ICR mice was assessed in experiments where chloroform ($CHCl_3$, 99+%, stabilized with 0.75% ethanol, Aldrich Chemical Co., Milwaukee, WI) was dissolved in peanut oil and administered by intraperitoneal (i.p., 5 ml/kg) or subcutaneous (s.c., 2 ml/kg) injections to male and

female mice in doses ranging from 50 to 1000 µl/kg. Control animals received peanut oil injections. The injections were all made within a 2 hr period between 0730 and 0930 and animal cages were placed in a fume hood after $CHCl_3$ injection. Mice were killed after 24 hr for assessment of renal and hepatic toxicity.

2. Time Course of $CHCl_3$ Toxicity in Male and Female ICR Mice

The time course of $CHCl_3$-induced renal and hepatic toxicity was assessed in male and female ICR mice injected with 250 µl $CHCl_3$/kg (s.c., 2 ml/kg) or with peanut oil alone. Again, the injections were all made within a 2 hr period between 0730 and 0930 to minimize the effect of diurnal variations and animal cages were placed in a chemical fume hood after $CHCl_3$ injection. Mice were killed at various time intervals during the subsequent 24 hr period after $CHCl_3$ administration for assessment of renal and hepatic toxicity.

3. Effect of Decreasing Renal Cortical Non-Protein Sulfhydryl Concentrations on $CHCl_3$ Toxicity in Male and Female ICR Mice

The effect of decreasing renal cortical and hepatic non-protein sulfhydryl concentrations on $CHCl_3$ toxicity was determined in male and female ICR mice injected with diethyl maleate (Aldrich Chemical Co., Milwaukee, WI). Mice were injected with peanut oil or with 0.6 ml/kg diethyl maleate (i.p., 5 ml/kg in peanut oil) 0.5 hr prior to administration of $CHCl_3$ (0, 50 or 500 µl/kg in peanut oil, s.c., 2 ml/kg) and were killed after 24 hr for assessment of renal and hepatic toxicity.

4. Effect of Partial Hepatectomy on $CHCl_3$ Nephrotoxicity in Male ICR Mice

Adult male ICR mice were anesthetized with diethyl ether and a 50-70% partial hepatectomy was performed. A midline incision of approximately 1 cm was made and the 2 main lobes of the liver were exteriorized and ligated near the hilum. The liver lobes distal to the ligature were excised and weighed. The incision was closed with 1 or 2 sutures and a wound clip was used to close the skin. Controls consisted of control mice and sham-operated mice in which the liver was exteriorized, replaced in the body cavity and the incision closed. Mice were allowed to recover for at least 1 hour after surgery under a heat lamp at 37°C. Animals had free access to water, but not food.

Following the recovery, mice received peanut oil or $CHCl_3$ dissolved in peanut oil by subcutaneous injection (2 ml/kg). $CHCl_3$ doses were 10, 50 and 250 µl/kg. Four hours after $CHCl_3$ dosing, mice were killed and assessed for $CHCl_3$ nephrotoxicity and hepatotoxicity as described previously. At the time the mice were killed, the weight of the remaining liver was determined and used to calculate the percent liver removed during the partial hepatectomy.

5. Effect of Sex Hormone Status on $CHCl_3$ Toxicity and Renal and Hepatic Mixed Function Oxidases in ICR Mice

Male mice were castrated or underwent sham-surgery under light ether anesthesia using two parallel incisions on either side of the scrotal septa, in the apex of the scrotum. In the castrated mice, the spermatic cord was ligated by a single suture and excised. The incisions were closed with a single suture. Groups of castrated and sham-operated mice were allowed to recover for 3 weeks before $CHCl_3$

administration or microsome preparation. Individual treatment groups consisted of 18 castrated, 13 sham-operated and 13 control male mice. Kidneys and livers from 10 control, 10 sham-operated or 15 castrated male mice were pooled for microsome preparation. The remaining mice were assessed for $CHCl_3$ toxicity as described above. For statistical purposes, a treatment group as described represents a single replicate; all experiments were replicated four times.

In another series of experiments, groups of male and female mice were injected subcutaneously with 1 mg testosterone propionate (0.1 ml) in peanut oil, or with peanut oil alone, on alternate days for 3 weeks. Treatment groups were composed of 18 female peanut oil-, 13 male peanut oil-, 13 male testosterone-, and 13 female testosterone-treated mice. Kidneys and livers from 10 mice of each group were pooled for microsome preparation, except for female peanut oil-treated mice where 15 animals were used. The remaining mice were assessed for $CHCl_3$ toxicity as described above. For statistical purposes, a treatment group as described represents a single replicate; all experiments were replicated four times.

Mixed function oxidase activities were determined in microsomal fractions as described above.

6. Assessment of $CHCl_3$ Nephrotoxicity In Vitro in Mouse Renal Cortical Slices

a. Preincubation - In vitro $CHCl_3$ toxicity

Mice were killed by cervical dislocation and kidneys removed rapidly and placed in ice-cold 0.9% NaCl. Thin renal cortical slices prepared from male or female mice (100 ± 10 mg wet weight tissue)

were placed in a 25 ml Erlenmeyer flask containing 4 ml of an isotonic phosphate-buffered medium (97 mM NaCl, 40 mM KCl, 0.74 mM $CaCl_2$ and 7.4 mM sodium phosphate, pH 7.4). The flasks containing medium and slices, were gassed with 100% O_2 or other gasses for 5 min as indicated. After gassing, $CHCl_3$ or deuterated-$CHCl_3$ ($CDCl_3$, Aldrich Chemical Co., Milwaukee, WI) was added as indicated with a 5 µl Hamilton microsyringe directly to the preincubation medium. Alterations of this procedure are noted in the figure legends. The flasks were stoppered immediately and the closed vessels were incubated at 37°C for 90 min, unless otherwise indicated, in a Dubnoff metabolic shaker moving at a rate of approximately 100 cycles per minute. After preincubation, slices were removed carefully from the preincubation medium with forceps, rinsed in $CHCl_3$-free medium of the same composition, and transferred to a 30 ml beaker containing medium for the assessment of organic ion accumulation.

b. Incubation - Organic ion accumulation

Slices were incubated in 4 ml of medium composed of 97 mM NaCl, 40 mM KCl, 0.74 mM $CaCl_2$, and 7.4 mM sodium phosphate (pH 7.4) as described by Cross and Taggart (1950). The medium was supplemented with 10 mM lactate and also contained 7.4×10^{-5}M p-aminohippurate (PAH) and 1×10^{-5}M [1-^{14}C]tetraethylammonium-bromide (TEA), specific activity 2.0 mCi/mmol (New England Nuclear, Boston, MA). Organic ion accumulation was determined as described previously.

7. Assessment of Nephrotoxicity of Hepatic $CHCl_3$ Metabolites in Male Mouse Renal Cortical Slices

Mice were killed by cervical dislocation and kidneys removed rapidly and placed in ice-cold 0.9% NaCl. Thin renal cortical slices prepared from male mice (100±10 mg wet weight tissue) were placed in a 25 ml Erlenmeyer flask containing 2 ml of an isotonic phosphate-buffered medium (97 mM NaCl, 40 mM KCl, 0.74 mM $CaCl_2$ and 7.4 mM sodium phosphate, pH 7.4) which also contained 6.25 μmol 2 oxothiazolidine-4-carboxylic acid (OTZ) or 6.25 μmol diglutathionyl dithiocarbonate (GSCOSG) where indicated. (OTZ and GSCOSG were obtained from Dr. L. Pohl.) The flasks containing medium and slices, were gassed with 100% O_2 for 5 min. After gassing, $CHCl_3$ (0.5 or 1.0 μl; 6.25 or 12.5 μmol) was added as indicated with a 5 μl Hamilton microsyringe directly to the preincubation medium. The flasks were stoppered immediately and the sealed vessels were incubated at 37°C for 90 min in a Dubnoff metabolic shaker moving at a rate of approximately 100 cycles per minute. After preincubation, slices were removed carefully from the preincubation medium with forceps, rinsed in $CHCl_3$- or metabolite-free medium of the same composition, and transferred to a 30 ml beaker containing medium for the assessment of organic ion accumulation as described previously.

8. Effect of Mouse Strain Differences on $CHCl_3$ Toxicity In Vitro and on Mixed Function Oxidases and $^{14}CHCl_3$ Metabolism by Microsomes

Mixed function oxidase activities and the metabolism of $^{14}CHCl_3$ were determined in renal cortical and hepatic microsomes prepared from several strains of mice as described previously. Kidney

cortex and liver were pooled from sexually mature mice; 10 male ICR (≈30 g), 15 female ICR (≈28 g), 15 male C57Bl/6 (≈20 g) and 15 male DBA/2 (≈20 g) mice were used.

$CHCl_3$ nephrotoxicity *in vitro* was assessed in renal cortical slices prepared from adult male and female ICR, male C57Bl/6 and male DBA/2 mice as described previously.

F. Statistics

All data were expressed as mean ± standard error of the mean (S.E.M.). Where appropriate, data were analyzed by analysis of variance, completely random design. Treatment differences were detected using the Student-Newman-Keul's tests (Steel and Torrie, 1960). The 0.05 level of probability was chosen as the criterion of significance.

RESULTS

A. In Vivo Studies

1. Effect of Dose and Route of Administration on $CHCl_3$ Toxicity in Male and Female ICR Mice

$CHCl_3$ produced dose-related hepatotoxicity in both male and female mice; nephrotoxicity was markedly different between the two sexes (Figure 5). $CHCl_3$ (50 to 1000 µl/kg) was not nephrotoxic to female mice 24 hr after injection; there was no effect on the ability of renal cortical slices to accumulate PAH and TEA nor on BUN concentration (Figure 5, A-C). Maximum nephrotoxicity in male mice occurred at a dose of 250 µl $CHCl_3$/kg whereas hepatotoxicity (SGPT), continued to increase with the higher doses of $CHCl_3$ in both male and female mice (Figure 5D). The extent of hepatic and renal toxicity, in general, was greater after s.c. than after i.p. administration of $CHCl_3$.

2. Time Course of $CHCl_3$ Toxicity in Male and Female ICR Mice

The time course of $CHCl_3$-induced renal and hepatic toxicity was examined in male and female mice receiving a 250 µl/kg s.c. injection of $CHCl_3$, a dose producing maximal nephrotoxicity in male mice and moderate hepatotoxicity in both sexes. In the liver, the time course and extent of hepatotoxicity were nearly identical in male and female mice as measured by SGPT activity, tissue non-protein

Figure 5. Effect of route of administration of $CHCl_3$ on hepatic and renal toxicity in male and female ICR mice. $CHCl_3$ (50 to 1000 µl/kg) or vehicle (peanut oil) was administered s.c. (2 ml/kg) or i.p. (5 ml/kg). (A) Renal cortical p-aminohippurate accumulation *in vitro* (PAH S/M), (B) renal cortical tetraethylammonium accumulation *in vitro* (TEA S/M), (C) blood urea nitrogen (BUN), and (D) serum glutamic pyruvic transaminase (SGPT) activity were measured 24 hr after $CHCl_3$ injections. Values are mean ± S.E.M., n=4.

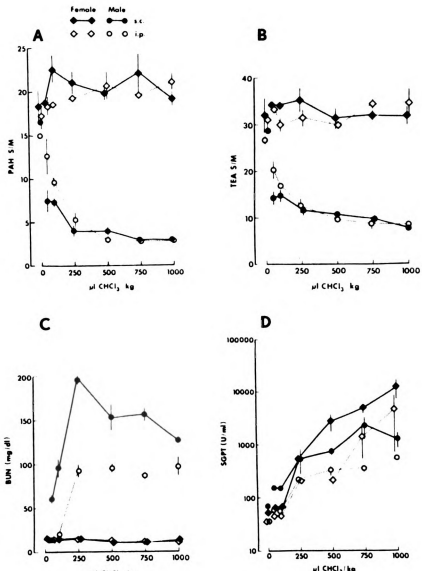

Female
Male
s.c.
i.p.
A
B
C
D
PAH S.M.
TEA S.M.
BUN (mg/dl)
SGPT (U/ml)
μl CHCl3 kg
0
250
500
750
1000

Figure 5

sulfhydryl concentrations and histological observations (Figures 6-8). The earliest changes were detected as decreases of tissue non-protein sulfhydryl concentrations by 1.5 to 2 hr after $CHCl_3$ administration (Figure 6). These biochemical changes preceded the rise of SGPT activity (Figure 7), which was not apparent until 4 hr, the time when hepatic non-protein sulfhydryl concentrations reached a nadir of 40 to 50% of control concentrations. Likewise, histopathological alterations in liver (swelling of centrilobular hepatocytes) were not detected until 4 to 5 hr after $CHCl_3$ administration (Figure 8). By 12 hr, hepatic non-protein sulfhydryl concentrations had returned to control values, though SGPT remained elevated and histopathological lesions were apparent. Hepatic histopathologic changes were first seen by 4 hr in male mice and 5 hr in females and consisted of swelling of centrilobular hepatocytes. Necrosis of hepatocytes was detected in male and female mice by 8 and 12 hr, respectively, after $CHCl_3$ administration (Figure 8A-D).

The onset of biochemical and functional changes in the kidney paralleled those occurring in the liver. Renal cortical non-protein sulfhydryl concentrations in male mice were decreased 1.5 hr after $CHCl_3$ administration and continued to decline until reaching the nadir of approximately 40% of control by 4 hr (Figure 9). Non-protein sulfhydryl concentrations in renal cortex from female mice were decreased by 20% 3 hr after $CHCl_3$ administration, but this was not associated with nephrotoxicity as there were no histological nor functional changes observed (Figures 9-13). In contrast to the liver, renal cortical non-protein sulfhydryl concentrations did not return to

Figure 6. Time course of non-protein sulfhydryl (NPSH) decrease in liver after 250 μl $CHCl_3$/kg, s.c., in male and female ICR mice. Values are mean ± S.E.M., n=4.

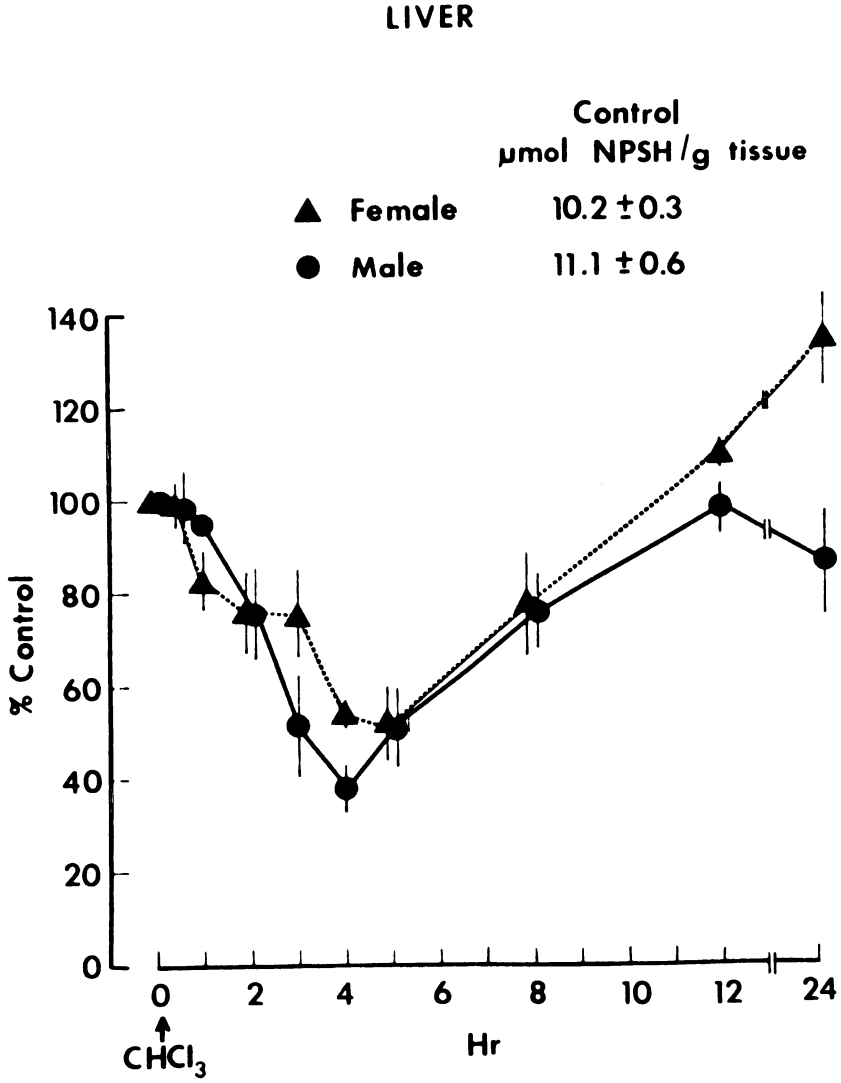

LIVER
Control
μmol NPSH/g tissue
Female 10.2 ± 0.3
Male 11.1 ± 0.6
% Control
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
24
Hr
CHCl3

Figure 6

Figure 7. Time course of serum glutamic pyruvic transaminase (SGPT) activity after 250 μl $CHCl_3$/kg, s.c., in male and female ICR mice. Values are mean ± S.E.M., n=4.

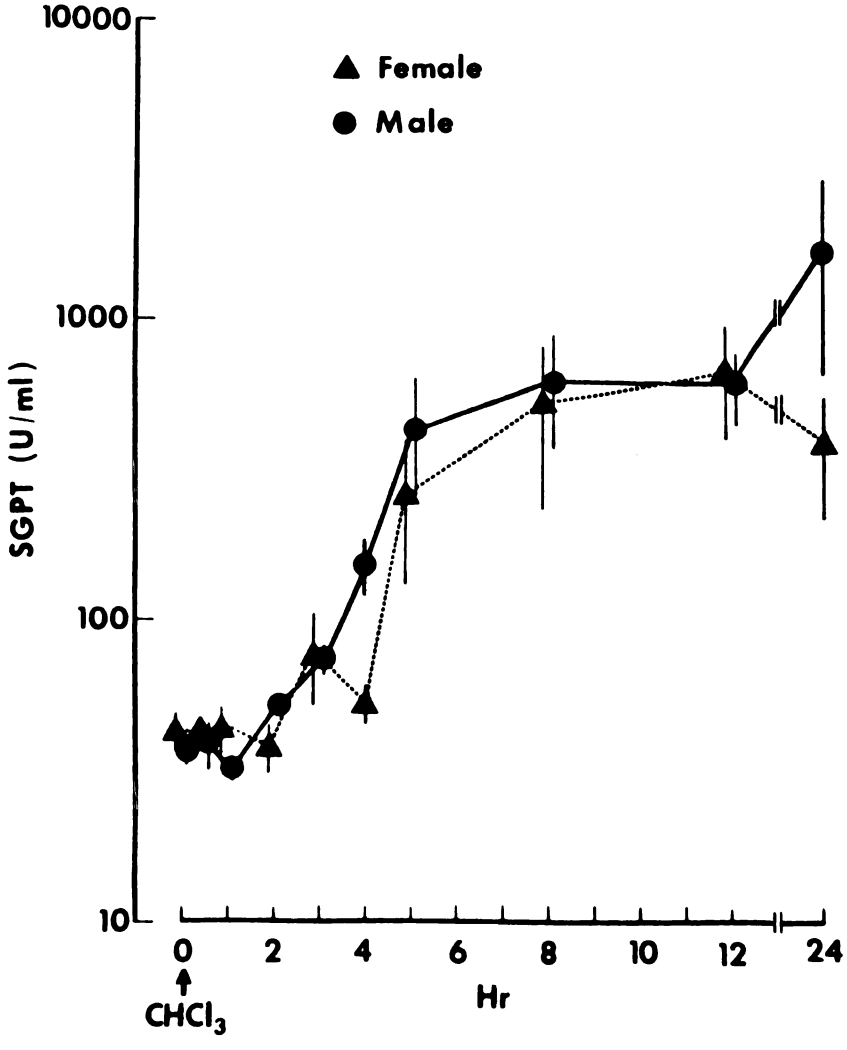


Figure 7

Figure 7. Time course of serum glutamic pyruvic transaminase (SGPT) activity after 250 μl $CHCl_3$/kg, s.c., in male and female ICR mice. Values are mean ± S.E.M., n=4.

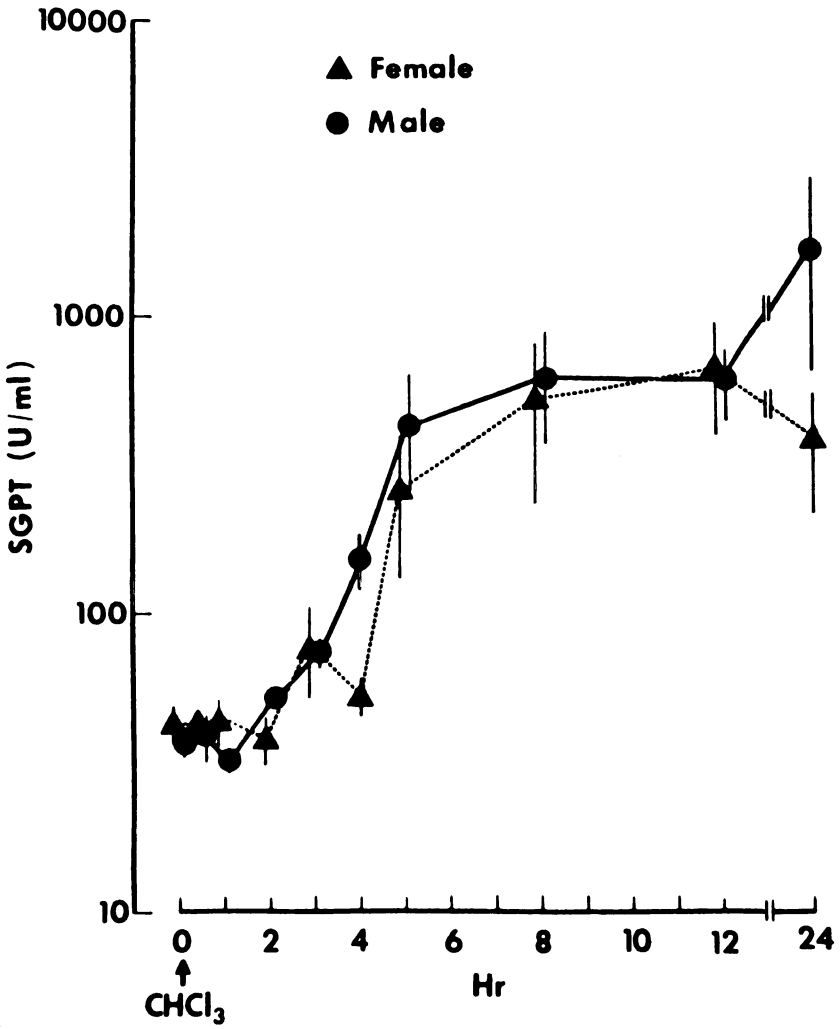


Figure 7

Figure 8. Representative hepatocellular morphology after 250 μl $CHCl_3$/kg, s.c. in male and female ICR mice. (A) Male, control liver. (B) Male, 5 hr after $CHCl_3$; centrilobular swelling of hepatocytes. This was first apparent 4 to 5 hr after $CHCl_3$ injection. (C) Female, 8 hr after $CHCl_3$; centri- and midlobular swelling of hepatocytes; some hepatocytes are necrotic. (D) Male, 24 hr after $CHCl_3$, marked hepatocyte swelling, ballooning of cells and of individual hepatocytes. Hematoxylin and eosin stain, x100.

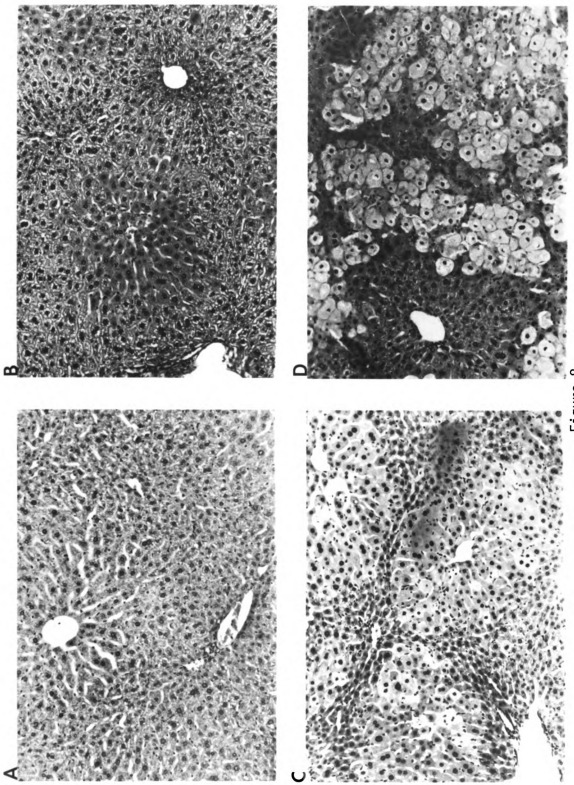


Figure 8

Figure 9. Time course of decrease of renal cortical non-protein sulfhydryl (NPSH) concentration after 250 μl $CHCl_3$/kg, s.c., in male and female ICR mice. Values are mean ± S.E.M., n=4.

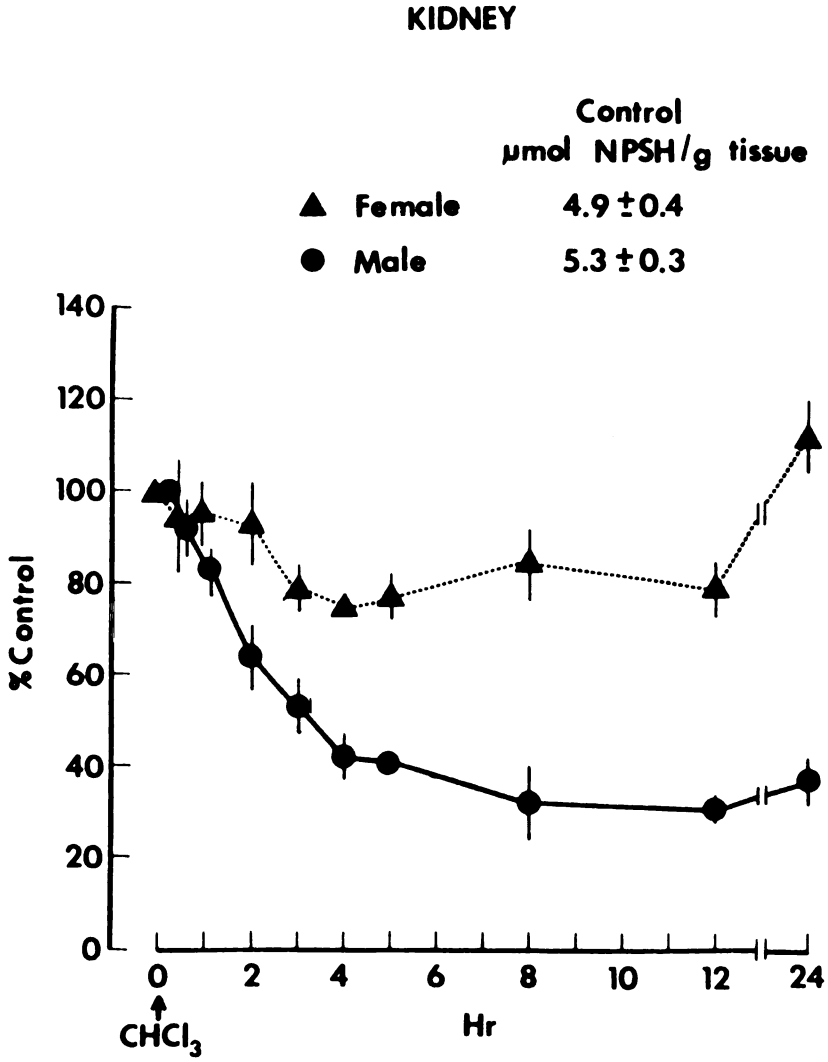

KIDNEY
Control
μmol NPSH/g tissue
Female 4.9 ± 0.4
Male 5.3 ± 0.3
% Control
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
24
Hr
CHCl3

Figure 9

control values during the 24 hr period. Decreases of the steady-state accumulation of PAH and TEA could be detected 2 hr after $CHCl_3$ administration in male mice, and were maximal by 5 hr (Figure 10). PAH and TEA S/M ratios remained decreased during the 24 hr time course. These functional changes paralleled the alterations detected in renal cortical non-protein sulfhydryl concentrations in male mice. In female mice, the accumulation of PAH and TEA was variable at the early time points, but there were no significant decreases of the S/M ratios during the 24 hr period to indicate nephrotoxicity. Slight increases of BUN concentration occurred in male mice by 5 hr after $CHCl_3$ administration (Figure 11). By 24 hr, the increase in BUN was more dramatic. There was no increase of BUN at any time point in female mice, again indicating a lack of susceptibility to $CHCl_3$ nephrotoxicity.

