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thesis entitled

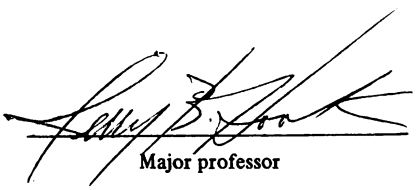
HALOGENATED ALIPHATIC HYDROCARBON
NEPHROTOXICITY

presented by

William Michael Kluwe

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Pharmacology
& Toxicology



Major professor

Date 5-1-79

HALOGENATED ALIPHATIC HYDROCARBON NEPHROTOXICITY

By

William Michael Kluwe

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology & Toxicology

1979

ABSTRACT

Halogenated Aliphatic Hydrocarbon Nephrotoxicity

by

William Michael Kluwe

Dietary ingestion of polybrominated biphenyls (PBB) and polychlorinated biphenyls (PCB) increased renal and hepatic aryl hydrocarbon hydroxylase (AHH) activities in a dietary concentration-dependent manner. Mixed-function oxidase (MFO) activities were also induced in liver and kidney by i.p. administration of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 3-methylcholanthrene (3MC), but sodium phenobarbital (NaPb) increased hepatic MFO activities only.

Renal and hepatic AHH activities and cytochrome P-450 (P-450) concentrations in male, Fischer 344 rats were increased by treatments with PBB, PCB and 3MC. NaPb increased hepatic AHH activity and P-450 content only. The rates of increase (and decline to normal values) of AHH activities following single oral doses of PBB, PCB and 3MC were much greater in the kidney than in the liver.

Treatment with 3MC increased the susceptibilities of renal and hepatic AHH to inhibition by α-napthflavone (ANF) in vitro while NaPb increased the susceptibility of hepatic but not renal AHH to inhibition by metyrapone (MET). PBB and PCB increased the susceptibility of renal AHH to inhibition by ANF but did not alter the susceptibility of hepatic AHH to inhibition by ANF or MET. Renal AHH was significantly

less susceptible than hepatic AHH to inhibition by SKF 525-A and MET. Low concentrations of ANF stimulated hepatic AHH activity in vitro but inhibited renal AHH activity. Renal AHH activities were less susceptible than hepatic AHH activities to reduction by i.p. administration of SKF 525-A and piperonyl butoxide (PB).

Treatments of mice with PBB and PCB potentiated the nephrotoxicity and hepatotoxicity of carbon tetrachloride (CCl_4) in rats and mice. PBB, PCB and HCB also increased total lipid content of the liver but not the kidney.

Treatments of mice with PBB and NaPb increased the hepatotoxicity of chloroform ($CHCl_3$). $CHCl_3$ nephrotoxicity was increased by PBB but decreased by PCB, 3MC and TCDD.

Preadministration of PB reduced the toxicity of $CHCl_3$ in mice. Administration of SKF 525-A before (120 min) $CHCl_3$, and SKF 525-A or PB after (60 min) $CHCl_3$, potentiated $CHCl_3$ toxicity.

$CHCl_3$ depleted renal and hepatic glutathione (GSH) in intact mice in a dose-dependent manner. PBB enhanced $CHCl_3$ depletion of renal and hepatic GSH. PCB blocked $CHCl_3$ depletion of renal GSH but did not alter $CHCl_3$ depletion of hepatic GSH. Diethyl maleate reduced renal and hepatic GSH concentrations and increased the susceptibility of mice to $CHCl_3$ toxicity.

Incubation of (^{14}C)-$CHCl_3$ with renal and hepatic microsomes resulted in the covalent binding of radioactivity to microsomal protein (290 pmoles/mg protein/5 min, liver; 15 pmoles/mg protein/5 min, kidney). Hepatic microsomes from PBB and PCB treated mice bound more radioactivity than hepatic microsomes from control mice. Renal

microsomes from PCB treated mice bound more radioactivity than renal microsomes from control and PBB treated mice.

Radioactivity was covalently bound to renal and hepatic endoplasmic reticulum (ER), mitochondria (M), cytoplasmic protein (CP) and nuclear RNA and DNA following i.p. administration of (^{14}C)-$CHCl_3$ to mice. The magnitude of binding to hepatic M and CP was increased by pretreatment with PBB and PCB but binding to renal ER, M, CP, RNA and DNA was decreased. Clearance of radioactivity from venous blood was a first-order process and occurred more rapidly in PBB and PCB pretreated mice than in control mice.