Histological comparison of kidneys from male and female mice showed equally profound results -- no lesions were observed in female mouse kidneys at any time (Figure 12). In male mice, the earliest evidence of necrosis as detected by light microscopy was in proximal tubular cells 5 hr after $CHCl_3$ aministration. Nuclei were pyknotic and there was loss of reticular cytoplasmic structure (Figure 12A). The lesions progressed to frank necrosis by 8 hr and increased in distribution and severity with time in the proximal tubular region only. By 12 hr, the tubular lumens were occluded with hyaline casts (Figure 12B). Epithelial cells of Bowman's capsules and distal tubules still appeared normal at 24 hr (Figure 12C).

Figure 10. Effect of 250 μl $CHCl_3$/kg, s.c., on *in vitro* renal cortical slice accumulation of p-aminohippurate (PAH S/M) and tetraethylammonium (TEA S/M) in male and female ICR mice. Values are mean ± S.E.M., n=4.

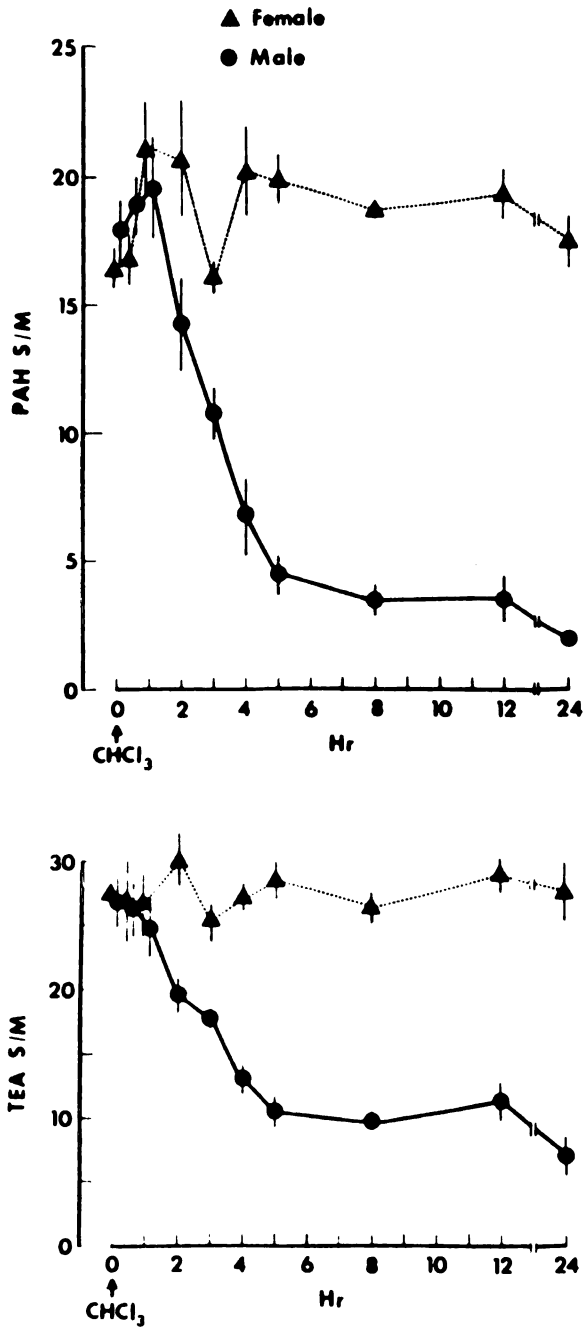

▲ Female
● Male
PAH S/M
25
20
15
10
5
0
0
2
4
6
8
10
12
24
Hr
CHCl3
TEA S/M
30
20
10
0
0
2
4
6
8
10
12
24
Hr
CHCl3

Figure 10

Figure 11. Time course of blood urea nitrogen (BUN) concentration after 250 μl $CHCl_3$/kg, s.c., in male and female ICR mice. Values are mean ± S.E.M., n=4.

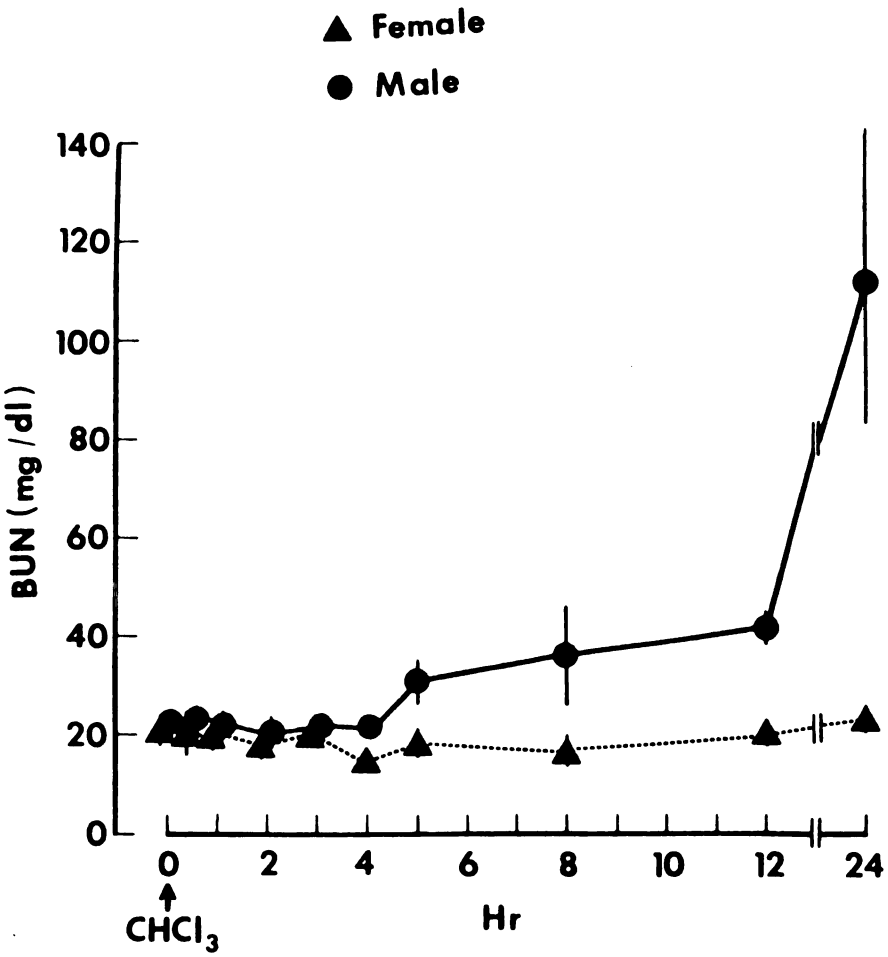

▲ Female
● Male
140
120
100
80
60
40
20
0
BUN (mg/dl)
0
2
4
6
8
10
12
24
Hr
CHCl3

Figure 11

Figure 12. Representative renal tubular morphology after 250 μl $CHCl_3$/kg, s.c., in male and female ICR mice. (A) Male, 5 hr after $CHCl_3$; pyknotic nuclei and loss of brush border in a small number of proximal tubular cells. (B) Male, 8 hr after $CHCl_3$; massive proximal tubular necrosis. (C) Male, 24 hr after $CHCl_3$; more extensive proximal tubular necrosis; epithelial cells of Bowman's capsule remain intact. (D) Female, 24 hr after $CHCl_3$; no histopathological alterations occurred after $CHCl_3$ administration. Hematoxylin and eosin stain, x100.

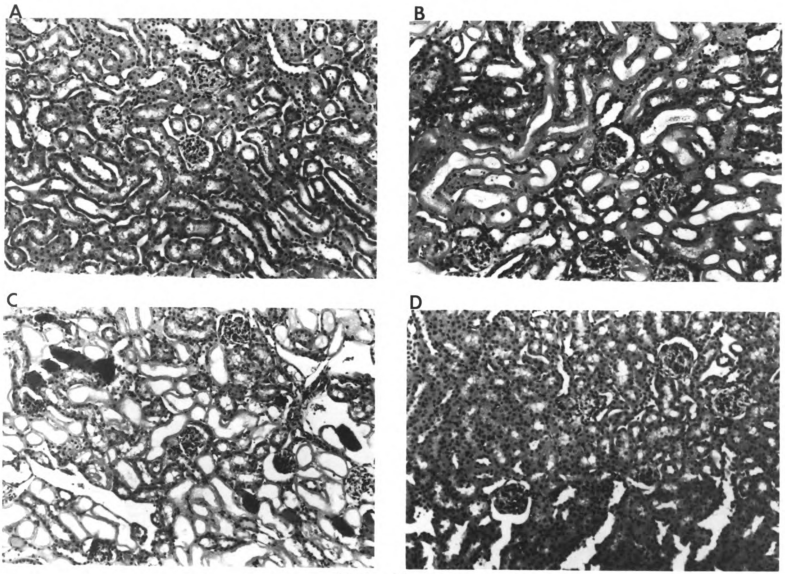


Figure 12

3. Effect of Decreasing Renal Cortical Non-Protein Sulfhydryl Concentration on $CHCl_3$ Toxicity in Male and Female ICR Mice

The role of non-protein sulfhydryl concentrations in the susceptibility of male and female mice to $CHCl_3$ nephrotoxicity was evaluated in mice pretreated with diethyl maleate to reduce renal glutathione concentrations. Male and female renal cortical non-protein sulfhydryl concentrations were reduced by 66 and 80%, respectively, and by approximately 90% in the liver of both sexes 30 min after an i.p. injection of 0.6 ml diethyl maleate/kg (Table 2). Administration of diethyl maleate 30 min prior to $CHCl_3$ (50 or 500 µl/kg, s.c.) did not alter the susceptibility of female mice to nephrotoxicity but did potentiate nephrotoxicity in male mice (Table 3).

4. Effect of Partial Hepatectomy on $CHCl_3$ Nephrotoxicity in Male ICR Mice

Partial hepatectomy of male mice did not alter the nephrotoxic response to $CHCl_3$ compared to control or sham-operated mice (Figures 13-15). Four hours after the s.c. injection of 250 µl $CHCl_3$/kg, accumulation of PAH and TEA and renal cortical non-protein sulfhydryl concentrations were significantly decreased in all three groups (control, sham-operated and partial hepatectomized) (Figure 13A-C). There was no difference in the nephrotoxic response to $CHCl_3$ of partial hepatectomized mice compared to control. Since 250 µl $CHCl_3$/kg, s.c., produced a maximal nephrotoxic response in the intact mouse (Figure 5), the effect of partial hepatectomy on the nephrotoxic effect of two lower doses of $CHCl_3$ was assessed. Four hours after 50 µl $CHCl_3$/kg, s.c., accumulation of PAH and TEA was significantly decreased in all three groups, but again there was no

TABLE 2

Effect of Diethyl Maleate on Renal Cortical and Hepatic Nonprotein Sulfhydryl Concentrations[a]

Organ	Sex	Pretreatment		% Decrease From Control
		Peanut Oil	Diethyl Maleate	
		NPSH concentration, μmol/g tissue		
Liver	Male	9.88±1.30	1.06±0.54	89%
	Female	8.13±0.58	0.66±0.34	92%
Kidney	Male	5.16±0.35	1.77±0.19	66%
	Female	3.56±0.30	0.72±0.26	80%

[a]Peanut oil or 0.6 ml/kg diethyl maleate dissolved in peanut oil was administered by i.p. injection (5 ml/kg) to male and female ICR mice. Mice were killed 30 min after injection and renal cortical and hepatic nonprotein sulfhydryl (NPSH) concentrations were determined as described in METHODS. Values are mean ± S.E.M., n=4.

TABLE 3

Effect of Diethyl Maleate on Chloroform-Induced Nephrotoxicity in Male and Female Mice[a]

$CHCl_3$ (μl/kg)	Male		Female	
	Control	Diethyl Maleate	Control	Diethyl Maleate
	BUN (mg/dl)			
0	27± 1	24± 3	29±4	23±2
50	61±13	136±34[b]	27±2	24±1
500	95±12[b]	187±34[b]	28±1	24±2

[a]Male and female ICR mice were injected with 0.6 ml/kg diethyl maleate dissolved in peanut oil or with vehicle (5 ml/kg, i.p.). Chloroform (50 or 500 μl/kg) or peanut oil was injected subcutaneously (2 ml/kg) 30 min after diethyl maleate. Mice were killed 24 hr later and blood urea nitrogen (BUN) concentrations were determined as described in METHODS. Values are mean ± S.E.M., n=4 mice.

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

Figure 13. Effect of partial hepatectomy on $CHCl_3$ nephrotoxicity in male ICR mice. $CHCl_3$ (250 µl/kg) or vehicle (peanut oil, P.O.) was administered s.c. (2 ml/kg) to control, sham-operated, or partially hepatectomized male mice. Surgical procedures are described in METHODS. (A) Renal cortical p-aminohippurate accumulation *in vitro* (PAH S/M), (B) renal cortical tetraethylammonium accumulation *in vitro* (TEA S/M), (C) renal cortical non-protein sulfhydryl (NPSH) concentration, (D) blood urea nitrogen (BUN), (E) serum glutamic pyruvic transaminase (SGPT) activity, and (F) hepatic NPSH concentrations were measured 4 hr after $CHCl_3$ injection. Values are mean ± S.E.M., n=3. * Significantly different from mice in the same treatment group not receiving $CHCl_3$, $p<0.05$. *Significantly different from control mice receiving the same dose of $CHCl_3$, $p<0.05$.

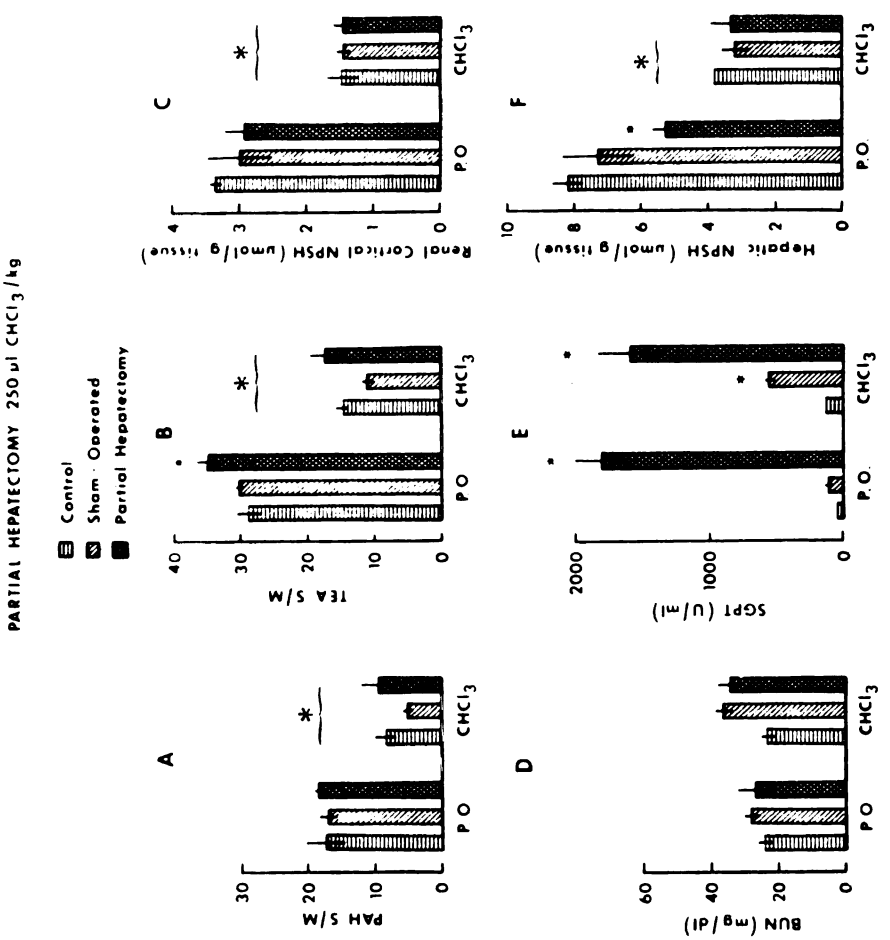

PARTIAL HEPATECTOMY 250 µl $CHCl_3$/kg
Control
Sham - Operated
Partial Hepatectomy
A
PAH S/M
B
TEA S/M
C
Renal Cortical NPSH (µmol/g tissue)
D
BUN (mg/dl)
E
SGPT (U/ml)
F
Hepatic NPSH (µmol/g tissue)
P.O.
$CHCl_3$

Figure 13

difference in the degree of nephrotoxicity in partially hepatectomized mice (Figure 14). Decreasing the $CHCl_3$ dose to 10 µl/kg did not produce nephrotoxicity within the 4 hr period (Figure 15).

There was no increase of BUN by 4 hr after $CHCl_3$ administration, consistent with the data from the time course study (Figure 11). SGPT was increased after 4 hr in the sham-operated and partially hepatectomized mice. This increase probably reflects damage to the liver during the surgical procedure since there were increases of SGPT in mice not receiving $CHCl_3$. The decrease of hepatic non-protein sulfhydryl concentrations in mice receiving 250 µl $CHCl_3$/kg probably is the result of $CHCl_3$ administration.

5. Effect of Sex Hormone Status on $CHCl_3$ Toxicity and Renal and Hepatic Mixed Function Oxidases in ICR Mice

a. Mixed function oxidase activity

Concentrations of cytochromes P-450 and b5 and ethoxycoumarin-O-deethylase activity were greater in male than in female mouse kidney microsomes (Table 4). Treatment of male and female mice with testosterone increased these mixed function oxidase components to similar activities; whereas castration of male mice reduced renal mixed function oxidase activity to that observed in untreated female mouse kidneys (Tables 4 and 5). Altering testosterone concentrations in mice did not affect renal NADPH cytochrome-c reductase or ethoxycoumarin-O-deethylase activities, nor were there any alterations of hepatic cytochrome content or hepatic mixed function oxidase activities (Tables 4 and 5).

Figure 14. Effect of partial hepatectomy on $CHCl_3$ nephrotoxicity in male ICR mice. $CHCl_3$ (50 µl/kg) or vehicle (peanut oil, P.O.) was administered s.c. (2 ml/kg) to control, sham-operated, or partially hepatectomized male mice. Surgical procedures are described in METHODS. (A) Renal cortical p-aminohippurate accumulation *in vitro* (PAH S/M), (B) renal cortical tetraethylammonium accumulation *in vitro* (TEA S/M), (C) renal cortical non-protein sulfhydryl (NPSH) concentration, (D) blood urea nitrogen (BUN), (E) serum glutamic pyruvic transaminase (SGPT) activity, and (F) hepatic NPSH concentrations were measured 4 hr after $CHCl_3$ injection. Values are mean ± S.E.M., n=4. *Significantly different from mice in the same treatment group not receiving $CHCl_3$, $p<0.05$. *Significantly different from control mice receiving the same dose of $CHCl_3$, $p<0.05$.

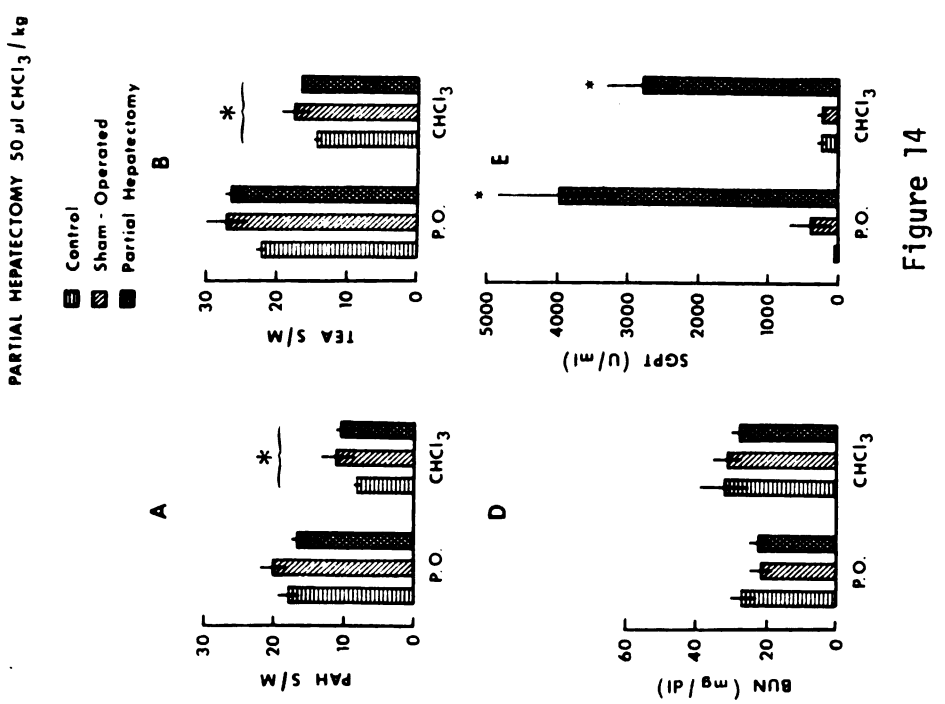

PARTIAL HEPATECTOMY 50 µl $CHCl_3$ / kg
Control
Sham - Operated
Partial Hepatectomy
A
PAH S/M
B
TEA S/M
C
Renal Cortical NPSH (µmol/g tissue)
D
BUN (mg/dl)
E
SGPT (U/ml)
F
Hepatic NPSH (µmol/g tissue)
P.O.
$CHCl_3$
*

Figure 14

Figure 15. Effect of partial hepatectomy on $CHCl_3$ nephrotoxicity in male ICR mice. $CHCl_3$ (10 µl/kg) or vehicle (peanut oil, P.O.) was administered s.c. (2 ml/kg) to control, sham-operated, or partially hepatectomized male mice. Surgical procedures are described in METHODS. (A) Renal cortical p-aminohippurate accumulation in vitro (PAH S/M), (B) renal cortical non-protein sulfhydryl (NPSH) concentration, (C) blood urea nitrogen (BUN), (D) serum glutamic pyruvic transaminase (SGPT) activity, and (E) hepatic NPSH concentrations were measured 4 hr after $CHCl_3$ injection. Values are mean ± S.E.M., n=3. *Significantly different from mice in the same treatment group not receiving $CHCl_3$, $p<0.05$. *Significantly different from control mice receiving the same dose of $CHCl_3$, $p<0.05$.

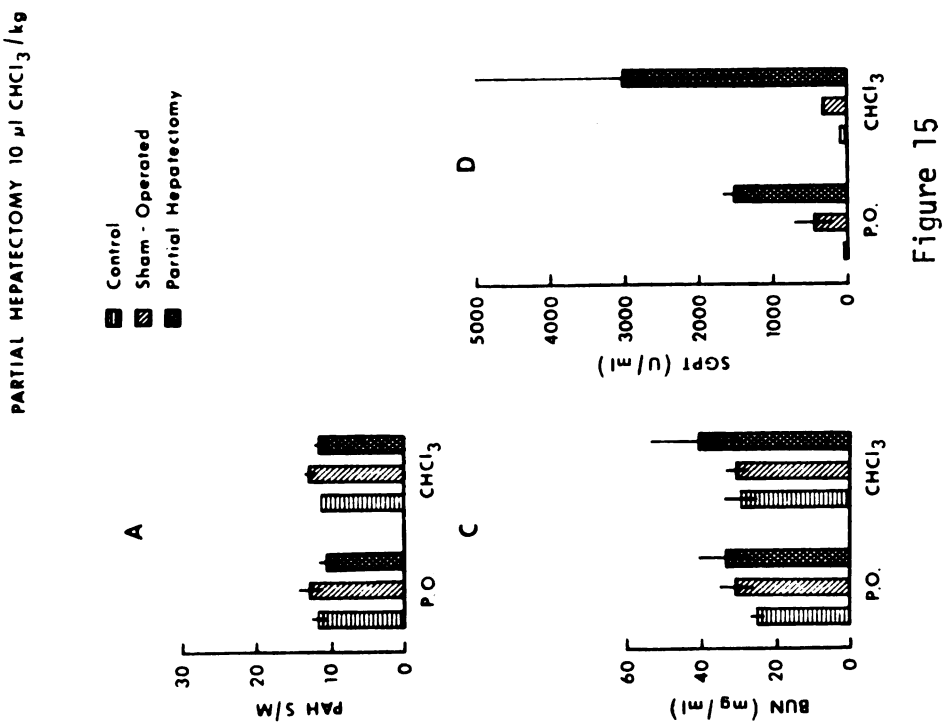

PARTIAL HEPATECTOMY 10 µl $CHCl_3$ / kg
Control
Sham - Operated
Partial Hepatectomy
A
PAH S/M
P.O
$CHCl_3$
B
Renal cortical NPSH (µmol / g tissue)
C
BUN (mg / ml)
D
SGPT (U / ml)
E
Hepatic NPSH (µmol / g tissue)

Figure 15

TABLE 4

Effect of Testosterone Pretreatment on Renal and Hepatic Cytochrome Content and Mixed Function Oxidases in Mice[a]

Treatment	Cytochrome P-450	Cytochrome b5	NADPH Cytochrome-c Reductase	Ethoxycoumarin O-Deethylase	Ethoxyresorufin O-Deethylase
	(nmol/mg protein)		(nmol/min/mg protein)	(nmol/min/mg protein)	
			RENAL		
Female	0.06±0.01	0.15±0.02[b]	33.3± 7.1	0.029±0.005[b]	0.004±0.001
Male	0.29±0.06[b]	0.29±0.04[b]	32.3± 3.9	0.127±0.020[b]	0.012±0.005
Female, Testosterone	0.40±0.08[b]	0.32±0.05[b]	46.2± 4.7	0.179±0.026[b]	0.008±0.003
Male, Testosterone	0.41±0.08[b]	0.34±0.06[b]	53.8± 5.2	0.207±0.017[b,c]	0.009±0.002
			HEPATIC		
Female	1.14±0.11	0.52±0.09	123.3±12.0	1.457±0.437	0.671±0.094
Male	1.20±0.12	0.46±0.06	101.3± 9.8	1.429±0.427	0.752±0.114
Female, Testosterone	1.24±0.15	0.44±0.04	107.9±11.2	1.455±0.529	0.592±0.08
Male, Testosterone	1.27±0.15	0.43±0.04	90.5± 5.6	1.324±0.443	0.605±0.089

[a]Female and male ICR mice pretreatment groups were injected s.c. for 3 weeks on alternate days with 0.1 ml of testosterone (1 mg) or peanut oil. Kidneys and livers from 15 mice were pooled for female peanut oil-treated mice and from 10 mice for the other 3 treatment groups. Values are mean ± S.E.M., n=4.

[b]Significantly different from female peanut oil-treated mice, $p<0.05$.

[c]Significantly different from male peanut oil-treated mice, $p<0.05$.

TABLE 5

Effect of Castration on Renal and Hepatic Cytochrome Content and Mixed Function Oxidases in Mice[a]

Treatment	Cytochrome P-450	Cytochrome b5	NADPH Cytochrome-c Reductase	Ethoxycoumarin O-Deethylase	Ethoxyresorufin O-Deethylase
	(nmol/mg protein)		(nmol/min/mg protein)	(nmol/min/mg protein)	
			RENAL		
Control	0.24±0.06	0.19±0.01	30.6±4.4	0.098±0.006	0.012±0.001
Sham	0.27±0.04	0.25±0.06	36.9±6.7	0.111±0.010	0.012±0.001
Castrate	0.05±0.01[b]	0.13±0.02	32.7±1.4	0.031±0.003[b]	0.008±0.002
			HEPATIC		
Control	1.14±0.04	0.41±0.01	83.6±8.8	0.798±0.059	0.854±0.139
Sham	1.13±0.06	0.40±0.03	97.6±5.8	0.861±0.166	0.862±0.090
Castrate	0.98±0.05	0.47±0.02	107.4±5.4	0.779±0.070	0.589±0.040

[a]Male ICR mice were castrated, or underwent sham surgery, or were untreated (control) as described in METHODS. After a 3 week surgical recovery period, kidneys and livers from 10 mice were pooled for control and sham treatment groups and from 15 mice for castrate treatment groups. Values are mean ± S.E.M., n=4.

[b]Significantly different from control and sham-operated male mice, $p<0.05$.

b. $CHCl_3$ toxicity

Kidney weight to body weight ratios (KW/BW) were greater in male than in female mice (Table 6). Testosterone treatment of male and female mice increased the KW/BW to greater than that of peanut oil-treated male mice, but had no significant effect on liver weight to body weight ratios (LW/BW). Castration reduced the KW/BW below that seen in sham-operated and control male mice (Table 7) to a value similar to that occurring in untreated female mice (Table 6). The change of KW/BW was the result of changes in the actual kidney weight.

Female and male mice treated with testosterone displayed a greater degree of nephrotoxicity to a given dose of $CHCl_3$ than did peanut oil-treated male mice; female mice were not susceptible to the nephrotoxicity (Figure 16; Tables 6 and 8). $CHCl_3$ nephrotoxicity was observed in susceptible mice 24 hr after a subcutaneous injection of $CHCl_3$ at doses of 50 or 500 µl/kg. There was a significant increase of KW/BW and BUN (Table 6; Figure 16C), a decreased ability of renal cortical slices to accumulate PAH and TEA (Figures 16A and B) and decreased renal cortical non-protein sulfhydryl concentrations (Table 8). Castration of male mice completely prevented the nephrotoxic effects of $CHCl_3$; this treatment group resembled female mice in their lack of susceptibility to $CHCl_3$ nephrotoxicity (Figure 17, Tables 7 and 9).

The effects of testosterone on $CHCl_3$ hepatotoxicity were not as clear. Testosterone treatment appeared to increase the degree of hepatotoxicity, particularly at the lower dose of $CHCl_3$

TABLE 6

Effect of Testosterone Pretreatment and $CHCl_3$ on Organ Weight/body Weight Ratios[a]

Treatment	Organ Weight/Body Weight x 100		
	$CHCl_3$ (µl/kg)		
	0	50	500
		RENAL	
Female	1.13±0.03[b]	1.18±0.04[b,c]	1.12±0.04[b,c]
Male	1.44±0.08[b]	1.60±0.08[b,c]	1.84±0.10[b,c]
Female, Testosterone	1.92±0.10[d]	2.44±0.07[c,d]	2.52±0.02[c,d]
Male, Testosterone	1.84±0.07[d]	2.45±0.08[c,d]	2.53±0.15[c,d]
		HEPATIC	
Female	4.32±0.13	4.97±0.21	5.17±0.03
Male	4.98±0.24	5.32±0.54	4.87±0.27
Female, Testosterone	5.22±0.39	4.68±0.55	4.46±0.08
Male, Testosterone	5.38±0.37	5.34±0.27	4.30±0.11

[a] $CHCl_3$ (50 or 500 µl/kg) or vehicle (peanut oil) was administered s.c. (2 ml/kg) to pretreated male and female ICR mice injected s.c. for 3 weeks on alternate days with 0.1 ml of testosterone (1 mg) or peanut oil. Organ weight to body weight ratios were determined 24 hr after $CHCl_3$ injections. Values are mean ± S.E.M., n=4.

[b] Significantly different from female mice with the same pretreatment, $p<0.05$.

[c] Significantly different from mice within the same pretreatment group receiving 0 µl $CHCl_3$/kg, $p<0.05$.

[d] Significantly different from mice of the same sex without testosterone pretreatment, $p<0.05$.

TABLE 7

Effect of Castration and $CHCl_3$ on Organ Weight/Body Weight Ratios[a]

Treatment	Organ Weight/Body Weight x 100		
	$CHCl_3$ (µl/kg)		
	0	50	500
		RENAL	
Control	1.50±0.11	1.75±0.12	1.73±0.04
Sham	1.61±0.14	1.83±0.17	1.76±0.12
Castrate	1.07±0.03[b]	1.22±0.06[b]	1.18±0.04[b]
		HEPATIC	
Control	5.61±0.51	4.81±0.38	4.77±0.19
Sham	5.41±0.49	5.67±0.16	4.78±0.23
Castrate	5.40±0.16	5.24±0.19	5.13±0.19

[a]Male ICR mice were castrated, or underwent sham surgery, or were untreated (control) as described in METHODS. $CHCl_3$ (50 or 500 µl/kg) or vehicle (peanut oil) was administered s.c. (2 ml/kg) after a 3 week surgical recovery period. Organ weight to body weight ratios were determined 24 hr after $CHCl_3$ injections. Values are mean ± S.E.M., n=4.

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

Figure 16. Effect of testosterone on renal and hepatic toxicity to $CHCl_3$ in male and female ICR mice. $CHCl_3$ (50 or 500 µl/kg) or vehicle (peanut oil) was administered s.c. (2 ml/kg) to pretreated male and female mice injected s.c. for 3 weeks on alternate days with 0.1 ml of testosterone (1 mg) or peanut oil. (A) Renal cortical p-aminohippurate accumulation in vitro (PAH S/M), (B) renal cortical tetraethylammonium accumulation in vitro (TEA S/M), (C) blood urea nitrogen (BUN), and (D) serum glutamic pyruvic transaminase (SGPT) activity were measured 24 hr after $CHCl_3$ injections. Values are mean ± S.E.M., n=4. *Significantly different from mice of the same sex pretreated with testosterone, $p<0.05$.

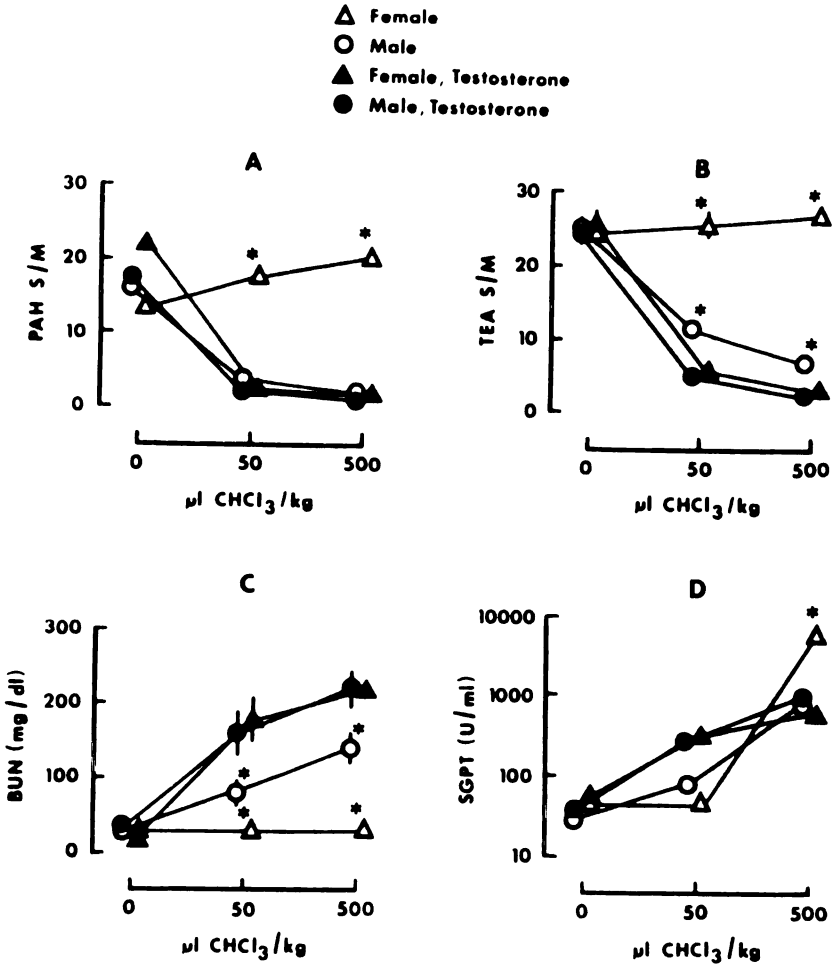

Female
Male
Female, Testosterone
Male, Testosterone
A
PAH S/M
30
20
10
0
0
50
500
µl CHCl3/kg
B
TEA S/M
30
20
10
0
0
50
500
µl CHCl3/kg
C
BUN (mg/dl)
300
200
100
0
0
50
500
µl CHCl3/kg
D
SGPT (U/ml)
10000
1000
100
10
0
50
500
µl CHCl3/kg

Figure 16

TABLE 8

Effect of Testosterone Pretreatment and $CHCl_3$ on Renal and Hepatic Non-Protein Sulfhydryl Concentrations[a]

Treatment	Non-Protein Sulfhydryl Concentration (μmol/g tissue)		
	$CHCl_3$ (μl/kg) 0	50	500
		RENAL	
Female	4.86±0.24	5.47±0.19[b,c]	5.13±0.27[b,c]
Male	5.71±0.18	2.50±0.32[c,d]	1.82±0.31[c,d]
Female, Testosterone	6.50±0.20	2.26±0.42[c]	1.60±0.04[c]
Male, Testosterone	6.88±0.40	2.04±0.30	1.53±0.25
		HEPATIC	
Female	10.19±0.57	11.60±0.64	10.51±1.33
Male	9.04±1.22	8.48±1.20[d]	8.69±1.08[c,d]
Female, Testosterone	10.45±0.76	5.87±1.28	4.31±0.57[c,d]
Male, Testosterone	10.00±1.33	7.45±0.22	5.20±1.34[c,d]

[a] $CHCl_3$ (50 or 500 μl/kg) or vehicle (peanut oil) was administered s.c. (2 ml/kg) to pretreated male and female ICR mice injected s.c. for 3 weeks on alternate days with 0.1 ml of testosterone (1 mg) or peanut oil. Organ weight to body weight ratios were determined 24 hr after $CHCl_3$ injections. Values are mean ± S.E.M., n=4.

[b] Significantly different from female mice with the same pretreatment, $p<0.05$.

[c] Significantly different from mice within the same pretreatment group receiving 0 μl $CHCl_3$/kg, $p<0.05$.

[d] Significantly different from mice of the same sex without testosterone pretreatment, $p<0.05$.

Figure 17. Effect of castration on renal and hepatic toxicity to $CHCl_3$ in male mice. Male ICR mice were castrated, or underwent sham surgery, or were untreated (control), as described in METHODS. $CHCl_3$ (50 or 500 µl/kg) or vehicle (peanut oil) was administered s.c. (2 ml/kg) after a 3 week surgical recovery period. (A) Renal cortical p-aminohippurate accumulation *in vitro* (PAH S/M), (B) renal cortical tetraethylammonium accumulation *in vitro* (TEA S/M), (C) blood urea nitrogen (BUN), and (D) serum glutamic pyruvic transaminase (SGPT) activity were measured 24 hr after $CHCl_3$ injections. Values are mean ± S.E.M., n=4). *Significantly different from control and sham-operated male mice, $p<0.05$.

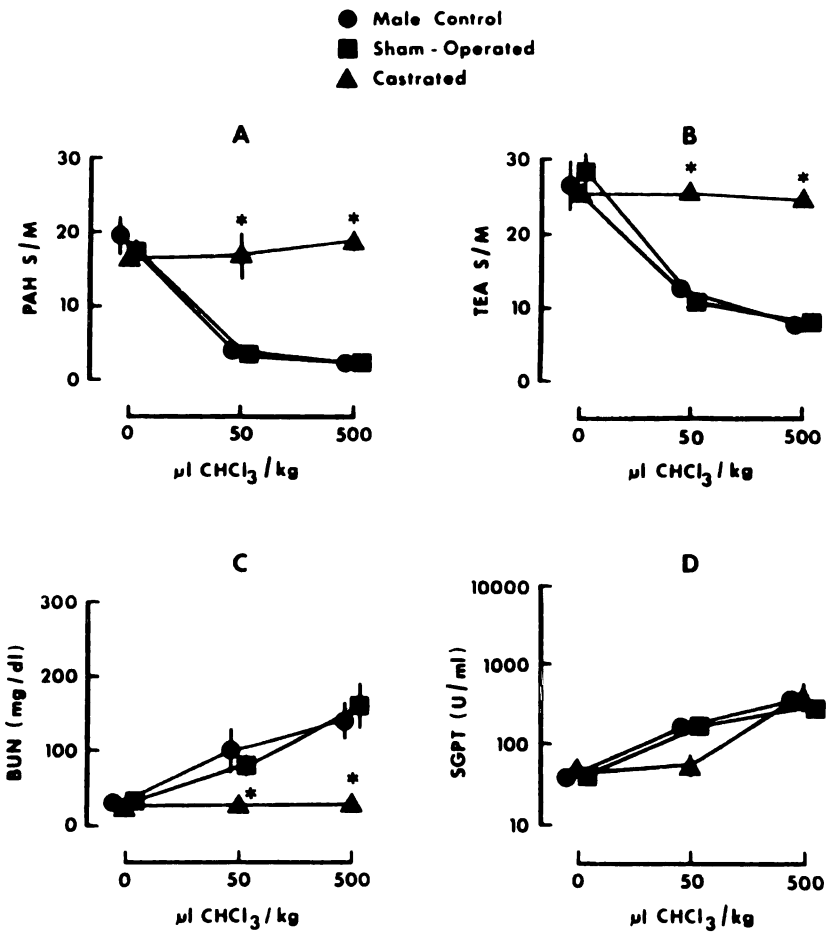

Male Control
Sham - Operated
Castrated
A
B
C
D
PAH S/M
TEA S/M
BUN (mg/dl)
SGPT (U/ml)
30
20
10
0
300
200
100
10000
1000
50
500
µl CHCl3/kg
*

Figure 17

TABLE 9

Effect of Castration and $CHCl_3$ on Renal and Hepatic Non-Protein Sulfhydryl Concentrations[a]

Treatment	Non-Protein Sulfhydryl Concentration (μmol/g tissue)		
	$CHCl_3$ (μl/kg) 0	50	500
		RENAL	
Control	6.63±0.050	3.93±0.19[b]	2.48±0.19[b]
Sham	7.16±0.062	3.65±0.28[b]	2.33±0.07[b]
Castrate	5.95±0.068	5.72±1.12[c]	6.39±0.62[c]
		HEPATIC	
Control	14.30±2.78	9.17±1.64	9.68±1.38
Sham	14.11±2.06	10.28±1.77	8.29±1.59
Castrate	8.45±2.61	14.41±1.36	12.94±2.28

[a]Male ICR mice were castrated, or underwent sham surgery, or were untreated (control) as described in METHODS. $CHCl_3$ (50 or 500 μl/kg) or vehicle (peanut oil) was administered s.c. (2 ml/kg) after a 3 week surgical recovery period. NPSH concentrations were determined 24 hr after $CHCl_3$ injection in hepatic and renal cortical tissue. Values are mean ± S.E.M., n=3.

[b]Significantly different from mice within the same pretreatment group receiving 0 μl $CHCl_3$/kg, $p<0.05$.

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

where there was a trend for SGPT to be increased to a greater extent than in peanut oil-treated male or female mice (Figure 16D) and for hepatic non-protein sulfhydryl concentrations to remain decreased 24 hr after exposure to $CHCl_3$ (Table 8).

c. Histology

Testosterone treatment of female mice produced dramatic morphological changes in the kidney and in the renal response to $CHCl_3$. Testosterone increased the size of proximal tubular cells in the outer cortex but not in the cortico-medullary region (Figure 18). After testosterone treatment of female mice the extent and degree of renal cortical necrosis produced by $CHCl_3$ was similar in severity to lesions in male mice (Figure 19). The marked hypertrophy of renal cortical cells and the increased severity of histological lesions were not detected in kidneys of male mice treated with testosterone, though KW/BW and the biochemical indices of nephrotoxicity suggested there may have been some hypertrophy of male mouse kidney after testosterone treatment (Figure 16, Tables 6 and 8). No renal lesions were detected in castrated male mice at either dose of $CHCl_3$ (Figure 20). No reduction of proximal tubular cell size was observed in castrated male mice.

Castration of male mice did not affect the hepatic lesions produced by $CHCl_3$. The degree of hepatic lesions was greater in untreated female than in male mice or in testosterone-treated female mice.

Figure 18. Sex differences in ICR mice renal proximal tubule morphology. Male and female mice were injected s.c. for 3 weeks on alternate days with 0.1 ml of testosterone (1 mg) or peanut oil. (A) Male. (B) Female. (C) Female treated with testosterone; increased size of proximal tubular cells in outer cortex. Hematoxylin and eosin stain, x100.

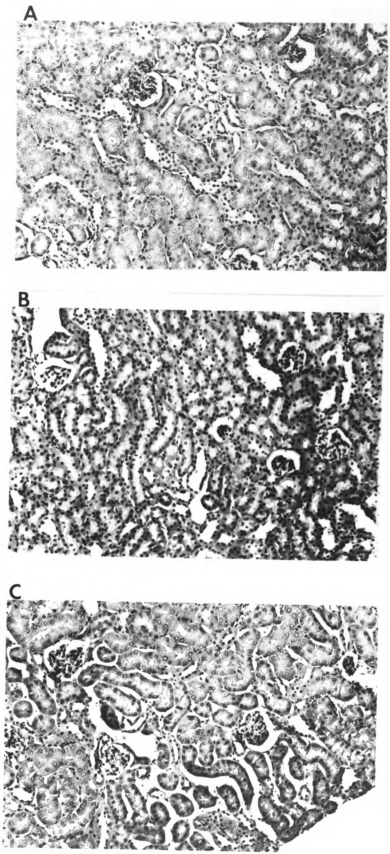


Figure 18

Figure 19. Effect of testosterone on susceptibility of female ICR mice to $CHCl_3$ nephrotoxicity. $CHCl_3$ (500 µl/kg) or vehicle (peanut oil) was administered s.c. (2 ml/kg) to pretreated female mice injected s.c. for 3 weeks on alternate days with 0.1 ml of testosterone (1 mg) or peanut oil. Mice were killed 24 hr after $CHCl_3$ injections for histological examination. (A) Female pretreated with peanut oil 24 hr after 500 µl $CHCl_3$/kg, no histopathological alterations occurred after $CHCl_3$. (B) Female pretreated with testosterone 24 hr after 500 µl $CHCl_3$/kg; extensive proximal tubular necrosis. Hematoxylin and eosin stain, x100.

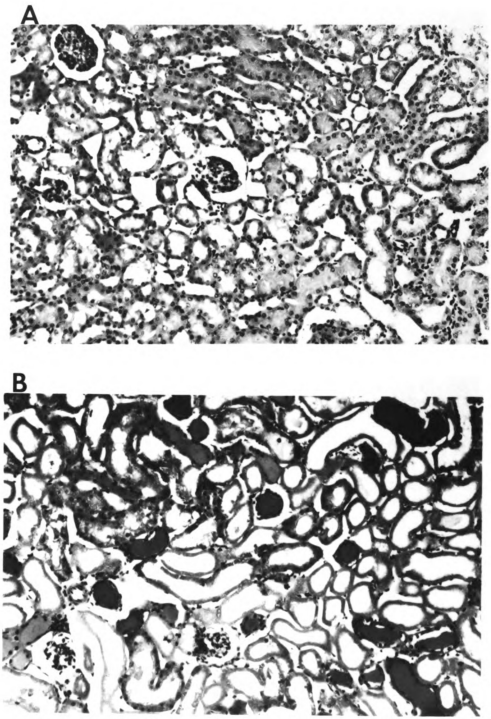


Figure 19

Figure 20. Effect of castration on susceptibility of male ICR mice to $CHCl_3$ nephrotoxicity. $CHCl_3$ (500 µl/kg) or vehicle (peanut oil) was administered s.c. (2 ml/kg) to male mice which were castrated, or underwent sham surgery, or were untreated (control) as described in METHODS. Mice were killed 24 hr after $CHCl_3$ injections for histological examination. (A) Control male 24 hr after 500 µl $CHCl_3$/kg; extensive proximal tubular necrosis. (B) Castrated male 24 hr after 500 µl $CHCl_3$/kg; no histopathological alterations occurred after $CHCl_3$. Hematoxylin and eosin stain, x100.

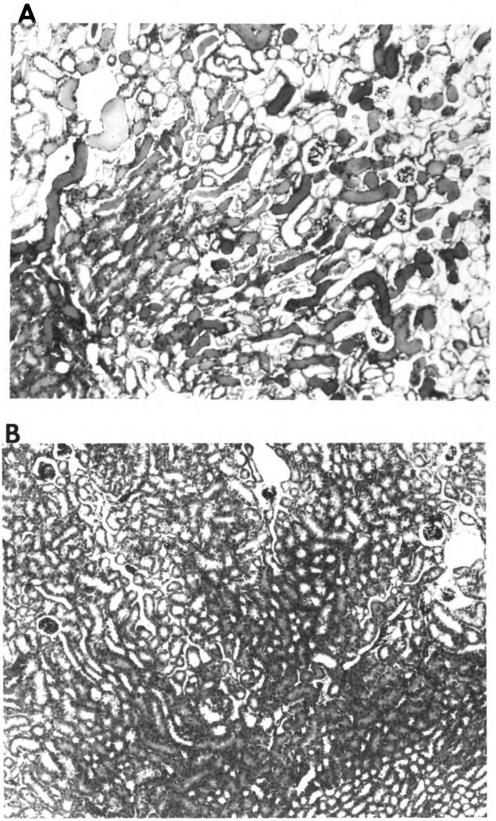


Figure 20

B. In Vitro Studies - Toxicity of $CHCl_3$ in Renal Cortical Slices

1. Assessment of $CHCl_3$ Nephrotoxicity In Vitro in ICR Mouse Renal Cortical Slices

Preincubation of renal cortical slices from male and female mice with 0.5 µl (≈6.25 µmol) $CHCl_3$ for up to 2 hr produced decreases in organic ion accumulation in male kidney slices only (Figure 21). TEA S/M ratios were decreased after 60 min of preincubation while the decrease of PAH S/M ratios was not observed until 90 min. The effect on organic ion accumulation was near maximal after a 2 hr incubation at this concentration of $CHCl_3$.

Preincubation with up to 4 µl (≈50 µmol) $CHCl_3$ for 90 min in vitro produced a concentration-related decrease in the ability of slices from males to accumulate PAH and TEA, while slices from female mice were only affected at the highest $CHCl_3$ concentrations (Figure 22). $CHCl_3$ (4 µl/ ≈50 µmol) reduced the PAH and TEA S/M ratios in slices from female mice less than the reduction in slices from male mice incubated with only 0.25 µl (≈3.1 µmol) $CHCl_3$.

Organic ion accumulation was decreased less after preincubation of male kidney slices with $CDCl_3$ (1 µl; ≈12.5 µmol) than with an equimolar concentration of $CHCl_3$ (Figure 23).

Preincubation of slices with $CHCl_3$ under an atmosphere of carbon monoxide:oxygen (80:20) or at 0°C prevented the $CHCl_3$-induced decrease of PAH and TEA accumulation (Figures 24 and 25). Carbon monoxide alone decreased the PAH S/M (15.1 versus 10.3), but there was no further decrease after the addition of $CHCl_3$. In general, the accumulation of TEA appeared to be less sensitive to alterations of

Figure 21. Effect of preincubation with 0.5 μl $CHCl_3$ for 30 to 120 min on PAH and TEA accumulation by renal cortical slices (100±10 mg) prepared from male or female ICR mice. Control slices were preincubated for 90 min. Preincubation and the assessment of PAH and TEA accumulation were conducted as described in METHODS. Values are mean ± S.E.M., n=4.

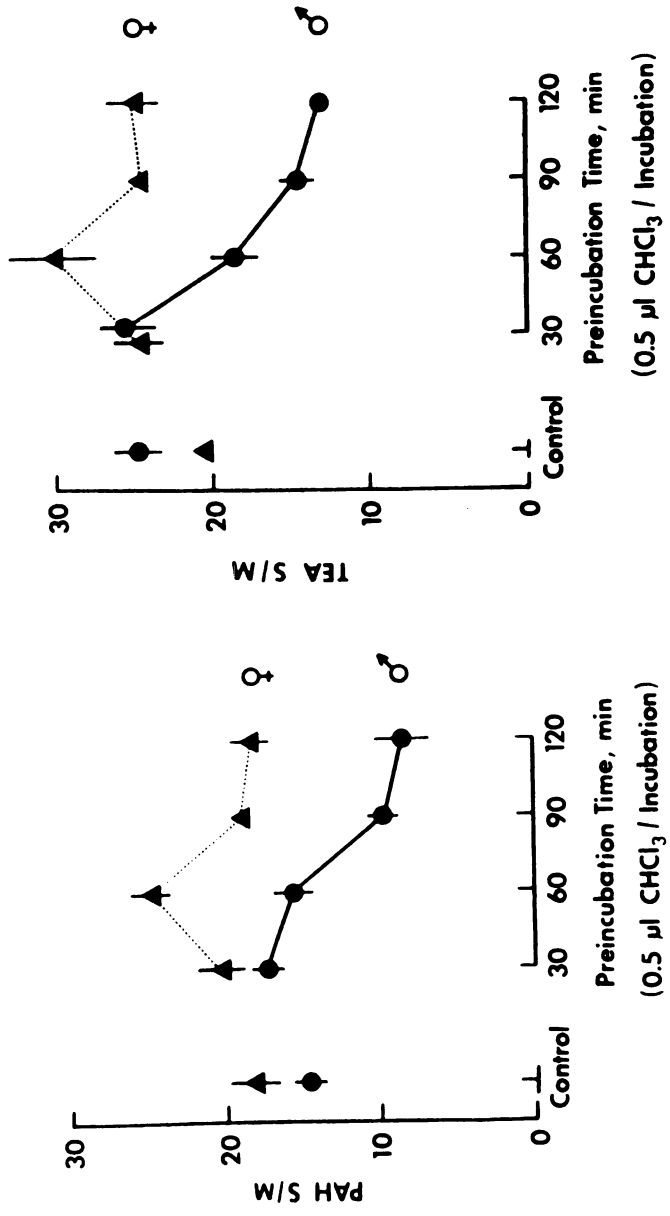

TEA S/M
PAH S/M
30
20
10
0
Control
30
60
90
120
Preincubation Time, min
(0.5 µl $CHCl_3$ / Incubation)
♀
♂

Figure 21

Figure 22. Effect of preincubation with $CHCl_3$ concentrations ranging from approximately 0-50 μmol (0-4 μl) on PAH and TEA accumulation by renal cortical slices prepared from male or female ICR mice. Slices (100±10 mg) were placed in a 25 ml Erlenmeyer flask containing 4 ml of medium and gassed for 5 min with O_2:CO_2 (95:5). $CHCl_3$ was added with a 5 μl microsyringe. The flasks were stoppered and incubated at 37°C for 90 min in a Dubnoff metabolic shaker. After preincubation, the assessment of PAH and TEA accumulation was conducted as described in METHODS. Values are mean ± S.E.M., n=4.

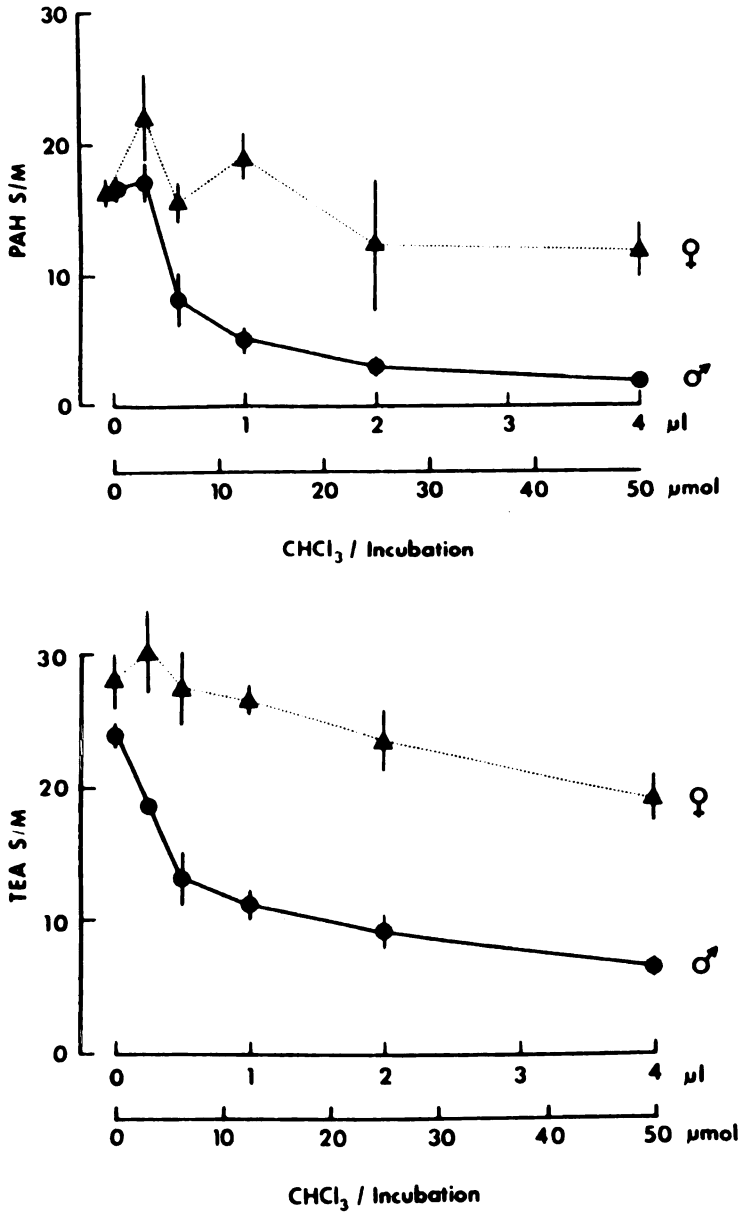

30
20
10
0
PAH S/M
♀
♂
0 1 2 3 4 µl
0 10 20 30 40 50 µmol
CHCl3 / Incubation
30
20
10
0
TEA S/M
♀
♂
0 1 2 3 4 µl
0 10 20 30 40 50 µmol
CHCl3 / Incubation

Figure 22

Figure 23. Effect of deuterium on toxicity of $CHCl_3$ *in vitro* in male ICR mouse kidney slices. Slices (100±10 mg) were preincubated with 1 µl $CDCl_3$ and assessed for PAH and TEA accumulation as described in METHODS. Values are mean ± S.E.M., n=8. *Significantly different from control slices, $p<0.05$. *Significantly different from slices preincubated with $CHCl_3$, $p<0.05$.

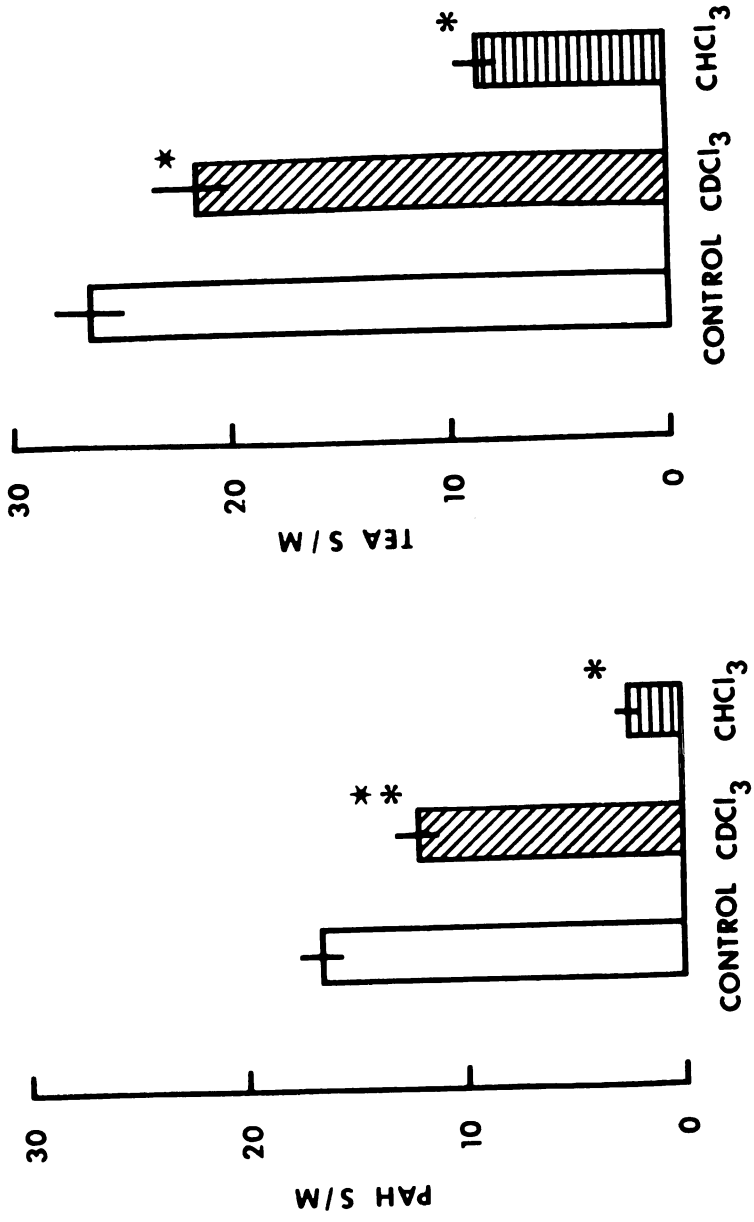
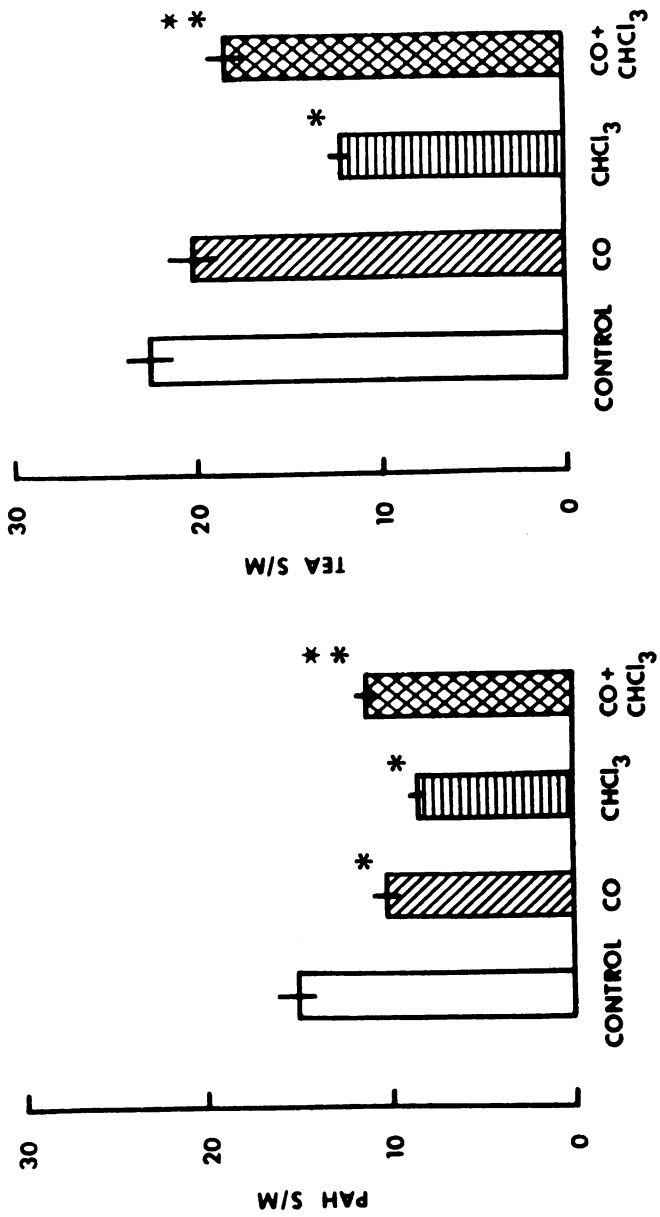

TEA S/M
30
20
10
0
CONTROL
$CDCl_3$
$CHCl_3$
*
*
PAH S/M
30
20
10
0
CONTROL
$CDCl_3$
$CHCl_3$
**
*

Figure 23

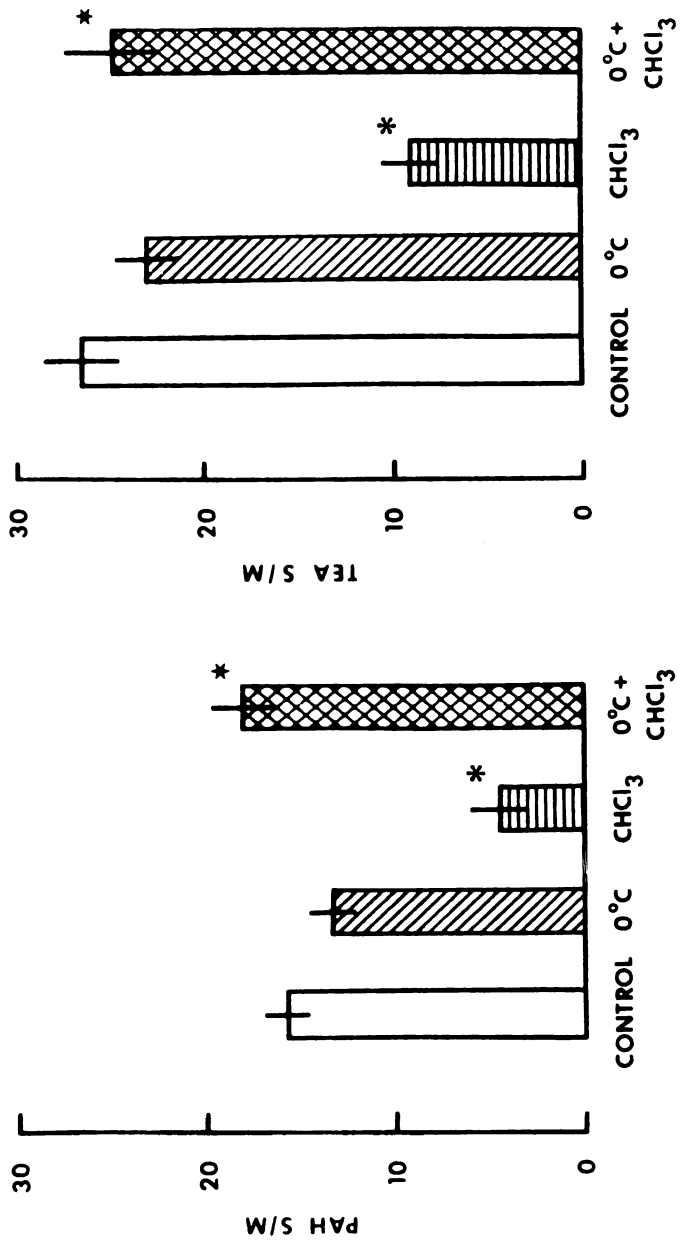
Figure 24. Effect of carbon monoxide (CO) on toxicity of $CHCl_3$ *in vitro* in male ICR mouse kidney slices. Preincubation of slices with $CHCl_3$ (1 µl) was conducted as described in METHODS with the exception that CONTROL and $CHCl_3$ flasks were gassed for 5 min with O_2 and CO and CO + $CHCl_3$ flasks were gassed for 5 min with $CO:O_2$ (80:20). Assessment of PAH and TEA accumulation was conducted as described in METHODS. Values are mean ± S.E.M., n=4. *Significantly different from control, $p<0.05$. *Significantly different from $CHCl_3$, $p<0.05$.



$CHCl_3$ - 1.0 µl (12.5 µmol)
CO - CO : O_2 (80 : 20)

Figure 24

Figure 25. Effect of 0°C incubation temperature on toxicity of $CHCl_3$ *in vitro* in male ICR mouse kidney slices. Preincubation of slices with $CHCl_3$ (1 µl) was conducted as described in METHODS with the exception that CONTROL and $CHCl_3$ flasks were preincubated at 37°C and 0°C and 0° + $CHCl_3$ flasks were preincubated in an ice bath for 90 min. Assessment of PAH and TEA accumulation was conducted as described in METHODS. Values are mean ± S.E.M., n=5. *Significantly different from control, $p<0.05$. *Significantly different from $CHCl_3$, $p<0.05$.



$CHCl_3$ - 1.0 µl (12.5 µmol)

Figure 25

incubation conditions than that of PAH. Preincubation of slices under a nitrogen atmosphere resulted in PAH and TEA S/M ratios near 1, indicating there was no active accumulation of organic ions, presumably due to anoxia and cell death (Figure 26).

Pretreatment of mice with diethyl maleate potentiated $CHCl_3$ toxicity in renal cortical slices (Figure 27).

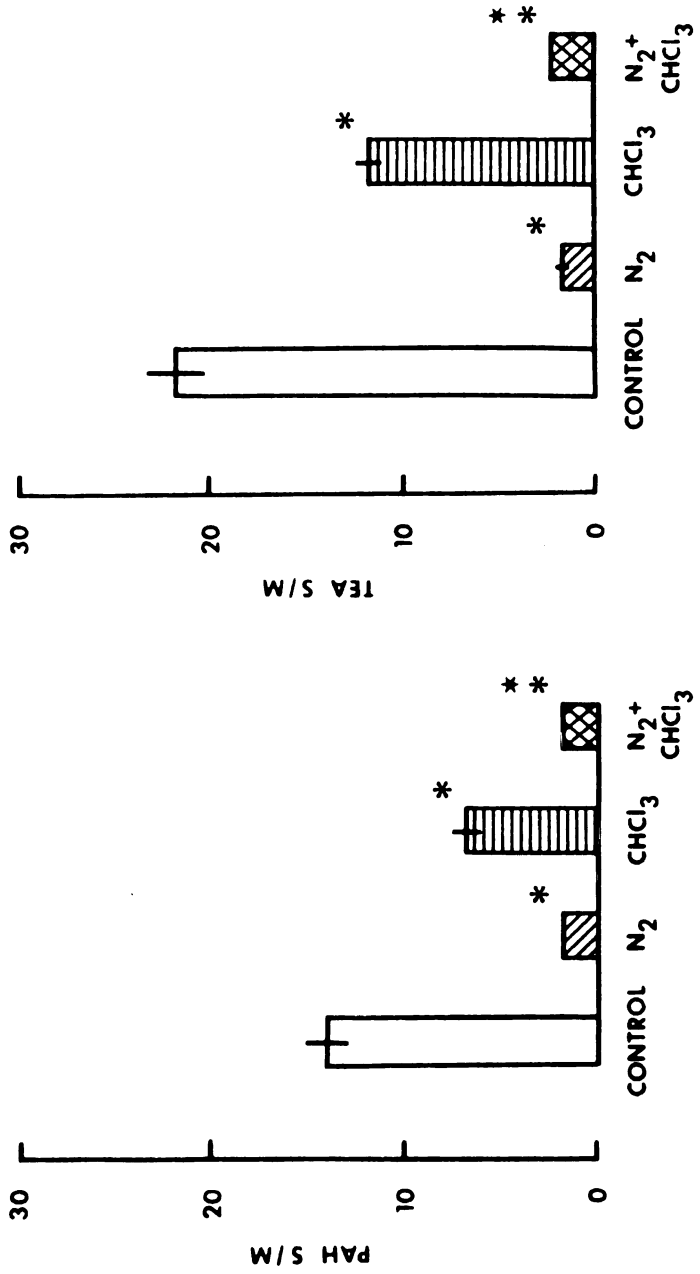
2. Assessment of the Nephrotoxicity of Hepatic $CHCl_3$ Metabolites in Male ICR Mouse Renal Cortical Slices

Preincubation of male mouse renal cortical slices with 6.25 or 12.5 μmol $CHCl_3$ for 90 min at 37°C decreased the ability of slices to accumulate PAH and TEA (Table 10). Preincubation of slices with 6.25 μmol 2-oxothiazolidine-4-carboxylic acid (OTZ) or diglutathionyl dithiocarbonate (GSCOSG) under the same conditions had no effect on PAH and TEA S/M ratios.

3. Effect of Mouse Strain Differences on $CHCl_3$ Toxicity In Vitro

$CHCl_3$ toxicity was assessed in renal cortical slices prepared from three different mouse strains reported to exhibit different susceptibilities to $CHCl_3$ nephrotoxicity *in vivo* (Figure 28). Previous investigations have indicated that male mice of the C57Bl/6 strain are relatively resistant and those of the DBA/2 strain are relatively sensitive to the nephrotoxic effects of $CHCl_3$ (Hill *et al.*, 1975; Hill, 1977; Clemens *et al.*, 1979). Based on the *in vitro* effect of $CHCl_3$ on the ability of renal cortical slices to accumulate PAH and TEA, male ICR mice were most susceptible to nephrotoxicity. Male C57Bl/6 and DBA/2 mice showed similar susceptibilities to $CHCl_3$-induced nephrotoxicity, slightly less than that observed in male ICR mice.

Figure 26. Effect of nitrogen on toxicity of $CHCl_3$ *in vitro* in male ICR mouse kidney slices. Preincubation of slices with $CHCl_3$ (1 µl) was conducted as described in METHODS with the exception that CONTROL and $CHCl_3$ flasks were gassed for 5 min with O_2 and N_2 and N_2 + $CHCl_3$ flasks were gassed for 5 min with 100% N_2. Assessment of PAH and TEA accumulation was conducted as described in METHODS. Values are mean ± S.E.M., n=4. *Significantly different from control, $p<0.05$. *Significantly different from $CHCl_3$, $p<0.05$.



$CHCl_3$ - 1.0 µl (12.5 µmol)

N_2 - 100 % N_2

Figure 26

Figure 27. Effect of diethyl maleate pretreatment *in vivo* on toxicity of $CHCl_3$ *in vitro* in male ICR mouse kidney slices. Preincubation of slices with $CHCl_3$ (0.25 µl) was conducted as described in METHODS with the exception that slices were prepared from male mice pretreated with peanut oil (CONTROL and $CHCl_3$) or 0.6 ml diethyl maleate (5 ml/kg, i.p.) (DEM and DEM + $CHCl_3$) 0.5 hr prior to kill. Assessment of PAH and TEA accumulation was conducted as described in METHODS. Values are mean ± S.E.M., n=4. *Significantly different from control, $p<0.05$. *Significantly different from DEM, $p<0.05$. †Significantly different from $CHCl_3$, $p<0.05$.

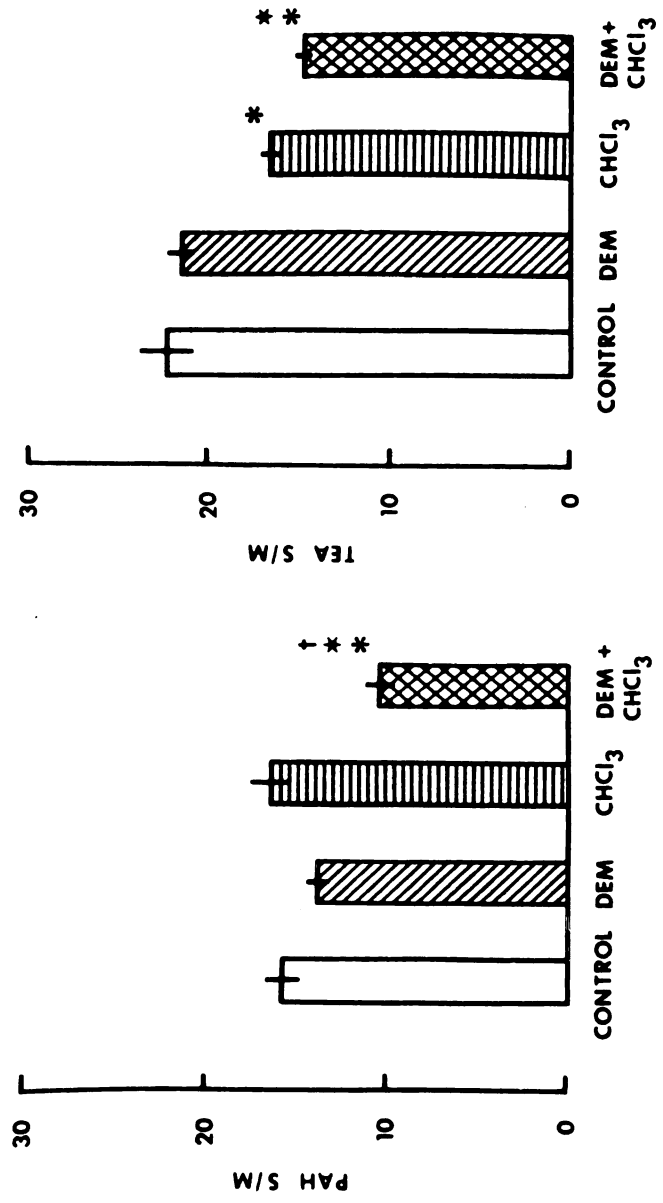

PAH S/M
TEA S/M
0
10
20
30
CONTROL
DEM
CHCl3
DEM + CHCl3
*
**
***
CHCl3 - 0.25 µl (3.12 µmol)
DEM - 0.6 ml/kg

Figure 27

TABLE 10

In Vitro Toxicity of $CHCl_3$ and Hepatic Metabolites in Male Mouse Kidney Slices[a]

	PAH S/M	TEA S/M
Control	15.5±0.1	24.2±0.8
6.25 μmol $CHCl_3$ (0.5 μl)	9.8±0.8*	14.7±0.9*
12.5 μmol $CHCl_3$ (1.0 μl)	7.2±1.4*	13.6±1.5*
6.25 μmol OTZ	16.0±0.8	24.6±1.1
6.25 μmol GSCOSG	18.9±1.6	26.7±1.8

[a]Male ICR mouse renal cortical slices (100±10 mg) were incubated in 2 ml of an isotonic phosphate-buffered medium (pH 7.4) with $CHCl_3$ or its metabolites, 2-oxothiazolidine-4-carboxylic acid (OTZ) or diglutathionyl dithiocarbonate (GSCOSG), for 90 min at 37°C in an oxygenated, sealed 25 ml Erlenmeyer flask. After preincubation, slices were assessed for their ability to accumulate PAH and TEA as described in METHODS. Values are mean ± S.E.M., n=3.

*Significantly different from control, $p<0.05$.

Figure 28. Effect of mouse strain differences on $CHCl_3$ nephrotoxicity *in vitro*. Renal cortical slices were prepared from male or female ICR, male C57BL/6 and male DBA/2 mice. Slices (100±10 mg) were placed in a 25 ml Erlenmeyer flask containing 4 ml of medium and gassed for 5 min with oxygen. $CHCl_3$ was added with a 5 µl microsyringe. The flasks were stoppered and incubated at 37°C for 90 min in a Dubnoff metabolic shaker. After preincubation, the assessment of PAH and TEA accumulation was conducted as described in METHODS. Values are expressed as percent decrease of S/M compared to slices of the same strain with no $CHCl_3$. Values are mean ± S.E.M., n=4.

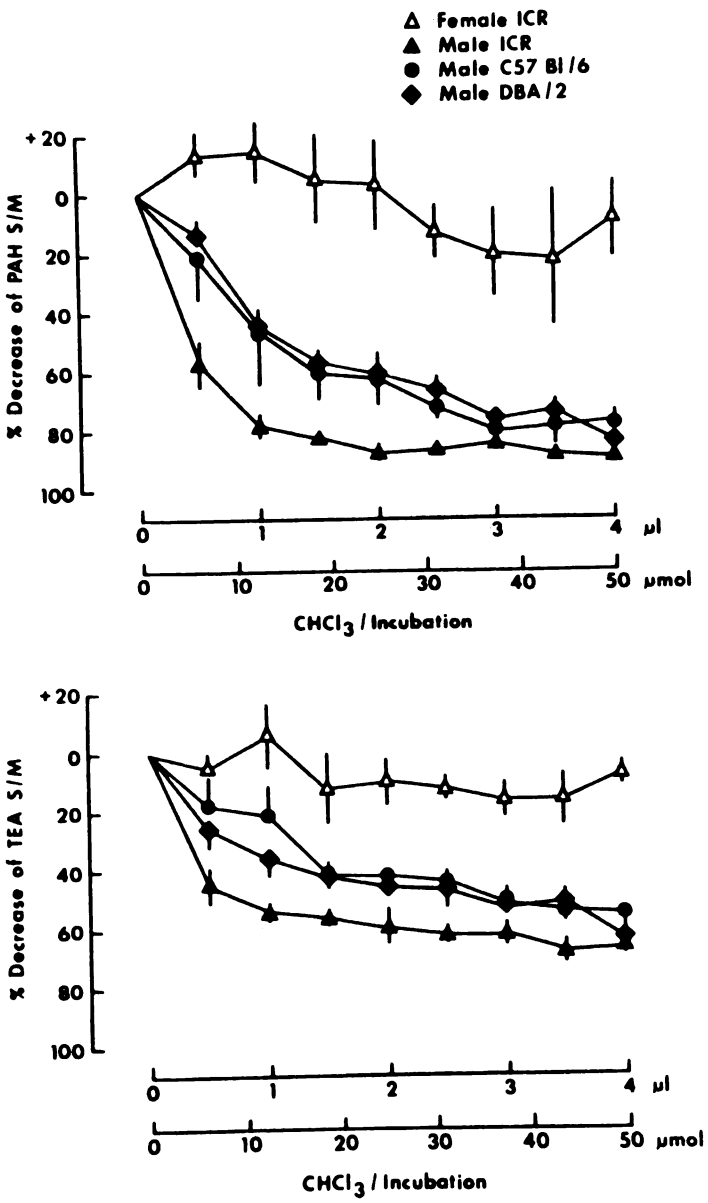

Female ICR
Male ICR
Male C57 Bl/6
Male DBA/2
+20
0
20
40
60
80
100
% Decrease of PAH S/M
0
1
2
3
4 µl
0
10
20
30
40
50 µmol
CHCl3 / Incubation
+20
0
20
40
60
80
100
% Decrease of TEA S/M
0
1
2
3
4 µl
0
10
20
30
40
50 µmol
CHCl3 / Incubation

Figure 28

Renal cortical slices prepared from female ICR mice did not appear to be susceptible to $CHCl_3$-induced nephrotoxicity.

C. In Vitro Studies - Metabolism of $^{14}CHCl_3$ by Renal Cortical and Hepatic Slices from ICR Mice

$^{14}CHCl_3$ was metabolized to $^{14}CO_2$, covalently bound radioactivity and aqueous soluble metabolites by slices prepared from male liver and renal cortex and female renal cortex of ICR mice (Table 11). A concentration of $^{14}CHCl_3$ was used that reduced PAH and TEA accumulation near maximally (Figures 22 and 28). Following a 2 hr incubation at 37°C, the production of $^{14}CO_2$ was greatest in slices from liver, with smaller amounts formed in renal cortical slices from male and female mice. The extent of covalent binding to liver and kidney did not parallel the evolution of $^{14}CO_2$. Covalent binding was greatest in male kidney slices followed by liver slices and then female kidney slices. The production of aqueous soluble metabolites paralleled the evolution of $^{14}CO_2$, with liver producing more than male and female kidney.

The extent of $^{14}CO_2$ production and covalent binding was determined over a 3 hr incubation period in male kidney and liver slices incubated with 3.12 μmol $CHCl_3$ (Figure 29). Again, the amount of covalent binding was greater in male kidney slices than in liver slices at all incubation times (Figure 29). The degree of covalent binding to both liver and kidney was linear for approximately 1 hr. $^{14}CO_2$ production in the 100 mg of slices was much greater in the liver

TABLE 11

In Vitro Metabolism of $^{14}CHCl_3$ to $^{14}CO_2$, Covalently Bound Radioactivity and Aqueous Soluble Metabolites by Male Hepatic and Renal Cortical Slices and Female Renal Cortical Slices from ICR Mice[a]

	Tissue		
	Male Liver	Male Kidney	Female Kidney
$^{14}CO_2$ Detected (nmol/100 mg tissue)	65.46± 5.04	14.36±0.95	8.30±0.54
Covalent Binding (nmol/mg protein)	1.84± 0.30	3.04±0.20	0.51±0.12
Aqueous (nmol/100 mg tissue)	59.63±20.00	11.94±1.01	10.02±0.76

[a]Reaction vessels contained 100 mg of tissue slices and 6.25 µmol $^{14}CHCl_3$ (specific activity ≈0.5 µCi/µmol added in a volume of 5 µl dimethyl formamide) in 2.0 ml of phosphate buffer, pH 7.4, composed of 96.7 mM NaCl, 7.4 mM sodium phosphate buffer, 40 mM KCl, and 0.74 mM $CaCl_2$. Reaction vessels were gassed with 100% O_2 for 5 min prior to adding $^{14}CHCl_3$ and incubations were conducted at 37°C in an oscillating water bath (100 cycles/min). Incubations were terminated by the injection of 2.0 ml 10% TCA. Values have been corrected for non-enzymatic metabolism of $^{14}CHCl_3$ using boiled slices. Values are mean ± S.E.M., n=4.

Figure 29. Time course of $^{14}CHCl_3$ metabolism to covalently bound radioactivity and $^{14}CO_2$ by male hepatic and renal cortical slices from ICR mice. Reaction vessels contained 100 mg of tissue slices and 3.12 μmol $^{14}CHCl_3$ (specific activity ≈0.5 μCi/μmol added in a volume of 2.5 μl dimethylformamide) in 2.0 ml of phosphate buffer, pH 7.4, composed of 96.7 mM NaCl, 7.4 mM sodium phosphate buffer, 40 mM KCl and 0.74 mM $CaCl_2$. Reaction vessels were gassed with 100% O_2 for 5 min prior to adding $^{14}CHCl_3$ and incubations were conducted at 37°C in an oscillating water bath (100 cycles/min). Incubations were terminated at the indicated time by the injection of 2.0 ml 10% TCA. Values are mean ± S.E.M., n=4.

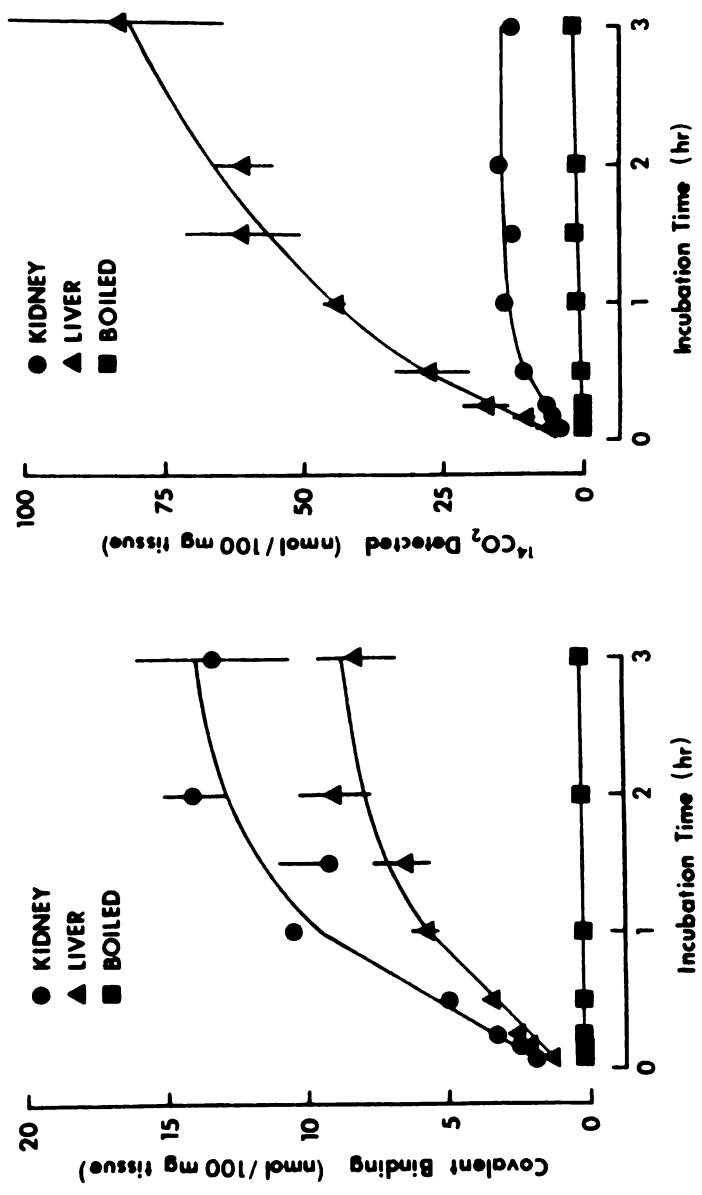

KIDNEY
LIVER
BOILED
Covalent Binding (nmol/100 mg tissue)
20
15
10
5
0
0
1
2
3
Incubation Time (hr)
KIDNEY
LIVER
BOILED
$^{14}CO_2$ Detected (nmol/100 mg tissue)
100
75
50
25
0
0
1
2
3
Incubation Time (hr)

Figure 29

than the kidney at all time points (Figure 29). $^{14}CO_2$ production continued to increase in liver slices up to 3 hr, while a plateau was reached in kidney slices after incubation for 30 min.

The metabolism of $^{14}CHCl_3$ to covalently bound radioactivity and $^{14}CO_2$ was reduced by carbon monoxide in liver slices and in male and female renal cortical slices (Figure 30). A 4:1 mixture of CO and O_2 reduced covalent binding by 93% in male and by 82% in female kidney slices. The reduction of oxygen concentration in the incubation vessel could only account for a 40-50% reduction of covalent binding in all of the tissues, as indicated by the incubations conducted under a 4:1 mixture of N_2 and O_2. Likewise, incubation of slices under an atmosphere of carbon monoxide reduced the metabolism of $^{14}CHCl_3$ to $^{14}CO_2$ in all tissues similar to the reduction of covalent binding.

D. In Vitro Studies - Microsomal Metabolism of $^{14}CHCl_3$

1. Metabolism of $^{14}CHCl_3$ In Vitro by Renal Cortical and Hepatic Microsomes - Effect of Time, Microsomal Protein Concentration and Substrate Concentration

$^{14}CHCl_3$ was metabolized to $^{14}CO_2$ and covalently bound metabolites by microsomes prepared from male mouse kidney cortex and liver (Table 12). Metabolism of $^{14}CHCl_3$ by microsomes prepared from female kidney cortex was similar to non-enzymatic $^{14}CHCl_3$ decomposition in heat-denatured (boiled) microsomes. There was little or no metabolism of $^{14}CHCl_3$ to $^{14}CO_2$ or covalently bound radioactivity by female renal cortical microsomes up to 2 hr incubation (Figure 31). $^{14}CHCl_3$ metabolism by male renal cortical microsomes to $^{14}CO_2$ was linear with time for approximately 15 min; metabolism to covalently bound radioactivity

Figure 30. Effect of carbon monoxide on metabolism of $^{14}CHCl_3$ to covalently bound radioactivity and $^{14}CO_2$ by male hepatic and renal cortical slices and female renal cortical slices from ICR mice. Reaction vessels contained 100 mg of tissue slices and 3.12 μmol $^{14}CHCl_3$ (specific activity ≃0.5 μCi/μmol added in a volume of 2.5 μl dimethylformamide) in 2.0 ml of phosphate buffer, pH 7.4, containing 96.7 mM NaCl, 7.4 mM sodium phosphate buffer, 40 mM KCl and 0.74 mM $CaCl_2$. Reaction vessels were gassed for 5 min prior to adding $^{14}CHCl_3$ with either 100% O_2, $CO:O_2$ (4:1 mixture) or $N_2:O_2$ (4:1 mixture). Incubations were conducted at 37°C in an oscillating water bath (100 cycles/min) and were terminated after 30 min by the injection of 2.0 ml of 10% TCA. Values are mean ± S.E.M., n=3.

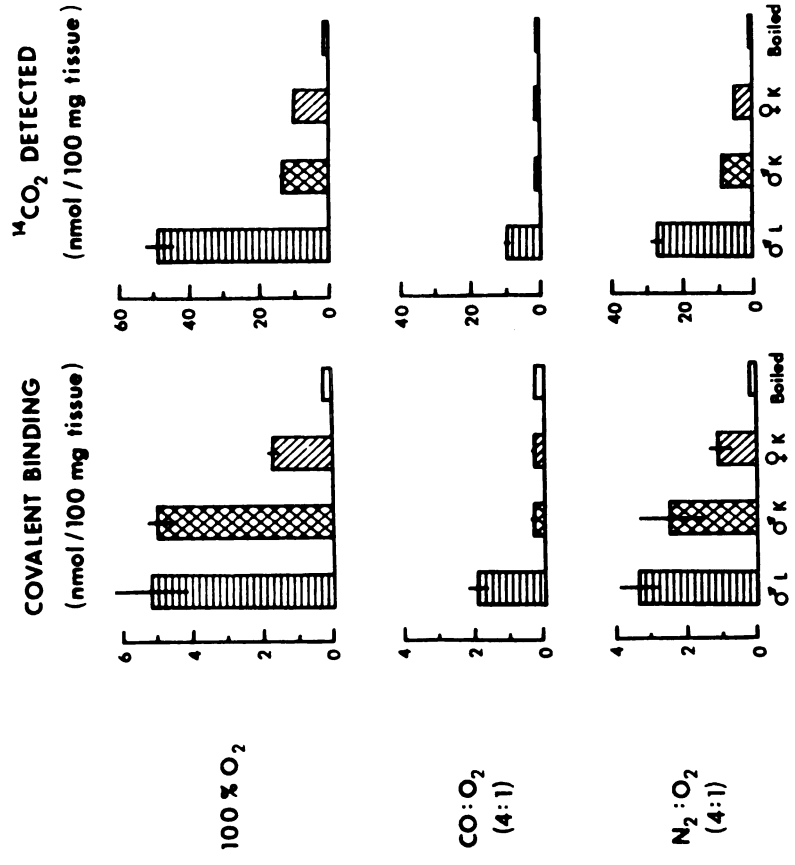

COVALENT BINDING
(nmol/100 mg tissue)
$^{14}CO_2$ DETECTED
(nmol/100 mg tissue)
100 % O_2
CO:O_2 (4:1)
N_2:O_2 (4:1)
♂ L
♂ K
♀ K
Boiled

Figure 30

TABLE 12

$^{14}CHCl_3$ Metabolism by Male Hepatic and Renal Cortical, Female Renal Cortical and Boiled Microsomes from ICR Mice[a]

Incubation Time (min)	Male Liver	Male Kidney	Female Kidney	Boiled
		COVALENT BINDING (nmol/mg protein)		
5	1.387±0.256	0.930±0.067	0.180±0.014	0.206±0.025
15	3.636±0.801	1.258±0.301	0.199±0.037	0.187±0.014
30	5.958±1.123	1.631±0.277	0.227±0.029	0.179±0.015
		$^{14}CO_2$ DETECTED (nmol/reaction vessel)		
5	8.828±0.576	6.561±0.289	0.913±0.060	0.852±0.032
15	20.517±2.807	9.030±0.947	1.152±0.191	0.965±0.064
30	30.637±4.118	10.827±0.770	1.228±0.093	1.126±0.123

[a]Reaction vessels contained 2 mg microsomal protein, 3.12 µmol $^{14}CHCl_3$ (specific activity ≈0.5 µCi/µmol added in a volume of 2.5 µl dimethylformamide), 0.4 µmol NADPH, 1.2 µmol $NADP^+$, 1.2 µmol NADH, 18 µmol glucose-6-phosphate, 4 units glucose-6-phosphate dehydrogenase, 0.65 mmol $MgCl_2$ in 0.1 M sodium phosphate buffer, pH 7.4, in a total volume of 1.5 ml. Reaction vessels were gassed with 100% O_2 for 5 min prior to adding $^{14}CHCl_3$, and incubations were conducted in an oscillating water bath (100 cycles/min). Incubations were terminated by the injection of 1.5 ml of 10% TCA. Values are mean ± S.E.M., n=4.

Figure 31. Time course of $^{14}CHCl_3$ metabolism to $^{14}CO_2$ and covalently bound radioactivity by male and female renal cortical microsomes. Reaction vessels contained 2 mg microsomal protein, 3.12 μmol $^{14}CHCl_3$ (specific activity ≈0.5 μCi/μmol, added in a volume of 2.5 μl of dimethylformamide), 0.4 μmol, NADPH, 1.2 μmol $NADP^+$, 1.2 μmol NADH, 18 μmol glucose-6-phosphate, 4 units glucose-6-phosphate dehydrogenase and 0.65 mmol $MgCl_2$ in 0.1 M sodium phosphate buffer, pH 7.4, in a total volume of 1.5 ml. Reaction vessels were gassed with 100% O_2 for 5 min prior to adding $^{14}CHCl_3$, and incubations were conducted at 37°C in an oscillating water bath (100 cycles/min). Incubations were terminated at the indicated time with 1.5 ml of 10% TCA. Values have been corrected for non-enzymatic metabolism of $^{14}CHCl_3$ using boiled microsomes. Values are mean ± S.E.M., n=4.

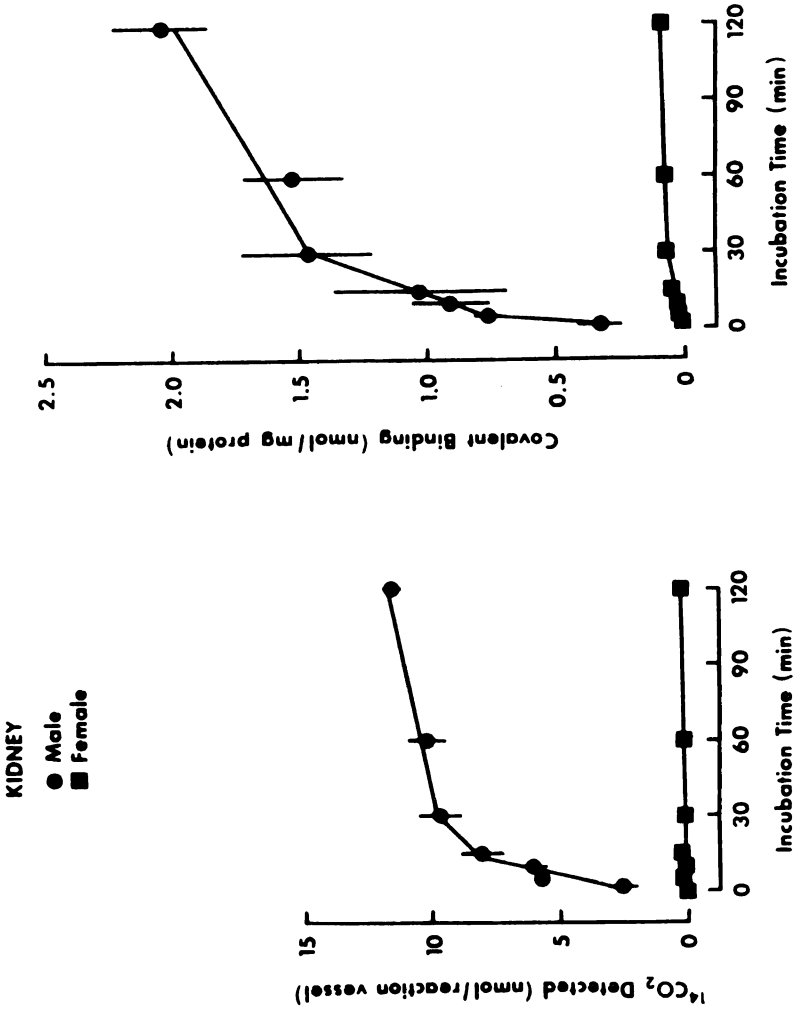

KIDNEY
Male
Female
$^{14}CO_2$ Detected (nmol/reaction vessel)
Covalent Binding (nmol/mg protein)
Incubation Time (min)
0
30
60
90
120
0
5
10
15
0.5
1.0
1.5
2.0
2.5

Figure 31

was linear with time from 5 to 30 min (Figure 31). Hepatic microsomal metabolism of $^{14}CHCl_3$ to $^{14}CO_2$ was linear with time for 15 min and metabolism to covalently bound radioactivity was linear for 30 min (Figure 32). In contrast to $^{14}CHCl_3$ metabolism by male hepatic and renal cortical slices (Table 11), $^{14}CHCl_3$ metabolism to $^{14}CO_2$ and covalently bound radioactivity always was greater by hepatic than by renal cortical microsomes from male mice (Table 12, Figures 31 and 32). Furthermore, there was an increase in the magnitude of difference between hepatic and renal cortical metabolism as incubation time increased.

Male renal cortical microsomal metabolism of $^{14}CHCl_3$ to $^{14}CO_2$ and covalently bound radioactivity was linear with protein concentration to at least 2 mg of microsomal protein during a 10 min incubation with 3.12 μmol $^{14}CHCl_3$ (Figure 33). Metabolism of $^{14}CHCl_3$ to $^{14}CO_2$ and covalently bound radioactivity by hepatic microsomes appeared linear up to 8 mg of microsomal protein, the highest concentration used in these studies (Figure 34).

On the basis of these data, an incubation period of 10 min with a microsomal protein concentration of 2 mg per 1.5 ml reaction was used for subsequent experiments.

The greater degree of hepatic microsomal metabolism of $CHCl_3$ relative to that of renal cortical microsomal metabolism was similar to the results observed for metabolism of $^{14}CHCl_3$ to $^{14}CO_2$ by hepatic and renal cortical slices. The cytochrome P-450 content in microsomes per mg microsomal protein was approximately four times greater in

Figure 32. Time course of $^{14}CHCl_3$ metabolism to $^{14}CO_2$ and covalently bound radioactivity by male hepatic microsomes. Reaction vessels contained 2 mg microsomal protein, 3.12 μmol $^{14}CHCl_3$ (specific activity ≃0.5 μCi/μmol, added in a volume of 2.5 μl of dimethylformamide), 0.4 μmol, NADPH, 1.2 μmol $NADP^+$, 1.2 μmol NADH, 18 μmol glucose-6-phosphate, 4 units glucose-6-phosphate dehydrogenase and 0.65 mmol $MgCl_2$ in 0.1 M sodium phosphate buffer, pH 7.4, in a total volume of 1.5 ml. Reaction vessels were gassed with 100% O_2 for 5 min prior to adding $^{14}CHCl_3$, and incubations were conducted at 37°C in an oscillating water bath (100 cycles/min). Incubations were terminated at the indicated time with 1.5 ml of 10% TCA. Values have been corrected for non-enzymatic metabolism of $^{14}CHCl_3$ using boiled microsomes. Values are mean ± S.E.M., n=4.

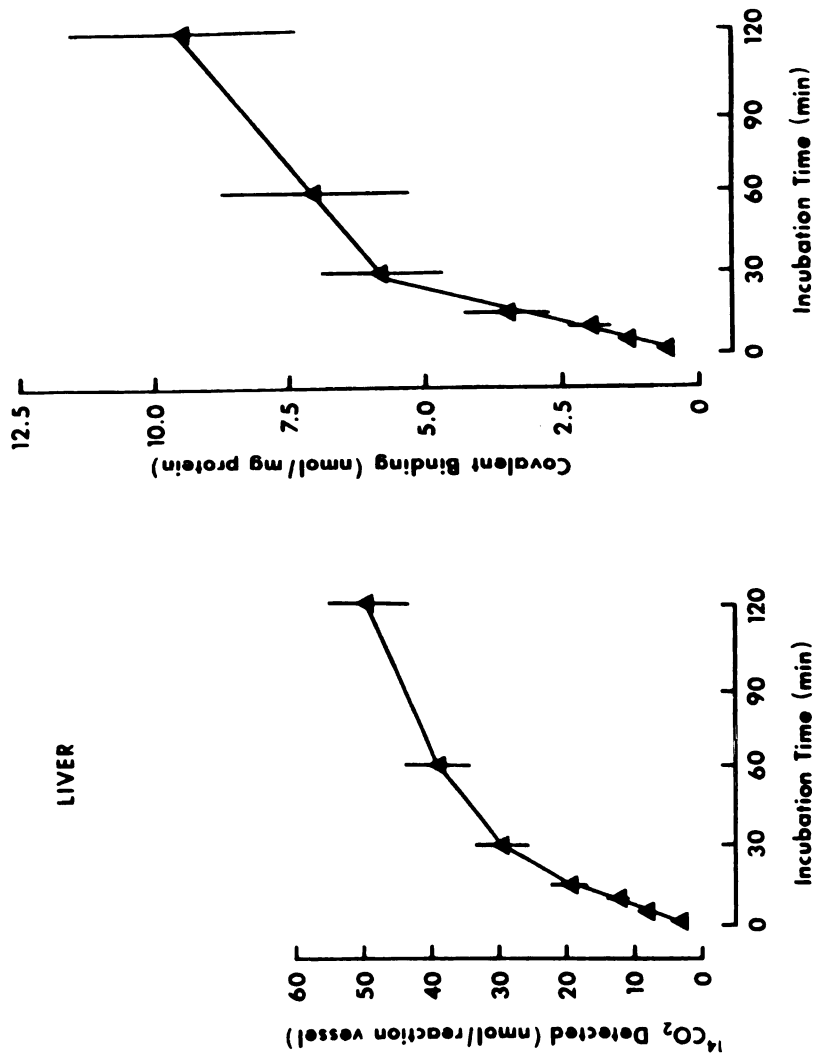

LIVER
$^{14}CO_2$ Detected (nmol/reaction vessel)
0
10
20
30
40
50
60
0
30
60
90
120
Incubation Time (min)
Covalent Binding (nmol/mg protein)
0
2.5
5.0
7.5
10.0
12.5
0
30
60
90
120
Incubation Time (min)

Figure 32

Figure 33. Effect of male renal cortical microsomal protein concentration on $^{14}CHCl_3$ metabolism to $^{14}CO_2$ and covalently bound radioactivity. Reaction vessels contained 0.5 to 8 mg microsomal protein, 3.12 μmol $^{14}CHCl_3$ (specific activity ≈0.5 μCi/μmol, added in a volume of 2.5 μl dimethylformamide), 0.4 μmol NADPH, 1.2 μmol $NADP^+$, 1.2 μmol NADH, 18 μmol glucose-6-phosphate, 4 units glucose-6-phosphate dehydrogenase and 0.65 mmol $MgCl_2$ in 0.1 M sodium phosphate buffer, pH 7.4, in a total volume of 1.5 ml. Reaction vessels were gassed with 100% $^{14}CO_2$ for 5 min prior to adding $^{14}CHCl_3$, and incubations were conducted at 37°C in an oscillating water bath (100 cycles/min) for 10 min. Incubations were terminated by the injection of 15 ml of 10% TCA. Values have been corrected for non-enzymatic metabolism of $^{14}CHCl_3$ and are extrapolated to nmoles of metabolite formed per reaction vessel. Values are mean ± S.E.M., n=3 to 5 for each data point.

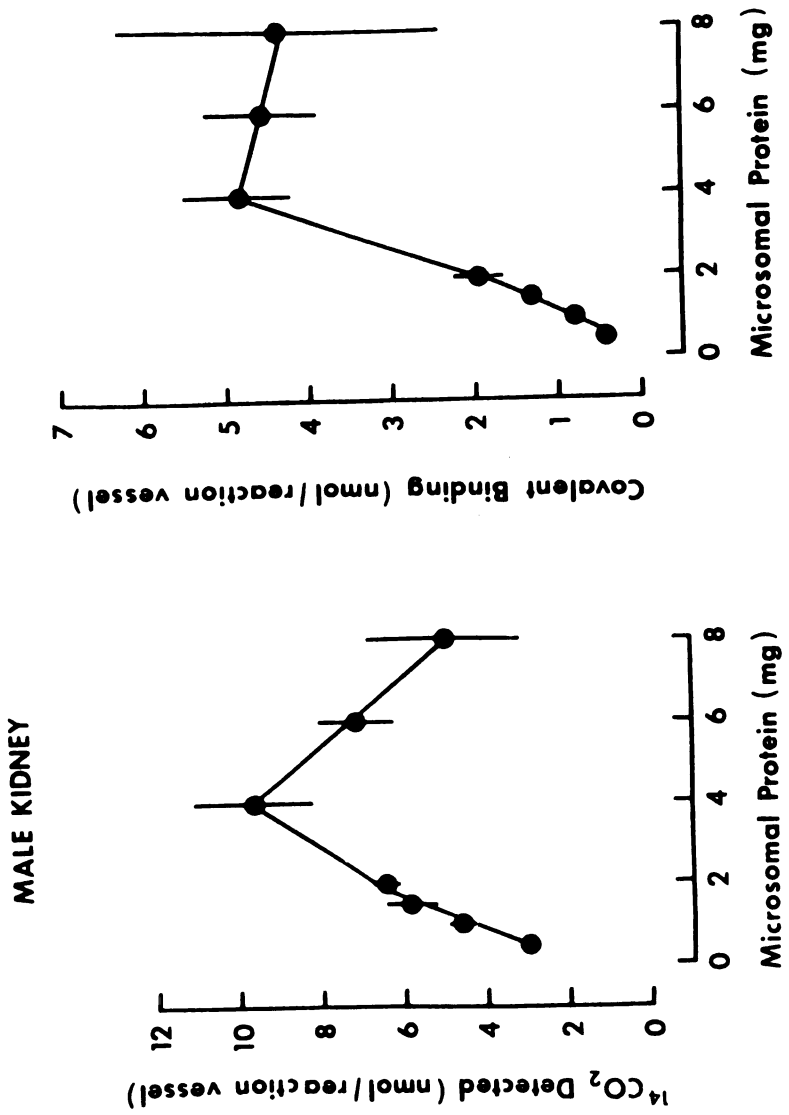

MALE KIDNEY
$^{14}CO_2$ Detected (nmol/reaction vessel)
0
2
4
6
8
10
12
0
2
4
6
8
Microsomal Protein (mg)
Covalent Binding (nmol/reaction vessel)
0
1
2
3
4
5
6
7
0
2
4
6
8
Microsomal Protein (mg)

Figure 33

Figure 34. Effect of male hepatic microsomal protein concentration on $^{14}CHCl_3$ metabolism to $^{14}CO_2$ and covalently bound radioactivity. Reaction vessels contained 0.5 to 8 mg microsomal protein, 3.12 μmol $^{14}CHCl_3$ (specific activity ≈0.5 μCi/μmol, added in a volume of 2.5 μl dimethylformamide), 0.4 μmol NADPH, 1.2 μmol $NADP^+$, 1.2 μmol NADH, 18 μmol glucose-6-phosphate, 4 units glucose-6-phosphate dehydrogenase and 0.65 mmol $MgCl_2$ in 0.1 M sodium phosphate buffer, pH 7.4, in a total volume of 1.5 ml. Reaction vessels were gassed with 100% O_2 for 5 min prior to adding $^{14}CHCl_3$, and incubations were conducted at 37°C in an oscillating water bath (100 cycles/min) for 10 min. Incubations were terminated by the injection of 15 ml of 10% TCA. Values have been corrected for non-enzymatic metabolism of $^{14}CHCl_3$ and are extrapolated to nmoles of metabolite formed per reaction vessel. Values are mean ± S.E.M., n=3 to 5 for each data point.

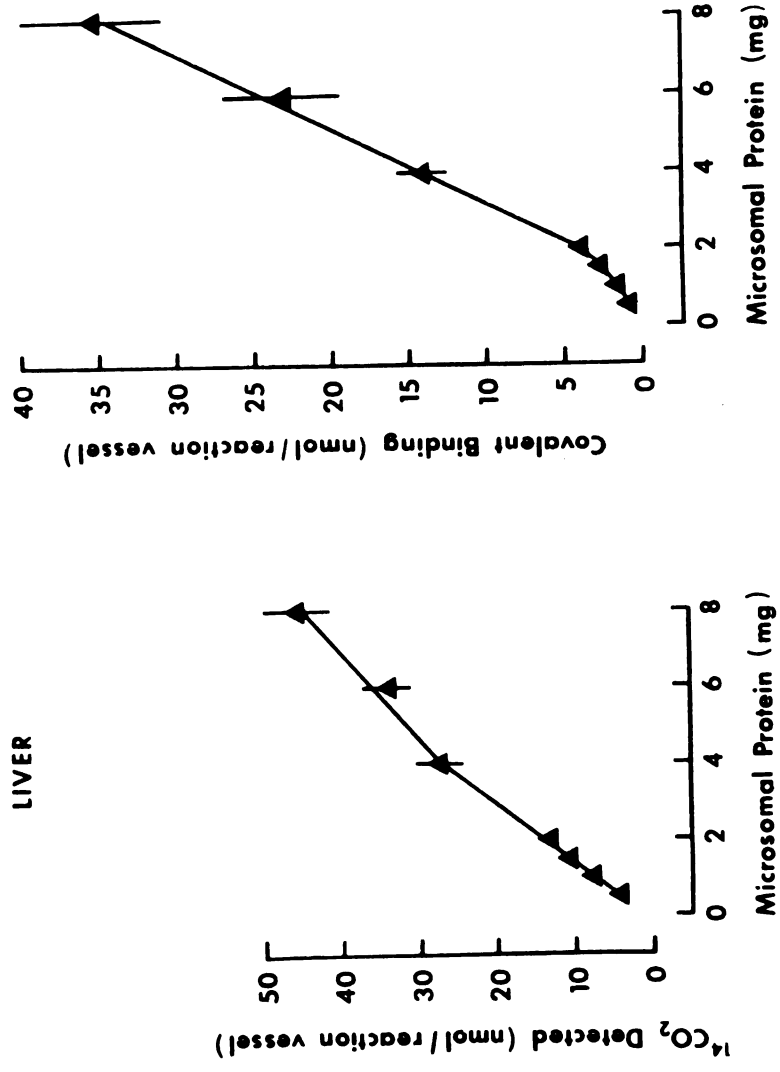

LIVER
$^{14}CO_2$ Detected (nmol / reaction vessel)
0
10
20
30
40
50
0
2
4
6
8
Microsomal Protein (mg)
Covalent Binding (nmol / reaction vessel)
0
5
10
15
20
25
30
35
40
0
2
4
6
8
Microsomal Protein (mg)

Figure 34

liver than male kidney cortex (Table 13). Microsomal metabolism of $^{14}CHCl_3$ to covalently bound radioactivity and $^{14}CO_2$ was approximately two times greater by liver than by kidney after a 10 min incubation of 3.12 μmol $^{14}CHCl_3$ with 2 mg microsomal protein. However, when microsomal metabolism was expressed in terms of nmol of metabolites per nmol cytochrome P-450, $CHCl_3$ metabolism was approximately two times greater in male mouse renal cortical microsomes than in hepatic microsomes (Table 13).

Male mouse renal cortical metabolism of $^{14}CHCl_3$ to $^{14}CO_2$ increased linearly with increasing substrate concentration up to 5.0 μmol of $CHCl_3$ (Figure 35). An Eadie-Hofstee plot was constructed to estimate the kinetic parameters for the renal cortical microsomal metabolism of $CHCl_3$ (Figure 36) (Eadie, 1952; Hofstee, 1952). The Michaelis constant (K_M) for the reaction was 2.78 μmol and the V_{max} was 0.782 nmol/2 mg microsomal protein/min. On the basis of these data, the substrate concentration was increased in subsequent reactions to decrease the potential that $CHCl_3$ concentrations would become rate-limiting.

The substrate concentrations used in these experiments (1.25 to 12.5 μmol $CHCl_3$) were not sufficiently high to estimate the kinetic parameters for hepatic microsomal metabolism of $CHCl_3$; a plot of velocity versus substrate concentration was parallel to the abscissa indicating the V_{max} had been reached (data not shown).

TABLE 13

Comparison of Renal Cortical and Hepatic Microsomal Metabolism of $^{14}CHCl_3$ in Relation to Cytochrome P-450 Concentrations[a]

	Male Kidney Cortex	Liver
Cytochrome P-450		
nmol/mg protein	0.453±0.029	1.627±0.065
Covalent Binding		
nmol/mg protein	1.086±0.167	2.092±0.264
nmol/nmol cytochrome P-450	2.435±0.410	1.237±0.114
$^{14}CO_2$ Detected		
nmol/mg protein	3.468±0.360	6.681±0.476
nmol/nmol cytochrome P-450	7.803±1.024	4.103±0.245

[a]Reaction vessels contained 2 mg microsomal protein, 3.12 μmol $^{14}CHCl_3$ (specific activity ≃0.5 μCi/μmol added in a volume of 2.5 μl dimethylformamide), 0.4 μmol NADPH, 1.2 μmol $NADP^+$, 1.2 μmol NADH, 18 μmol glucose-6-phosphate, 4 units glucose-6-phosphate dehydrogenase and 0.65 mmol $MgCl_2$ in 0.1 M sodium phosphate buffer, pH 7.4, in a total volume of 1.5 ml. Reaction vessels were gassed with 100% O_2 for 5 min prior to adding $^{14}CHCl_3$, and incubations were conducted at 37°C in an oscillating water bath (100 cycles/min) for 10 min. Incubations were terminated by the injection of 1.5 ml of 10% TCA. Values have been corrected for non-enzymatic metabolism of $^{14}CHCl_3$ and are expressed as nmol $^{14}CHCl_3$ metabolized per mg microsomal protein or per nmol cytochrome P-450. Values are mean ± S.E.M., n=5.

Figure 35. Effect of substrate concentration on $^{14}CHCl_3$ metabolism to $^{14}CO_2$ by male renal cortical microsomes. Reaction vessels contained 2 mg microsomal protein, 1.25 μmol $^{14}CHCl_3$ (specific activity ≈0.5 μCi/μmol) plus 0.25 to 11.25 μmol nonradioactive $CHCl_3$ to provide the indicated substrate concentration, 0.4 μmol NADPH, 1.2 μmol $NADP^+$, 1.2 μmol NADH, 18 μmol glucose-6-phosphate, 4 units glucose-6-phosphate dehydrogenase and 0.65 mmol $MgCl_2$ in 0.1 M sodium phosphate buffer, pH 7.4, in a total volume of 1.5 ml. Reaction vessels were gassed with 100% O_2 for 5 min prior to adding $CHCl_3$, and incubations were conducted at 37°C in an oscillating water bath (100 cycles/min) for 10 min. Incubations were terminated by the injection of 1.5 ml of 10% TCA. Values have been corrected for non-enzymatic metabolism of $^{14}CHCl_3$ using boiled microsomes. Values are mean ± S.E.M., n=3.

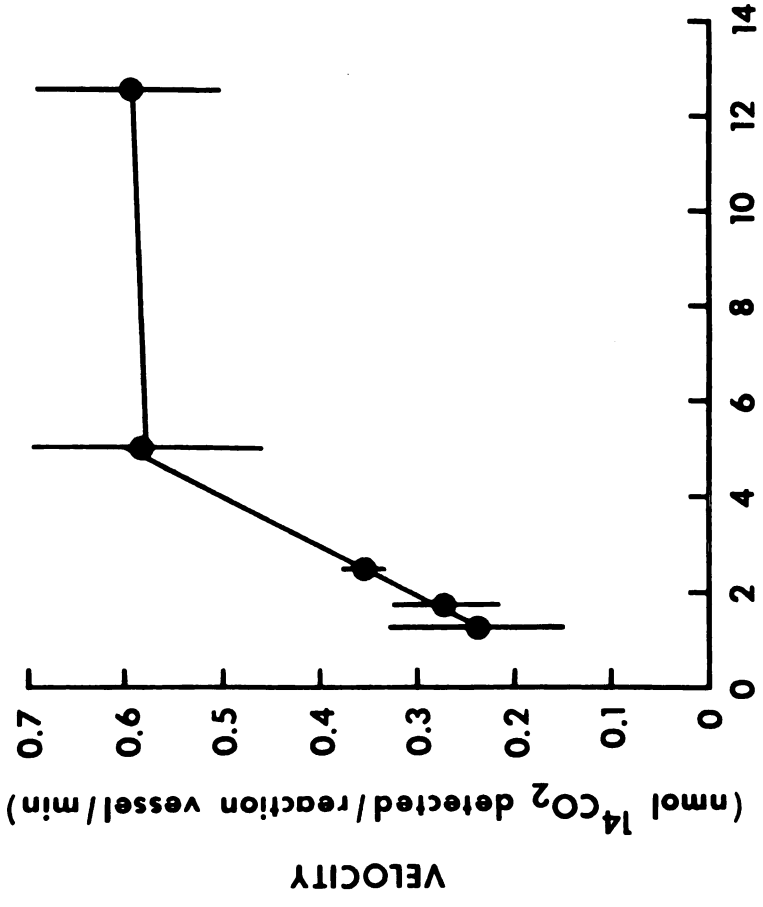

VELOCITY
(nmol $^{14}CO_2$ detected/reaction vessel/min)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
12
14
$CHCl_3$ (μmol)

Figure 35

Figure 36. Kinetics of the renal cortical microsomal metabolism of $^{14}CHCl_3$ to $^{14}CO_2$. Substrate concentrations ranged from 1.25 to 12.5 μmol. See Figure 35 for a description of the incubation condition. Data were plotted by the method of Eadie and Hofstee (Eadie, 1952; Hofstee, 1952). V = nmol $^{14}CO_2$ detected/reaction vessel/min and S = μmol $CHCl_3$ added to the incubation, n=3.

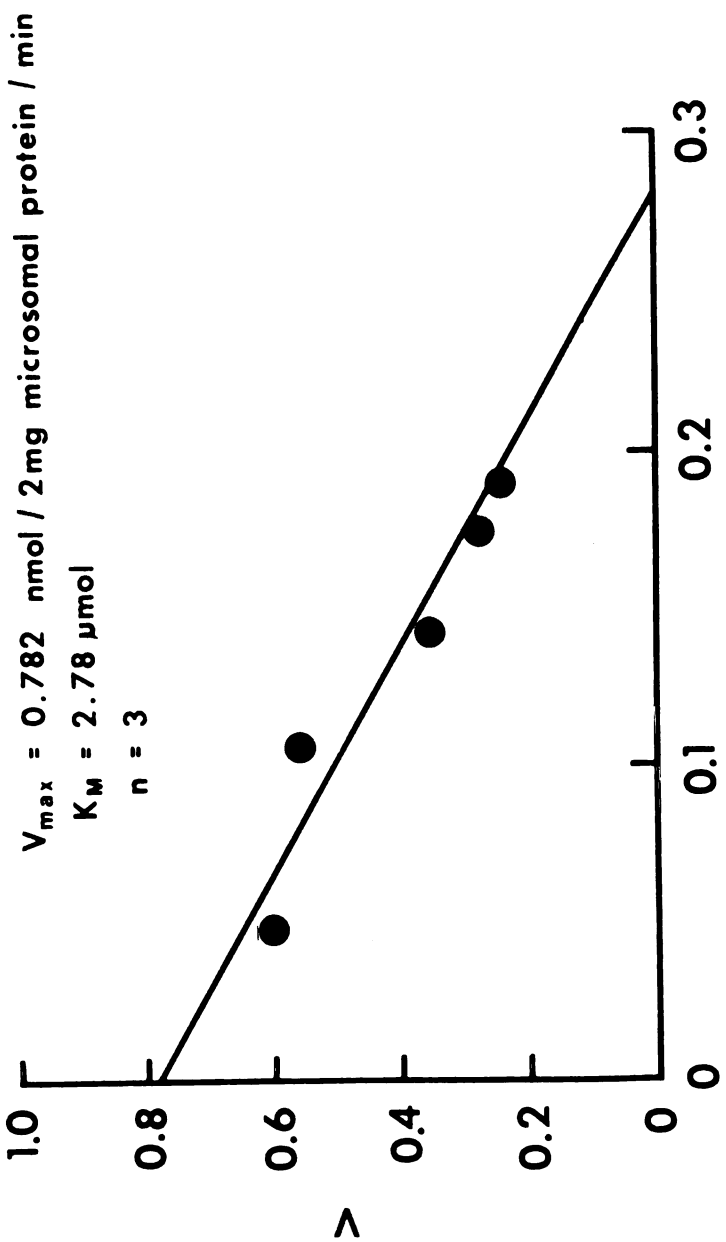

V_{max} = 0.782 nmol / 2mg microsomal protein / min
K_M = 2.78 µmol
n = 3
V
1.0
0.8
0.6
0.4
0.2
0
0
0.1
0.2
0.3
V/[S]

Figure 36

2. Effect of NADH and NADPH on Microsomal Metabolism of $^{14}CHCl_3$

Metabolism of $^{14}CHCl_3$ by renal cortical and hepatic microsomes required the presence of a NADPH regenerating system (Figures 37 and 38). The extent of $^{14}CHCl_3$ metabolism to $^{14}CO_2$ and covalently bound radioactivity was similar in incubations with heat-denatured microsomes or with microsomes in the absence of a NADPH regenerating system. Enzymatic metabolism of $^{14}CHCl_3$ by renal cortical microsomes in the presence of NADH alone was only about 5% of that observed in the presence of NADPH (Figure 37). In contrast, hepatic microsomal $^{14}CHCl_3$ metabolism in the presence of NADH alone was approximately 40% of that observed in the presence of NADPH alone (Figure 38). Addition of both NADH and NADPH to the incubation mixture increased the metabolism of $^{14}CHCl_3$ in an additive manner.

3. Subcellular Localization of $^{14}CHCl_3$ Metabolism

Hepatic and renal cortical 9000 g supernatant and the microsomal fraction from male ICR mice metabolized $^{14}CHCl_3$ to $^{14}CO_2$ and covalently bound radioactivity (Table 14). Metabolism of $^{14}CHCl_3$ by the cytosol fraction was very low. There was little or no conversion to aqueous soluble metabolites by the microsomal fraction alone, however when glutathione was added, the amount of aqueous soluble counts was increased. There was no metabolism of $^{14}CHCl_3$ by any of these fractions in the absence of a NADPH regenerating system (data not shown).

4. Binding Spectra of $CHCl_3$ with Renal Cortical and Hepatic Microsomes

The binding of $CHCl_3$ to oxidized cytochrome P-450 from male renal cortical and hepatic microsomes produced a typical type I binding spectrum (Figure 39).

Figure 37. Effect of NADH and NADPH on male renal cortical metabolism of $^{14}CHCl_3$ to covalently bound radioactivity and $^{14}CO_2$. All reaction vessels contained 1 mg microsomal protein and 3.12 μmol $^{14}CHCl_3$ (specific activity ≃0.5 μCi/μmol added in a volume of 2.5 μl dimethylformamide) in 0.1 M sodium phosphate buffer, pH 7.4, in a total volume of 0.75 ml (-NADH - NADPH). Reaction vessels also contained 1 mM NADH (NADH), 1 mM NADPH plus 1 mM $NADP^+$ (NADPH), 1 mM NADH plus 1 mM NADPH plus 1 mM $NADP^+$ (NADH + NADPH) as indicated, plus 3 mM $MgCl_2$, 5 mM glucose-6-phosphate and 1.5 units glucose-6-phosphate dehydrogenase. Boiled reaction vessels were similar to NADH + NADPH flasks, but were boiled for 5 min. Reaction vessels were gassed with 100% O_2 for 5 min prior to adding $^{14}CHCl_3$, and incubations were conducted at 37°C in an oscillating water bath (100 cycles/min) for 10 min. Incubations were terminated by the injection of 0.75 ml of 10% TCA. Values are mean ± S.E.M., n=4.

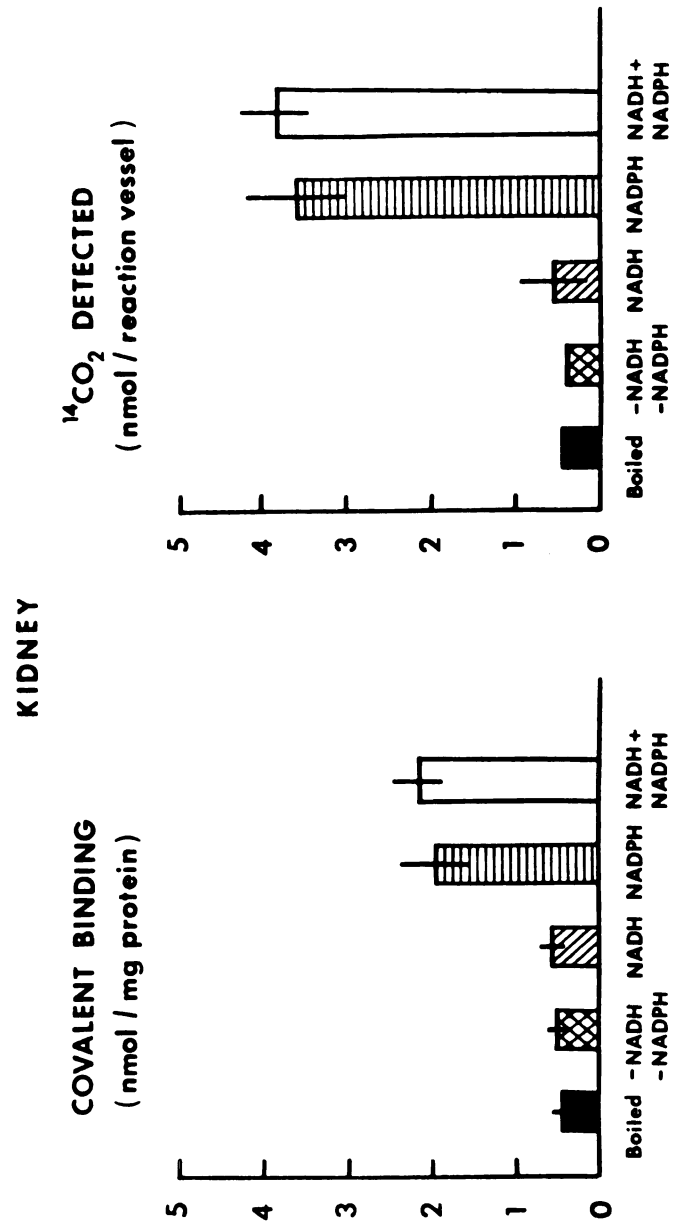

KIDNEY
COVALENT BINDING
(nmol / mg protein)
$^{14}CO_2$ DETECTED
(nmol / reaction vessel)
0
1
2
3
4
5
Boiled
-NADH -NADPH
NADH
NADPH
NADH+ NADPH

Figure 37

Figure 38. Effect of NADH and NADPH on male hepatic microsomal metabolism of $^{14}CHCl_3$ to covalently bound radioactivity and $^{14}CO_2$. All reaction vessels contained 1 mg microsomal protein and 3.12 μmol $^{14}CHCl_3$ (specific activity ≃0.5 μCi/μmol added in a volume of 2.5 μl dimethylformamide) in 0.1 M sodium phosphate buffer, pH 7.4, in a total volume of 0.75 ml (-NADH - NADPH). Reaction vessels also contained 1 mM NADH (NADH), 1 mM NADPH plus 1 mM $NADP^+$ (NADPH), 1 mM NADH plus 1 mM NADPH plus 1 mM $NADP^+$ (NADH + NADPH) as indicated, plus 3 mM $MgCl_2$, 5 mM glucose-6-phosphate and 1.5 units glucose-6-phosphate dehydrogenase. Boiled reaction vessels were similar to NADH + NADPH flasks, but were boiled for 5 min. Reaction vessels were gassed with 100% O_2 for 5 min prior to adding $^{14}CHCl_3$, and incubations were conducted at 37°C in an oscillating water bath (100 cycles/min) for 10 min. Incubations were terminated by the injection of 0.75 ml of 10% TCA. Values are mean ± S.E.M., n=4.

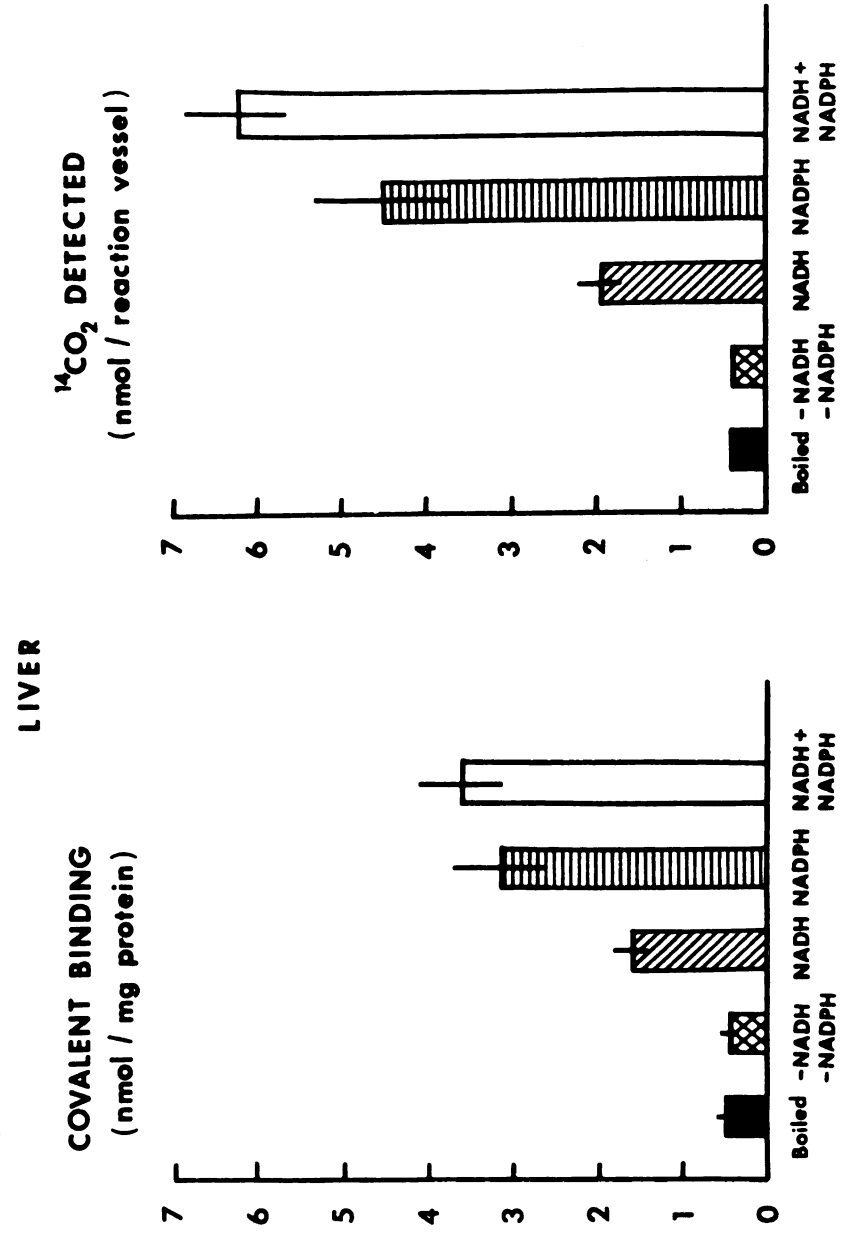

LIVER
COVALENT BINDING
(nmol / mg protein)
$^{14}CO_2$ DETECTED
(nmol / reaction vessel)
0
1
2
3
4
5
6
7
Boiled
-NADH -NADPH
NADH
NADPH
NADH+ NADPH

Figure 38

TABLE 14

Metabolism of $^{14}CHCl_3$ to $^{14}CO_2$, Covalently Bound Radioactivity and Aqueous Soluble Metabolites in the Presence and Absence of Glutathione (GSH) by Male Renal Cortical and Hepatic Subcellular Fractions[a]

	Renal Cortex		Liver	
	-GSH	+GSH	-GSH	+GSH
9000 g SUPERNATANT				
$^{14}CO_2$[b]	1.487±0.304	1.226±0.385	2.895±0.422	2.446±0.307
Covalent binding[c]	0.518±0.169	0.198±0.057	2.059±0.141	0.747±0.091
Aqueous[c]	0.092±0.041	0.778±0.239	0.503±0.135	2.186±0.316
CYTOSOL				
$^{14}CO_2$	0.009±0.005	0.110±0.110	0.252±0.226	0.132±0.052
Covalent Binding	0.016±0.004	0.006±0.006	0.098±0.066	0.027±0.018
Aqueous	0.012±0.012	0.053±0.043	0.011±0.008	0.212±0.061
MICROSOMES				
$^{14}CO_2$	1.672±0.344	0.939±0.225	4.104±0.477	3.715±0.654
Covalent Binding	0.365±0.109	0.116±0.025	1.929±0.334	0.379±0.104
Aqueous	0.012±0.008	1.582±0.470	0.014±0.014	6.287±1.000

continued on next page....

TABLE 14 (continued)....

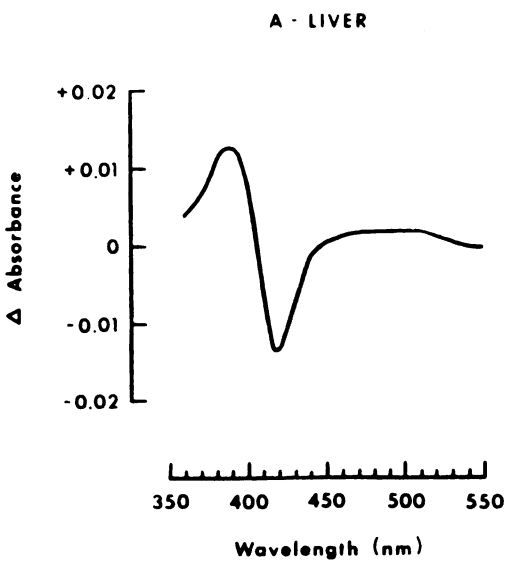
	Renal Cortex		Liver	
	-GSH	+GSH	-GSH	+GSH
MICROSOMES + CYTOSOL				
$^{14}CO_2$	1.651±0.458	1.175±0.358	5.419±0.333	4.376±0.342
Covalent Binding	0.396±0.165	0.222±0.054	3.054±0.463	0.790±0.071
Aqueous	0.074±0.024	1.112±0.392	0.392±0.123	5.512±0.878

[a]Reaction vessels contained 2 mg 9,000 g supernatant, or 1 mg cytosolic, or 1 mg microsomal, or 1 mg cytosolic plus 1 mg microsomal protein and 5 mM glutathione (GSH) as indicated. In addition, reaction vessels contained, 3.12 µmol $^{14}CHCl_3$ (specific activity ≈0.5 µCi/µmol added in a volume of 2.5 µl dimethylformamide) 0.1 µmol NADPH, 0.3 µmol $NADP^+$, 0.3 µmol NADH, 4.5 µmol glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase and 0.16 mmol $MgCl_2$ in 0.1 M sodium phosphate buffer, pH 7.4, in a total volume of 0.75 ml. Reaction vessels were gassed with 100% O_2 for 5 min prior to adding $^{14}CHCl_3$, and incubations were conducted at 37°C in an oscillating water bath (100 cycles/min) for 10 min. Incubations were terminated by the injection of 0.75 ml of 10% TCA. Values have been corrected for non-enzymatic metabolism of $^{14}CHCl_3$ using incubations without a NADPH regenerating system. Values are mean ± S.E.M., n=4.

[b]nmol detected/reaction vessel.

[c]nmol/reaction vessel.

Figure 39. Binding spectrum of $CHCl_3$ with hepatic (A) and renal cortical (B) microsomal cytochrome P-450. Each cuvette contained 2 ml of 0.1 M sodium phosphate buffer, pH 7.0, and 2 mg microsomal protein. The baseline was recorded and 12.5 μmol $CHCl_3$ (diluted 1:10 in ethanol) was added to the sample cuvette with a microliter syringe. An equal volume of ethanol was added to the reference cuvette. After mixing, the difference spectrum was recorded. The baseline was subtracted from the change in light absorbance caused by the addition of $CHCl_3$ to the sample cuvettes and the resultant difference spectra were plotted. Spectra were obtained at room temperature.


A - LIVER
+0.02
+0.01
0
-0.01
-0.02
Δ Absorbance
350
400
450
500
550
Wavelength (nm)

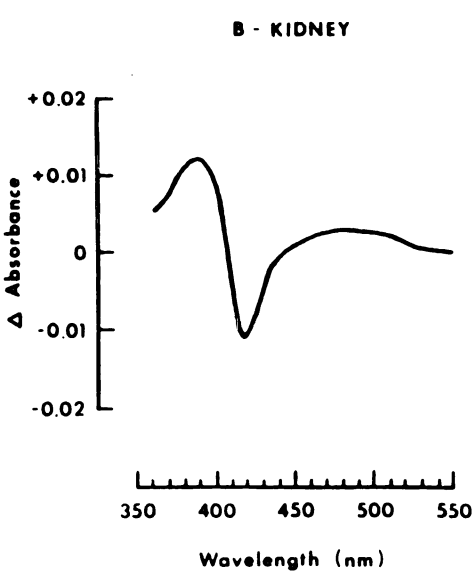

B - KIDNEY
+0.02
+0.01
0
-0.01
-0.02
Δ Absorbance
350
400
450
500
550
Wavelength (nm)

Figure 39

5. Effect of Oxygen Concentration and Carbon Monoxide on $^{14}CHCl_3$ Metabolism

The metabolism of $^{14}CHCl_3$ to $^{14}CO_2$ and covalently bound radioactivity was reduced when the oxygen concentration was reduced from 100% to 20% in the reaction vessel (Figure 40). A 4:1 mixture of N_2 and O_2 reduced microsomal metabolism by approximately 70% for renal cortex and by 30-50% for liver. A 4:1 mixture of CO and O_2 reduced both renal cortical and hepatic microsomal metabolism to a greater extent; metabolism was reduced by approximately 90% in renal cortical and by approximately 80% in hepatic microsomes.

6. Effect of Mixed Function Oxidase Inhibitors on $^{14}CHCl_3$ Metabolism

Inhibitors of cytochrome P-450 did not affect renal cortical microsomal metabolism of $^{14}CHCl_3$ to $^{14}CO_2$ and covalently bound radioactivity in a similar manner (Figure 41). SKF 525-A (10^{-3} and 10^{-4}M) appeared to reduce the metabolism of $^{14}CHCl_3$ to $^{14}CO_2$, but did not affect covalent binding. Metyrapone (10^{-4} and 10^{-5}M) had an opposite effect; covalent binding was decreased with no effect on $^{14}CO_2$ production. Piperonyl butoxide (10^{-4}M) and α-napththoflavone (10^{-4} and 10^{-5}M) also decreased the metabolism of $^{14}CHCl_3$ to covalently bound radioactivity with little or no effect on $^{14}CO_2$ production.

The same phenomenon was observed in hepatic microsomes; inhibition of $^{14}CHCl_3$ metabolism to $^{14}CO_2$ and covalently bound radioactivity was not similar (Figure 42). Covalent binding was decreased markedly by SKF 525-A (10^{-3} and 10^{-4}M) and metyrapone (10^{-4} and 10^{-5}M); there was a tendency to decrease $^{14}CO_2$ but not to a statistically

Figure 40. Effect of carbon monoxide and oxygen concentration on male renal cortical and hepatic microsomal metabolism of $^{14}CHCl_3$ to $^{14}CO_2$ and covalently bound radioactivity. Reaction vessels contained 1 mg microsomal protein, 3.12 μmol $^{14}CHCl_3$ (specific activity ≃0.5 μCi/μmol, added in a volume of 2.5 μl dimethylformamide), 0.1 μmol NADPH, 0.3 μmol $NADP^+$, 0.3 μmol NADH, 4.5 μmol glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 0.16 mmol $MgCl_2$ in 0.1 M sodium phosphate buffer, pH 7.4, in a total volume of 0.75 ml. Reaction vessels were gassed for 5 min prior to adding $^{14}CHCl_3$ with either 100% O_2, N_2:O_2 (4:1 mixture), or CO:O_2 (4:1 mixture). Incubations were conducted at 37°C in an oscillating water bath (100 cycles/min) for 10 min. Incubations were terminated by the injection of 0.75 ml of 10% TCA. Values have been corrected for non-enzymatic metabolism of $^{14}CHCl_3$ using incubations without a NADPH regenerating system. Values are mean ± S.E.M., n=6.

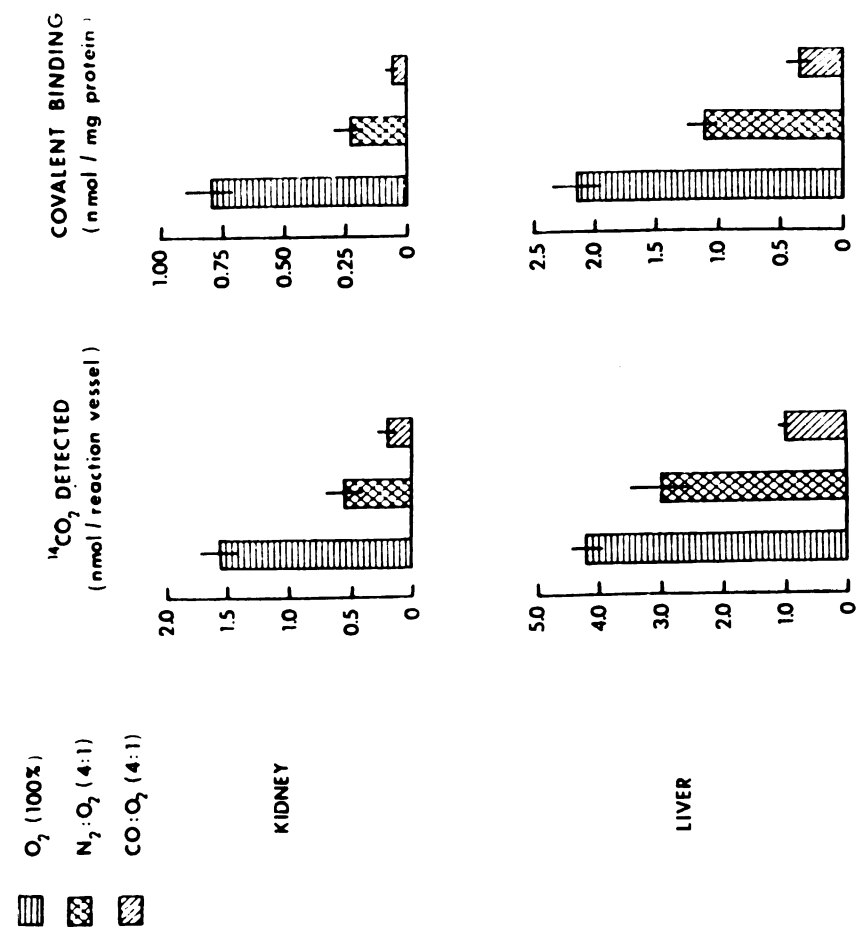

O_2 (100%)
N_2:O_2 (4:1)
CO:O_2 (4:1)
$^{14}CO_2$ DETECTED
(nmol / reaction vessel)
COVALENT BINDING
(nmol / mg protein)
KIDNEY
LIVER
2.0
1.5
1.0
0.5
0
1.00
0.75
0.50
0.25
5.0
4.0
3.0
2.0
1.0
2.5

Figure 40

Figure 41. Effect of cytochrome P-450 inhibitors on male renal cortical metabolism of $^{14}CHCl_3$ to $^{14}CO_2$ and covalently bound radioactivity. Reactions vessels contained 1 mg microsomal protein, 3.12 μmol $^{14}CHCl_3$ (specific activity ≃0.5 μCi/μmol added in a volume of 2.5 μl dimethylformamide), 0.1 μmol NADPH, 0.3 μmol $NADP^+$, 0.3 μmol NADH, 4.5 μmol glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase and 0.16 mmol $MgCl_2$ in 0.1 M sodium phosphate buffer, pH 7.4, in a total volume of 0.75 ml. Stock solutions of inhibitors or vehicle (H_2O or ethanol) were added as 1% of the total reaction volume to achieve the indicated inhibitor concentrations. SKF 525-A and metyrapone were formulated in H_2O; piperonyl butoxide and α-naphthoflavone were formulated in ethanol. Reaction vessels were gassed with 100% O_2 for 5 min prior to adding $^{14}CHCl_3$, and incubations were conducted at 37°C in an oscillating water bath (100 cycles/min) for 10 min. Incubations were terminated by the injection of 0.75 ml of 10% TCA. Values have been corrected for non-enzymatic metabolism of $^{14}CHCl_3$. Metabolism of $^{14}CHCl_3$ by microsomes plus vehicle (Control + H_2O and Control + Ethanol) was expressed as nmol $CHCl_3$ metabolized to $^{14}CO_2$ and covalently bound radioactivity. Metabolism of $^{14}CHCl_3$ by microsomes in the presence of inhibitors was expressed as % of the appropriate vehicle control. Values are mean ± S.E.M., n=5. Statistical significance was determined by calculating the 95% confidence interval for microsomal reactions containing inhibitors. ✱Significantly different from Control + appropriate vehicle, $p<0.05$.

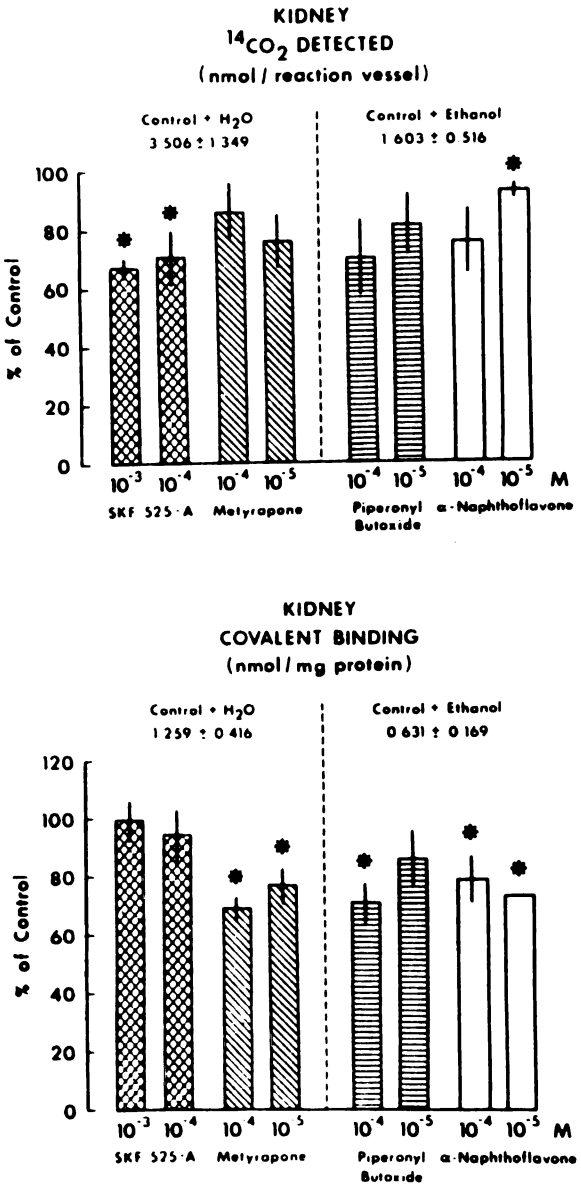

KIDNEY
$^{14}CO_2$ DETECTED
(nmol / reaction vessel)
Control + H_2O
3 506 ± 1 349
Control + Ethanol
1 603 ± 0 516
% of Control
100
80
60
40
20
0
10^{-3} 10^{-4} 10^{-4} 10^{-5} 10^{-4} 10^{-5} 10^{-4} 10^{-5} M
SKF 525-A
Metyrapone
Piperonyl Butoxide
α-Naphthoflavone
KIDNEY
COVALENT BINDING
(nmol / mg protein)
Control + H_2O
1 259 ± 0 416
Control + Ethanol
0 631 ± 0 169
% of Control
120
100
80
60
40
20
0
10^{-3} 10^{-4} 10^{-4} 10^{-5} 10^{-4} 10^{-5} 10^{-4} 10^{-5} M
SKF 525-A
Metyrapone
Piperonyl Butoxide
α-Naphthoflavone

Figure 41

Figure 42. Effect of cytochrome P-450 inhibitors on male hepatic metabolism of $^{14}CHCl_3$ to $^{14}CO_2$ and covalently bound radioactivity. Reactions vessels contained 1 mg microsomal protein, 3.12 μmol $^{14}CHCl_3$ (specific activity ≈0.5 μCi/μmol added in a volume of 2.5 μl dimethylformamide), 0.1 μmol NADPH, 0.3 μmol $NADP^+$, 0.3 μmol NADH, 4.5 μmol glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase and 0.16 mmol $MgCl_2$ in 0.1 M sodium phosphate buffer, pH 7.4, in a total volume of 0.75 ml. Stock solutions of inhibitors or vehicle (H_2O or ethanol) were added as 1% of the total reaction volume to achieve the indicated inhibitor concentrations. SKF 525-A and metyrapone were formulated in H_2O; piperonyl butoxide and α-naphthoflavone were formulated in ethanol. Reaction vessels were gassed with 100% O_2 for 5 min prior to adding $^{14}CHCl_3$, and incubations were conducted at 37°C in an oscillating water bath (100 cycles/min) for 10 min. Incubations were terminated by the injection of 0.75 ml of 10% TCA. Values have been corrected for non-enzymatic metabolism of $^{14}CHCl_3$. Metabolism of $^{14}CHCl_3$ by microsomes plus vehicle (Control + H_2O and Control + Ethanol) was expressed as nmol $CHCl_3$ metabolized to $^{14}CO_2$ and covalently bound radioactivity. Metabolism of $^{14}CHCl_3$ by microsomes in the presence of inhibitors was expressed as % of the appropriate vehicle control. Values are mean ± S.E.M., n=5. Statistical significance was determined by calculating the 95% confidence interval for microsomal reactions containing inhibitors. ✱Significantly different from Control + appropriate vehicle, $p<0.05$.

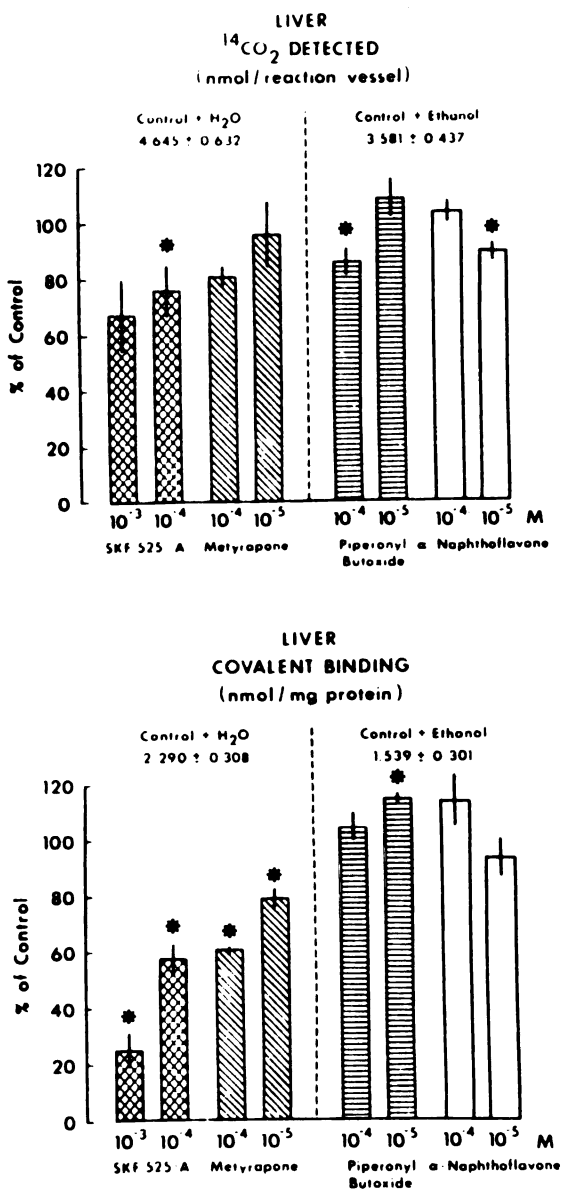

LIVER
$^{14}CO_2$ DETECTED
(nmol / reaction vessel)
Control + H_2O
4 645 ± 0 632
Control + Ethanol
3 581 ± 0 437
% of Control
120
100
80
60
40
20
0
10^{-3} 10^{-4} 10^{-4} 10^{-5} 10^{-4} 10^{-5} 10^{-4} 10^{-5} M
SKF 525 A
Metyrapone
Piperonyl Butoxide
α Naphthoflavone
LIVER
COVALENT BINDING
(nmol / mg protein)
Control + H_2O
2 290 ± 0 308
Control + Ethanol
1 539 ± 0 301
% of Control
120
100
80
60
40
20
0
10^{-3} 10^{-4} 10^{-4} 10^{-5} 10^{-4} 10^{-5} 10^{-4} 10^{-5} M
SKF 525 A
Metyrapone
Piperonyl Butoxide
α Naphthoflavone

Figure 42

significant degree. Neither piperonyl butoxide (10^{-4} and 10^{-5}M) nor α-naphthoflavone (10^{-4} and 10^{-5}M) decreased covalent binding, though there were similar decreases in $^{14}CO_2$ production.

7. Effect of Strain Differences on Renal Cortical and Hepatic Mixed Function Oxidases and $^{14}CHCl_3$ Metabolism by Microsomes

Renal cortical cytochrome content and mixed function oxidase activities (ethoxycoumarin-O-deethylase and $^{14}CHCl_3$ metabolism) were similar statistically in C57Bl/6, DBA/2 and ICR strains of male mice (Table 15). Female ICR mouse renal cortical cytochrome content and mixed function oxidase activities were significantly less than male mice of the three strains; there was a trend for increasing activities from C57Bl/6J to DBA/6 to male ICR mice.

In contrast to renal cortical microsomal measurements, the only statistically significant differences detected in hepatic microsomes was a greater content of cytochrome b5 in female ICR mice than in male C57Bl/6, DBA/2 and ICR mouse strains (Table 16).

TABLE 15

Strain Differences - Renal Cortical Cytochrome Content and Metabolism[a]

	Female ICR	Male C57Bl/6	Male DBA/2	Male ICR
Cytochrome P-450 (nmol/mg protein)	0.05 ±0.01	0.16 ±0.02	0.24 ±0.03	0.30 ±0.05
Cytochrome b-5 (nmol/mg protein)	0.17 ±0.02	0.30 ±0.02	0.38 ±0.02	0.38 ±0.02
Ethoxycoumarin-O-Deethylase (nmol/mg protein/min)	0.042±0.010	0.148±0.025	0.200±0.021	0.158±0.018
$^{14}CHCl_3$ METABOLISM				
$^{14}CO_2$ Detected (nmol/reaction vessel/10 min)	0.752±0.052	2.624±0.304	3.538±0.472	4.768±0.558
Covalent Binding (nmol/mg protein/10 min)	0.408±0.036	1.522±0.204	1.820±0.156	2.228±0.298

[a]Cytochromes P-450 and b5 concentrations and ethoxycoumarin-O-deethylase activities were determined as described in METHODS. $^{14}CHCl_3$ metabolism was determined in reaction vessels containing 1 mg microsomal protein, 3.12 µmol $^{14}CHCl_3$ (specific activity ≈0.5 µCi/µmol, added in a volume of 2.5 µl dimethylformamide), 0.1 µmol NADPH, 0.3 µmol $NADP^+$, 0.3 µmol NADH, 4.5 µmol glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 0.16 mmol $MgCl_2$ in 0.1 M sodium phosphate buffer, pH 7.4, in a total volume of 0.75 ml. Reaction vessels were gassed with 100% O_2 for 5 min prior to adding $^{14}CHCl_3$, and incubations were conducted at 37°C in an oscillating water bath (100 cycles/min) for 10 min. Incubations were terminated by the injection of 0.75 ml of 10% TCA. Values have been corrected for non-enzymatic metabolism of $^{14}CHCl_3$. Values underscored by the same line are not significantly different, $p<0.05$. Values are mean ± S.E.M., n=4.

TABLE 16

Strain Differences - Hepatic Cytochrome Content and Metabolism[a]

	Female ICR	Male C57Bl/6	Male DBA/2	Male ICR
Cytochrome P-450 (nmol/mg protein)	1.35 ±0.11	1.16 ±0.06	1.19 ±0.10	1.37 ±0.58
Cytochrome b-5 (nmol/mg protein)	0.65 ±0.03*	0.45 ±0.05	0.47 ±0.05	0.49 ±0.01
Ethoxycoumarin-O-Deethylase (nmol/mg protein/min)	3.743±0.356	3.150±0.346	2.592±0.239	2.758±0.224
$^{14}CHCl_3$ METABOLISM				
$^{14}CO_2$ Detected (nmol/reaction vessel/10 min)	6.652±1.006	6.872±0.554	8.072±0.806	6.794±1.130
Covalent Binding (nmol/mg protein/10 min)	4.096±0.738	3.664±0.550	4.636±0.728	3.516±0.850

[a]Cytochromes P-450 and b5 concentrations and ethoxycoumarin-O-deethylase activities were determined as described in METHODS. $^{14}CHCl_3$ metabolism was determined in reaction vessels containing 1 mg microsomal protein, 3.12 µmol $^{14}CHCl_3$ (specific activity ≈0.5 µCi/µmol, added in a volume of 2.5 µl dimethylformamide), 0.1 µmol NADPH, 0.3 µmol $NADP^+$, 0.3 µmol NADH, 4.5 µmol glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 0.16 mmol $MgCl_2$ in 0.1 M sodium phosphate buffer, pH 7.4, in a total volume of 0.75 ml. Reaction vessels were gassed with 100% O_2 for 5 min prior to adding $^{14}CHCl_3$, and incubations were conducted at 37°C in an oscillating water bath (100 cycles/min) for 10 min. Incubations were terminated by the injection of 0.75 ml of 10% TCA. Values have been corrected for non-enzymatic metabolism of $^{14}CHCl_3$. *Significantly different from male mice of the three strains, $p<0.05$. Values are mean ± S.E.M., n=4.

DISCUSSION

A. In Vivo Evidence for Renal Metabolism of $CHCl_3$

Assessments of acute toxicity commonly are made 24 hr after exposure to a toxicant. Such evaluations often include determination of structural changes or accumulation of toxic substances (such as creatinine or urea) and enzymes released into the blood (such as SGPT) or urine as a result of cell toxicity, lysis and death. The present studies indicated that nephrotoxicity could be detected as early as 2 hr after $CHCl_3$ administration as decreased ability of renal cortical slices to accumulate PAH and TEA (Figure 10).

$CHCl_3$ and other model toxicants, such as bromobenzene and acetaminophen, which are metabolized to reactive electrophilic intermediates result in hepatotoxicity subsequent to a marked decrease of normal cellular glutathione concentrations and subsequent to increased amounts of covalent binding to other cellular macromolecules (Docks and Krishna, 1976; Brown et al., 1974a; Ekström and Högberg, 1980; Mitchell et al., 1973; Jollow et al., 1974). In fact, one of the methods used to demonstrate hepatic metabolism of $CHCl_3$ to phosgene, the presumed electrophilic intermediate, has been to quantitate the production of diglutathionyl dithiocarbonate, which results from the interaction of phosgene with glutathione (Pohl et al., 1981).

Theoretically, at some time after cellular glutathione concentrations are decreased substantially, covalent binding of the electrophilic intermediate interferes with normal cellular function and results in toxicity. The sequence of events observed to occur in the liver after $CHCl_3$ administration in this study was consistent with this scheme (Figures 6-8).

The mechanism of $CHCl_3$ nephrotoxicity and metabolism by the kidney has not been established. Discrepancies between *in vivo* and *in vitro* covalent binding and the inability to alter renal covalent binding *in vitro* with conditions that alter hepatic cytochrome P-450-dependent metabolism suggested that the mechanism of $CHCl_3$ toxicity in liver and kidney is different (Ilett *et al.*, 1973). More specifically, prior to this investigation it had been suggested that the kidney may lack the ability to metabolize $CHCl_3$ directly (Ilett *et al.*, 1973). Nevertheless, the relationship of temporal events in liver and kidney observed in this study suggested that $CHCl_3$ was metabolized by both liver and kidney. For example, the decrease of renal cortical non-protein sulfhydryl concentrations after $CHCl_3$ *in vivo* paralleled and slightly preceded the decrease in renal cortical slice accumulation of PAH and TEA (Figures 9 and 10). This parallel decrease of non-protein sulfhydryl concentrations and organic ion accumulation in the renal cortex shortly after $CHCl_3$ administration suggested the *in situ* formation of a reactive intermediate within the kidney that resulted in toxicity, possibly similar to what has been observed in the liver (Pohl, 1979). Also, like the liver, there was no evidence of histopathological changes by light microscopy until

maximal decreases of non-protein sulfhydryl concentrations and functional changes were produced 4 to 5 hr after $CHCl_3$ administration (Figures 9, 10 and 12).

A decrease of tissue non-protein sulfhydryl concentrations subsequent to administration of a toxic chemical can occur by several mechanisms including conjugation with a reactive intermediate, cellular redox toxicity, and/or alterations in metabolism of glutathione. Decreases of hepatic non-protein sulfhydryl concentrations after $CHCl_3$ administration in rats coincided with the occurrence of hepatotoxicity (Brown et al., 1974a; Docks and Krishna, 1976), which probably occurred as a result of the interaction of phosgene with glutathione to form diglutathionyl dithiocarbonate (Pohl et al., 1981). Likewise, phenobarbital increased hepatic metabolism of $CHCl_3$, the rate of glutathione depletion and the amount of diglutathionyl dithiocarbonate formed (Pohl et al., 1981). Renal glutathione concentrations in male mouse kidney were decreased after $CHCl_3$ administration and nephrotoxicity could be potentiated when renal non-protein sulfhydryl concentrations were reduced by diethyl maleate administration (Figure 9, Table 3; Kluwe and Hook, 1981). Because the sequence and time course of events relating to $CHCl_3$ toxicity in liver and kidney of male mice appear to be similar, the mechanism of $CHCl_3$ metabolism to a reactive intermediate in the kidney may be similar to that occurring in the liver. While diethyl maleate pretreatment reduced hepatic and renal non-protein sulfhydryl concentrations by 90% and 73%, respectively, and potentiated hepatic and renal toxicity in male mice and hepatic toxicity in female mice, there was no indication of any nephrotoxicity

in female mice 24 hr after $CHCl_3$ administration (Tables 2 and 3). The lack of $CHCl_3$ nephrotoxicity in female mice suggested that the female mouse kidney did not have the metabolic capability to form reactive metabolites from $CHCl_3$. Thus, the use of female mouse renal cortical tissue provided a negative control with which to characterize the relationship between renal metabolism and toxicity in male mice.

The susceptibility of male and female mice to $CHCl_3$ nephrotoxicity appeared to be related to renal cytochrome P-450 concentrations, which were dramatically altered by testosterone. The effect of testosterone to induce susceptibility to $CHCl_3$ nephrotoxicity in female mice and the effect of castration to diminish or eliminate susceptibility to $CHCl_3$ nephrotoxicity are similar to previous reports (Table 4-9; Figures 16-20) (Eschenbrenner and Miller, 1945; Deringer et al., 1953; Culliford and Hewitt, 1957; Taylor et al., 1974). The inherent differences between male and female mice, the induction in female and male mice by testosterone, and the reduction in castrated male mice of renal mixed function oxidases are consistent with the concept of renal cytochrome P-450-dependent metabolism of $CHCl_3$ to a nephrotoxic metabolite. While testosterone is known to have a number of anabolic effects on mouse kidneys and conceivably could increase the activity of some other as yet unidentified enzymes involved in $CHCl_3$ metabolism, the correlation between renal cytochrome P-450 concentrations and susceptibility to $CHCl_3$ nephrotoxicity cannot be ignored.

The mouse kidney seems to be unique in its response to testosterone. Other species, notably the rat, more commonly show alterations of hepatic enzyme activity in response to testosterone administration or castration (Booth and Gillette, 1962; Kato et al., 1962; Kato and Onoda, 1970). Mouse hepatic cytochrome content and mixed function oxidase activity did not appear to be affected by testosterone treatment in these experiments (Table 4 and 5). The relationship of renal ethoxycoumarin-O-deethylase activity to testosterone concentrations suggested that testosterone may induce a phenobarbital-type cytochrome P-450 in mouse kidneys that is distinct from the hepatic form of phenobarbital-induced cytochrome P-450, since mouse renal mixed function oxidases are not induced by phenobarbital (Kluwe et al., 1978).

$CHCl_3$ nephrotoxicity was not altered in mice after a 60-70% partial hepatectomy when compared to intact male mice (Figures 13-15). These data provided further evidence in support of direct renal metabolism of $CHCl_3$ to produce nephrotoxicity independent of hepatic metabolism. If hepatic metabolism were important for the manifestation of $CHCl_3$-induced nephrotoxicity, it could be argued that the 30-40% of liver remaining in partially hepatectomized mice could produce sufficient $CHCl_3$ metabolites to result in nephrotoxicity. However, the results of the $CHCl_3$ metabolism and toxicity experiments in vitro indicated the kidney does metabolize $CHCl_3$ to nephrotoxic metabolites independent of the liver.

B. In Vitro Evidence for Renal Metabolism of $CHCl_3$

The nephrotoxicity of $CHCl_3$ *in vitro* assessed by the ability of renal cortical slices to accumulate organic ions was similar to that observed after the administration of $CHCl_3$ *in vivo* in several respects. Firstly, the time required for expression of toxicity *in vitro* was 1 to 1.5 hr (Figure 17); decreases of PAH and TEA S/M *in vivo* were evident by 2 hr after a 250 µl/kg (3.1 mmol/kg) subcutaneous injection of $CHCl_3$ (Figure 10). Secondly, the concentration of $CHCl_3$ required to produce an effect on organic ion accumulation *in vitro* (3 to 25 µmol) (Figure 22) were of the same order of magnitude as would be calculated to reach the kidney after *in vivo* administration of $CHCl_3$. For example, PAH and TEA accumulation were reduced in male mice in a dose-related manner after subcutaneous injection of 50 to 250 µl/kg (0.6 to 3.1 mmol/kg) (Figure 5). In a 30 g mouse, the dose of $CHCl_3$ administered would be approximately 18 to 94 µmol. Since the kidneys receive approximately 20-25% of the total cardiac output, approximately 4 to 24 µmol of $CHCl_3$ would be delivered to the two kidneys (approximately 500 mg including medulla and papilla) in a first pass assuming instantaneous and complete absorption of the dose. This amount of $CHCl_3$ was comparable to that producing a decrease of PAH and TEA S/M ratios *in vitro* (100 mg of renal cortical slices exposed to 3 to 25 µmol). Thirdly, the sex-related differences *in vitro* (Figures 21 and 22) were consistent with observations *in vivo* of $CHCl_3$-induced nephrotoxicity. PAH and TEA S/M ratios were reduced by $CHCl_3$ *in vitro* in slices from male mice with little or no effect on slices from

female mice. Therefore, the effects of $CHCl_3$ *in vitro* on organic ion accumulation do not appear to be mediated by non-specific solvent effects of $CHCl_3$ on tissue viability but, rather, reflect a specific biochemical lesion. Similarly, only male mice were susceptible to the nephrotoxic effects of $CHCl_3$ administered to the intact animal. Thus, the use of slices preincubated with $CHCl_3$ *in vitro* appeared to be an appropriate model to use in characterizing the mechanism(s) of renal $CHCl_3$ metabolism and toxicity without the influence of hepatic metabolism.

In vitro, $CDCl_3$ was less potent than $CHCl_3$ in decreasing the accumulation of PAH and TEA by renal cortical slices (Figure 23), consistent with lesser nephrotoxic effects of $CDCl_3$ following *in vivo* administration (Ahmadizadeh *et al.*, 1981). More importantly, the deuterium effect *in vitro* provided substantial evidence that the kidney could metabolize $CHCl_3$ *in situ* and that cleavage of the C-H bond may be a requisite metabolic step in renal metabolism of $CHCl_3$, as in the liver (Pohl, 1979). The PAH and TEA S/M ratios were not decreased in slices preincubated with 1 µl $CHCl_3$ at 0°C, also suggesting some type of enzymatic metabolism of $CHCl_3$ (Figure 25).

Further evidence that the toxicity of $CHCl_3$ *in vitro* in male kidney slices was the result of direct renal metabolism of $CHCl_3$ was provided by the observation that incubation of slices with $CHCl_3$ under an atmosphere of carbon monoxide diminished the toxic effects of $CHCl_3$ on PAH and TEA accumulation (Figure 24). Inhibition of the toxicity of $CHCl_3$ *in vitro* by carbon monoxide suggested oxidative metabolism of

$CHCl_3$ by cytochrome P-450. However, since these experiments were done in slices with intact cells, carbon monoxide could have bound to other cytochromes and interfered with oxidative metabolism indirectly. Thus, these experiments did not provide definitive evidence that $CHCl_3$ metabolism by renal cytochrome P-450 was necessary for $CHCl_3$-induced nephrotoxicity.

The nephrotoxic effect of $CHCl_3$ *in vitro* on PAH and TEA accumulation was potentiated in renal cortical slices prepared from male mice pretreated with diethyl maleate (Figure 27). This was similar to the potentiation of $CHCl_3$-induced nephrotoxicity *in vivo* when renal cortical glutathione concentrations were reduced by diethyl maleate prior to $CHCl_3$ administration (Kluwe and Hook, 1981; Table 3).

The hepatic $CHCl_3$ metabolites, 2-oxothiazolidine-4-carboxylic acid and diglutathionyl dithiocarbonate, were not nephrotoxic to male mouse renal cortical slices under incubation conditions where equimolar concentrations of $CHCl_3$ were clearly nephrotoxic (Table 10). Furthermore, these metabolites were not nephrotoxic when administered to mice either intravenously or orally at doses in which $CHCl_3$ produced nephrotoxicity (Branchflower and Pohl, 1982; Pohl, personal communication). These results suggested that metabolites of $CHCl_3$ formed in the liver did not contribute to the nephrotoxicity seen after the *in vivo* administration of $CHCl_3$ and that the nephrotoxicity was due to *in situ* renal metabolism of $CHCl_3$.

Both hepatic and renal cortical slices metabolized $^{14}CHCl_3$ as indicated by evolution of $^{14}CO_2$, covalently bound radioactivity and aqueous-soluble metabolites. Formation of $^{14}CO_2$ from $^{14}CHCl_3$ was 4.6

times greater in male liver than male kidney, similar to the magnitude of difference described by Paul and Rubinstein (1963) for rat liver and kidney slices (Table 11). Formation of $^{14}CO_2$ was 1.7 times greater in male than female kidney slices. The extent of covalent binding to liver and kidney did not parallel the degree of metabolism of $^{14}CHCl_3$ to $^{14}CO_2$. Covalent binding was greatest in male kidney slices, followed by male liver slices and then female kidney slices. Interestingly, the degree of covalent binding to tissue slices after a 2 hr *in vitro* incubation was very similar to that reported by Ilett *et al.* (1973) 6 hr after the *in vivo* administration of 3.72 mmol $^{14}CHCl_3$/kg to male and female mice. These binding data were the first direct indication that the kidney could metabolize $CHCl_3$ *in situ* to a reactive intermediate. The discrepancy between covalent binding and $^{14}CO_2$ formation, and metabolism of $^{14}CHCl_3$ to $^{14}CO_2$ by female kidney slices, suggested that the ability of a tissue to metabolize $^{14}CHCl_3$ to $^{14}CO_2$ may not be related quantitatively to the susceptibility of the tissue to toxicity. These differences between $^{14}CO_2$ formation and the extent of covalent binding will be discussed below.

Incubation of slices under an atmosphere of carbon monoxide decreased the metabolism of $^{14}CHCl_3$ (Figure 30) under similar experimental conditions as those observed to reduce the toxicity of $CHCl_3$ in renal cortical slices (Figure 24). The data suggested that renal metabolism of $CHCl_3$ to a nephrotoxic intermediate was mediated by cytochrome P-450.

In the pathway of $CHCl_3$ metabolism by the liver, $CHCl_3$ is metabolized by a cytochrome P-450-dependent mechanism, probably to trichloromethanol, which spontaneously decomposes to phosgene (Figure 2). Phosgene is believed to be the reactive intermediate that covalently binds to tissue macromolecules. The spontaneous decomposition of phosgene to CO_2 is probably the major source of CO_2 production in $CHCl_3$ metabolism, although the further metabolism of other $CHCl_3$ metabolites may contribute to the production of CO_2.

Several explanations were examined for the large discrepancy between $^{14}CO_2$ production in the liver and kidney in relation to the similar degree of covalent binding in the two organs. First, it was determined that 20 μmol of NaOH in the CO_2 trap was sufficient for the recovery of $^{14}CO_2$ in kidney slices. However, increasing the NaOH to 50 μmol resulted in greater $^{14}CO_2$ detection in incubations with liver slices (data not shown). Therefore, all subsequent reactions were conducted with 50 μmol NaOH in the CO_2 trap. Secondly, determination of the aqueous soluble $^{14}CHCl_3$ metabolites in liver and male kidney indicated that there were actually more aqueous soluble metabolites generated by the liver than the kidney (Table 10). This could not account for the apparent differences in $^{14}CHCl_3$ metabolism. Finally, the incorporation of $^{14}CO_2$ by liver and kidney slices was compared. At least 2 times more $^{14}CO_2$ was incorporated by male kidney than liver slices; female kidney slices incorporated more $^{14}CO_2$ than did male kidney slices (Table 17). Thus, if $^{14}CHCl_3$ is metabolized to $^{14}CO_2$,

TABLE 17

Assessment of $^{14}CO_2$ Incorporation by Male Hepatic and Renal Cortical Slices and Female Renal Cortical Slices from ICR Mice[a]

	$^{14}CO_2$ Incorporation (nmol/100 mg tissue)
Male Liver	0.134±0.030
Male Kidney	0.288±0.005
Female Kidney	0.514±0.047
Boiled	0.010±0.001

[a]Reaction vessels contained 100 mg of tissue slices in 2.0 ml of phosphate buffer, pH 7.4, containing 96.7 mM NaCl, 7.4 mM sodium phosphate buffer, 40 mM KCl and 0.74 mM $CaCl_2$. Reaction vessels were gassed with 100% O_2 for 5 min. Flasks were sealed with a sleeve-type rubber septum with a removable center well containing approximately 85 nmol (specific activity 10 μCi/μmol) ^{14}C-sodium carbonate. $^{14}CO_2$ was liberated in the sealed flask by injecting 100 μl of 0.1 M HCl directly into the center well. Incubations were conducted at 37°C in an oscillating water bath (100 cycles/min) for 2 hr. Incubations were terminated by the injection of 2.0 ml of 10% TCA. Slices and media were homogenized and extracted for determination of covalent binding as described in METHODS. Values are expressed as nmol $^{14}CO_2$ incoprorated per 100 mg of tissue slices and are mean ± S.E.M., n=4.

some of the apparent differences in covalent binding could be the result of different capacities of various tissues to incorporate $^{14}CO_2$.

Another potential difference could be the extent of radioactivity covalently bound to other cell fractions that were not assessed in this investigation, such as lipids and nucleic acids. However, the extent of covalent binding to these fractions in the kidney appears to be less than to protein (Clemens et al., 1979).

C. Evidence for Cytochrome P-450 Mediated $CHCl_3$ Metabolism in the Renal Cortex

A major finding of this study was that a microsomal fraction of renal cortex could metabolize $CHCl_3$ and that this metabolism appeared to be mediated by cytochrome P-450. Several lines of evidence support the involvement of cytochrome P-450 in the renal metabolism of $CHCl_3$. $^{14}CHCl_3$ was metabolized to $^{14}CO_2$ and covalently bound radioactivity by microsomes prepared from male mouse liver and renal cortex; little or no $^{14}CHCl_3$ metabolism by female renal cortical microsomes was detected (Table 13,; Figures 31 and 32). Comparison of $^{14}CHCl_3$ metabolism by subcellular fractions of male renal cortex and liver indicated that metabolism occurred in the microsomal fraction; very little metabolism was detected in the cytosol fraction alone (Table 14).

Renal cortical microsomal $^{14}CHCl_3$ metabolism was dependent on incubation time, microsomal protein concentration and substrate concentration (Figures 31-35). NADPH was required for metabolism of $^{14}CHCl_3$; only low rates of metabolism were observed in renal microsomes in the presence of NADH alone (Figure 37).

Further evidence for the involvement of cytochrome P-450 in the kidney was the inhibition of microsomal $^{14}CHCl_3$ metabolism when incubations were conducted under an atmosphere of carbon monoxide (Figure 40). Additionally, $CHCl_3$ produced a type I binding spectrum with oxidized microsomes from male renal cortex similar to the spectrum produced with oxidized hepatic microsomes (Figure 39).

Metabolism of $^{14}CHCl_3$ by renal cortical and hepatic microsomes was dependent on the presence of oxygen. In these studies, renal cortical and hepatic metabolism of $^{14}CHCl_3$ was assessed routinely under an atmosphere of 100% O_2. Decreasing the concentration of oxygen in the incubation vessel from 100 to 20% reduced the renal microsomal metabolism of $^{14}CHCl_3$ by approximately 70% (Figure 40). Metabolism of $^{14}CHCl_3$ by hepatic microsomes was reduced only by 30 to 50% when the oxygen concentration was decreased from 100 to 20%. This effect of oxygen concentration may explain partially the apparent discrepancy with previous investigations assessing microsomal metabolism of $^{14}CHCl_3$. In earlier studies, incubations were conducted under an atmosphere of air (approximately 20% O_2) and indicated little or no microsomal metabolism of $^{14}CHCl_3$ by the kidney in contrast to the liver (Ilett et al., 1973; Clemens et al., 1979).

The differences between renal cortical and hepatic microsomal metabolism of $^{14}CHCl_3$ suggested that different forms of cytochrome P-450 within the liver and kidney may mediate $CHCl_3$ metabolism. Differences in the degree of $CHCl_3$ metabolized in relation to the oxygen concentration has been discussed previously (Figure 40). Another striking difference was the observation that NADH alone could support

40% of the $CHCl_3$ metabolism observed in the presence of NADPH in liver microsomes; in renal cortical microsomes NADH supported only 5% of the metabolism observed in the presence of NADPH (Figures 37 and 38).

The effects of various cytochrome P-450 inhibitors on renal cortical and hepatic microsomal metabolism of $CHCl_3$ also suggested that different forms of cytochrome P-450 within the liver and kidney mediate $CHCl_3$ metabolism (Figures 41 and 42). Inhibitors often did not produce parallel decreases in $^{14}CHCl_3$ metabolism to $^{14}CO_2$ and covalent binding, which complicated interpretations of the observations. For example, piperonyl butoxide previously had been reported to reduce renal and hepatic toxicity and covalent binding of $^{14}CHCl_3$ in mice (Ilett et al., 1973; Kluwe and Hook, 1981). Covalent binding was significantly reduced by 10^{-4}M piperonyl butoxide in renal cortical microsomes and $^{14}CO_2$ production was significantly reduced in hepatic microsomes. SKF 525-A previously had been reported to enhance renal and hepatic toxicity of $CHCl_3$ in vivo (Kluwe and Hook, 1981). In these studies, SKF 525-A (10^{-3} and 10^{-4}M) markedly reduced covalent binding in hepatic microsomes with little or no significant effect on $^{14}CO_2$; $^{14}CO_2$ production was decreased in renal cortical microsomes with no effect on covalent binding. The concentrations of inhibitors used in these experiments had been reported to inhibit mouse renal and hepatic microsomal metabolism of model substrates (Kluwe et al., 1982). Perhaps less variability would have resulted if the inhibitors had been preincubated with the microsomal fractions prior to adding $^{14}CHCl_3$. Nevertheless, these inhibitor studies provided further

evidence for a role of cytochrome P-450 in the metabolism of $^{14}CHCl_3$ by renal cortical microsomes. The apparent inconsistencies in response to the inhibitors may have reflected the presence of multiple forms of cytochrome P-450 that occur in liver and kidney.

Mouse strain differences were assessed for further correlative evidence between renal cortical cytochrome content and the ability to metabolize $CHCl_3$. Male mice of the C57Bl/6 and DBA/2 strains were reported to be relatively resistant and sensitive, respectively, to the nephrotoxicity of $CHCl_3$ *in vivo* (Hill *et al.*, 1975; Hill, 1979; Clemens *et al.*, 1979). These strains were compared to female and male ICR mice which are resistant and sensitive, respectively, to the nephrotoxicity of $CHCl_3$ *in vivo* (Figure 1) and *in vitro* (Figure 22). Susceptibility of renal cortical slices to $CHCl_3$-induced nephrotoxicity *in vitro* and the ability of renal cortical microsomes to metabolize $^{14}CHCl_3$ were related (Figure 28; Table 15). However, C57Bl/6 and DBA/2 mice appeared to be similar and ICR male mice appeared to be somewhat greater in susceptibility to $CHCl_3$ nephrotoxicity and the ability to metabolize $^{14}CHCl_3$. This apparent discrepancy merely may reflect spontaneous genetic differences occurring in the strains of mice bred by various animal suppliers over the years. Again, there were basically no differences in hepatic cytochrome content or $^{14}CHCl_3$ metabolism among the three strains (Table 16). These data are consistent with observations *in vivo* of hepatic and renal toxicity and further support the independent metabolism *in situ* of $CHCl_3$ by target organs in the mechanism of toxicity. Furthermore, the absence of

testosterone effects and strain differences in hepatic cytochrome content and $^{14}CHCl_3$ metabolism (Tables 4, 5 and 16), in contrast to the kidney, suggested that $CHCl_3$ may be metabolized by a different form of cytochrome P-450 in the kidney than in the liver.

The presence of 5 mM glutathione in renal cortical and hepatic microsomal reactions decreased the amount of $^{14}CHCl_3$ covalently bound to the trichloroacetic acid-precipitable protein; there was a corresponding increase of radioactive metabolites soluble in the aqueous phase (Table 14). This increase of aqueous soluble metabolites in the presence of microsomes plus glutathione may represent the formation of a phosgene and glutathione conjugate, diglutathionyl dithiocarbonate (Pohl et al., 1981). This would be consistent with recent observations by Pohl and coworkers who have identified phosgene as a metabolite of $CHCl_3$ in kidney homogenates from DBA/2 male mice in the presence of glutathione (Pohl, personal communication). Thus, these data suggested that renal metabolism of $CHCl_3$ to phosgene by cytochrome P-450 is a requisite step in the mechanism of $CHCl_3$ toxicity in the kidney.

D. Speculation

Covalent binding after administration in vivo of a radiolabelled model toxicant often has been used as an indication of metabolism by and toxicity to a specific organ (Jollow et al., 1973; Ilett et al., 1973; Gillette, 1974; Boyd et al., 1975). In the case of $CHCl_3$, a similar degree of covalent binding to liver and kidney microsomal protein was observed after in vivo $^{14}CHCl_3$ administration (Ilett et

al., 1973); however, covalent binding to renal microsomal protein in vitro was only 6 to 12% of the covalent binding to hepatic microsomal protein in vitro (Ilett et al., 1973; Clemens et al., 1979).

The data in Figure 40 suggested that optimal metabolism of a xenobiotic by renal cortical cytochrome P-450 in vitro may require greater concentrations of oxygen than metabolism by hepatic cytochrome P-450. This may be similar to the situation that exists in vivo. The renal cortex receives a very high proportion of blood flow and, therefore, oxygen in relation to its mass (Maher, 1976). Furthermore, cytochrome P-450 activity in the kidney appears to be greatest in the renal cortex, particularly in the proximal tubules (Fowler et al., 1977; Zenser et al., 1978; Rush et al., 1983), the site of $CHCl_3$ toxicity. In the liver, the centrilobular region is the site of $CHCl_3$ toxicity. This region of the liver contains a high proportion of a phenobarbital-inducible form of cytochrome P-450, but is believed to receive a relatively lower oxygen concentration than that delivered to the periportal region where blood first enters the liver lobules (Baron et al., 1978; James et al., 1981; Sweeney, 1981; Matsumura and Thurman, 1982).

The data from these studies may suggest that specific cells within the proximal tubules can metabolize xenobiotics to a greater extent and are, therefore, more susceptible to toxicity than liver cells for certain toxicants. Traditionally, microsomal metabolism is expressed as nmol substrate metabolized per mg microsomal protein. By this calculation, hepatic metabolism of $^{14}CHCl_3$ was approximately twice that of renal cortical $^{14}CHCl_3$ metabolism (Table 13). However,

it is not surprising that more metabolism of $^{14}CHCl_3$ was measured in hepatic microsomes since there was approximately four times more hepatic cytochrome P-450 per mg microsomal protein than there was renal cytochrome P-450 per mg microsomal protein (Table 13). Thus, when metabolism was expressed as nmol $CHCl_3$ metabolized per nmol cytochrome P-450, there appeared to be greater renal metabolism of $CHCl_3$ (Table 13). Therefore, in those kidney cells with cytochrome P-450, there may be more $CHCl_3$ metabolism (and toxicity) than in liver.

In rabbits, induction of renal cytochrome P-450 concentrations with phenobarbital and 2,3,7,8-tetrachlorodibenzo-p-dioxin produced proliferation of smooth endoplasmic reticulum specifically in the pars recta (or S_3) segment of the proximal tubule (Zenser et al., 1978; Rush et al., 1983). Assuming that specific forms of cytochrome P-450 are localized in mouse proximal tubule cells, these calculations suggest that individual cells in the kidney may be equally or more responsive than liver cells. Therefore, nephrotoxicity of agents requiring metabolic activation actually may represent toxicity resulting from in situ metabolism, and not merely from a concentration phenomenon unique to the kidney.

Comparison of $^{14}CHCl_3$ metabolism by female renal cortical slices and microsomes may indicate there is an additional mechanism for $^{14}CHCl_3$ metabolism in the kidney that is not mediated by microsomal cytochrome P-450. The metabolism of $^{14}CHCl_3$ to $^{14}CO_2$ is similar for male and female renal cortical slices, yet there is little or no

metabolism of $^{14}CHCl_3$ by female renal cortical microsomes (Tables 11 and 12). However, if such an alternative metabolism pathway does exist, it does not appear to be related to toxicity (and/or to generation of a reactive intermediate) since female mice are not susceptible to $CHCl_3$-induced nephrotoxicity and since there is much less covalent binding in female than in male renal cortical slices.

E. Conclusions

The results from this study indicate that nephrotoxicity was related to the *in situ* metabolism of $CHCl_3$ by the kidney. $CHCl_3$ toxicity occurring in the liver and kidney appeared to be independent events. Furthermore, metabolic studies in renal cortical slices and microsomes indicated that $CHCl_3$ was metabolized by cytochrome P-450 in the renal cortex. Phosgene appeared to be a reactive metabolite of $CHCl_3$ in the kidney, as in the liver, based on the similarities between conditions that alter hepatic and renal cortical metabolism.

SUMMARY

The purposes of this investigation were two-fold: (1) To test the hypothesis that the kidney metabolizes $CHCl_3$; and (2) To characterize the mechanism of $CHCl_3$-induced nephrotoxicity. The mouse was used as the model species in order to utilize the dramatic sex differences in susceptibility to $CHCl_3$-induced nephrotoxicity.

$CHCl_3$ toxicity was assesed in male and female ICR mice as an indication of whether renal and hepatic toxicity were independent events. Nephrotoxicity in male mice could be detected as early as 2 hr after $CHCl_3$ administration (250 µl/kg, s.c.) as decreased ability of renal cortical slices to accumulate the organic ions p-aminohippurate (PAH) and tetraethylammonium (TEA). The decrease was preceded and paralleled by a reduction of renal cortical non-protein sulfhydryl concentrations (an index of tissue reduced glutathione concentrations). Histological alterations were not observed until non-protein sulfhydryl concentrations and PAH and TEA accumulation had reached the nadir, 5 hr after $CHCl_3$ administration. Female mice exhibited no evidence of nephrotoxicity, even when the dose was increased to 1000 µl/kg or when pretreated with diethyl maleate to reduce renal cortical non-protein sulfhydryl concentrations prior to $CHCl_3$ injection. The extent of hepatotoxicity was similar in male and female mice and

decreases of hepatic non-protein sulfhydryl concentrations also were detected by 1.5 hr after $CHCl_3$ administration. The rapid response of the kidney to $CHCl_3$ toxicity in male mice and the similarity of liver toxicity in both sexes suggested that nephrotoxicity occurred independently of hepatotoxicity. Furthermore, 50 to 70% partial hepatectomy did not alter the nephrotoxic response to $CHCl_3$.

There were sex-related differences in renal cytochrome P-450 and b5 concentrations and in ethoxycoumarin-O-deethylase activity in mouse kidneys; in all cases activity was higher in males. Castration of male mice eliminated susceptibility to $CHCl_3$ nephrotoxicity and reduced renal mixed function oxidases to concentrations observed in female mice. Treatment of male and female mice with testosterone increased the susceptibility to $CHCl_3$ nephrotoxicity and increased renal mixed function oxidases to similar activities in both sexes. These data were consistent with cytochrome P-450-dependent metabolism of $CHCl_3$ by the kidney.

The ability to detect early nephrotoxic changes *in vivo* following $CHCl_3$ administration was used to develop an *in vitro* model to evaluate the mechanism of $CHCl_3$ nephrotoxicity. Preincubation of renal cortical slices with $CHCl_3$ from male, but not female, mice resulted in a subsequent decrease of the ability of slices to accumulate PAH and TEA. These sex-related differences, the time required for manifestation of this effect (60-90 min), and the concentration dependency (0-50 μmol, 0-4 μl $CHCl_3$) were similar to *in vivo* observations on $CHCl_3$ nephrotoxicity in mice. Furthermore, an equimolar concentration of deuterated-$CHCl_3$ *in vitro* was less effective than $CHCl_3$ in decreasing

PAH and TEA accumulation in male renal cortical slices. The effects of $CHCl_3$ on PAH and TEA accumulation could be diminished or blocked by preincubation with $CHCl_3$ in the presence of carbon monoxide or at 0°C, respectively. The nephrotoxicity of $CHCl_3$ *in vitro* was increased in renal cortical slices from male mice pretreated with diethyl maleate. Thus, this *in vitro* model using mouse renal cortical slices and the sex-related differences in $CHCl_3$ nephrotoxicity suggested that the kidney may metabolize $CHCl_3$ *in situ* to a nephrotoxic metabolite.

Under similar incubation conditions, $^{14}CHCl_3$ was metabolized to $^{14}CO_2$, covalently bound radioactivity and aqueous soluble metabolites by male hepatic and renal cortical slices; a smaller degree of metabolism occurred in renal cortical slices from female mice. Metabolism was reduced when slices were incubated with $^{14}CHCl_3$ under an atmosphere of carbon monoxide (80% CO:20% O_2), suggesting cytochrome P-450-mediated metabolism.

Metabolism of $^{14}CHCl_3$ by microsomes prepared from renal cortex and liver provided definitive evidence for a role of cytochrome P-450 in the renal metabolism and toxicity of $CHCl_3$. $^{14}CHCl_3$ was metabolized to $^{14}CO_2$ and covalently bound radioactivity by male renal cortical microsomes; metabolism required oxygen, a NADPH regenerating system, was dependent on incubation time, microsomal protein concentration and substrate concentration, and was inhibited by carbon monoxide. No metabolism of $^{14}CHCl_3$ by female renal cortical microsomes was detected. $CHCl_3$ produced a type I binding spectrum with oxidized male renal cortical and hepatic microsomes. Incubation of

glutathione with microsomes and $^{14}CHCl_3$ increased the amount of aqueous soluble metabolites detected, suggesting the formation of a phosgene conjugate as has been described for hepatic $CHCl_3$ metabolism.

These data support the hypothesis that renal cytochrome P-450 metabolizes $CHCl_3$ to a nephrotoxic intermediate.

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