The results of this dissertation suggest that the nephrotoxicities of halogenated aliphatic hydrocarbons may depend, in part, on biotransformation to a metabolite which is responsible for the toxicity. The site of toxic metabolite formation may be liver, kidney or both.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to Drs. Steven Aust, Theodore Brody, Bernard Schwetz and Robert Roth for constructive criticism of the dissertation research and especially to Dr. Jerry Hook for moral support and intellectual guidance throughout my graduate studies at Michigan State University. I would also like to thank Forest Clark, Cathy Herrmann, Robert McNish, Harriett Sherman and Kenneth Smithson for their excellent technical assistance.

Most of all I would like to thank Cathy, who stood fast beside me through some exciting, but often difficult, times. Much of this dissertation is owed to her patience and soothing presence.

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INTRODUCTION

A. The Kidney as a Target Organ

1. Incidence and costs of human nephropathy

It is generally believed among health professionals that renal dysfunction is a major factor in human disease, either as the primary cause of disease, a contributing factor, or as a major symptom. Though accurate estimates of the overall incidence of kidney-related maladies are not currently available, more than 12 million persons in the United States alone are known to suffer from chronic debilitating diseases of the kidney and urinary tract (including prostatic enlargement, urolithiasis, chronic urinary tract infection and neuromuscular disorders of bladder control), and renal failure is the probable cause of death in an estimated 80,000 to 100,000 fatalities yearly (DHEW-NIH, 1978).

In addition to the human suffering wrought, kidney-related diseases are quite probably a significant contributing factor to rapidly rising health care costs. Though total expenditures for the diagnosis and treatment of kidney-related diseases are not known, the cost of a single federal program providing Medicare benefits to approximately 40,000 victims of end-stage renal disease (32,556 renal dialysis patients and 4,450 recipients of kidney transplants) was greater than 1 billion dollars in 1977 and is expected to rise to 3 billion dollars by 1984 (DHEW-NIH, 1978).

These indications of the high incidence and economic costs of human nephropathies suggest that laboratory and clinical investigations leading to the elucidation of the causes and consequences of renal injury as well as to its prevention may be of great benefit to mankind. Although the percentage of clinical nephropathies originating from or exacerbated by occupational and environmental exposure to nephrotoxicants is not known, the great variety of chemicals demonstrated to be nephrotoxic in experimental animals and their wide use in medicine, agriculture and manufacturing industries indicate that the potential for chemically induced nephrotoxicity is great.

2. Pathophysiology of toxic nephropathy

Chemically induced toxic nephropathy was defined by Schreiner and Maher (1965) as an "adverse functional or structural change in the kidney due to the effect of a chemical . . . inhaled, injected, ingested or absorbed, or which yields toxic metabolites with an identifiable effect on the kidney". While the consequences of such toxic insults to the kidney can be many and varied, the most important, perhaps, is renal failure, a condition in which the regulation of body fluid and solute balance is lost. Prolonged renal failure is incompatible with continued survival and artificial means (hemodialysis) must be employed if the victim is to survive.

Renal failure in humans is characterized by oliguria (less than 400 ml urine per day) or anuria, a low urine to plasma urea and creatinine ratio (urine is isotonic), and a high fractional sodium excretion (Levinsky, 1977). Renal resistance is increased with a concomitant decrease in renal blood flow. Such nonspecific signs

provide few hints of the etiology of this condition. Renal failure has been produced in experimental animals by administration of a variety of chemicals and mechanical constriction of the renal artery. Two general models of acute renal failure have emerged from studies on experimental animals. In the first, the vasoconstrictor model, increased resistance in the pre-glomerular vasculature appears to be responsible for tubular ischemia and resultant cellular hypoxia and the loss of energy-dependent tubular functions (Levinsky, 1977; Stein et al., 1978). In the other, the nephrotoxic model, tubular dysfunction appears to be caused by direct disruptive effects of nephrotoxicants on epithelial tubular cells, primarily those of the proximal tubule (Levinsky, 1977; Stein et al., 1978). It is generally believed that renal failure in humans, as a clinical entity, most likely encompasses aspects of both experimental models and that renal ischemia and tubular necrosis are interdependent phenomena (Levinsky, 1977). Acute renal failure may gradually revert to normal renal function when exposure to the toxicant is stopped, but the condition may be permanent if the initial injury is severe.

Although morphological and functional characteristics of experimentally induced toxic nephropathy have been described in detail (Biber et al., 1968; Dach and Kurtzman, 1976; McDowell et al., 1976), the animal studies reported to date have failed to clearly identify critical subcellular lesions and mechanisms of injury. However, it is likely that the initiating event in many cases occurs on the molecular level and that the overall response of the kidney to the initial lesion may be determined by the types of cells affected,

the quantitative amount of tissue damage produced and, in the case of immature animals, developmental status of the kidney at the time of exposure to the toxicant. The mature kidney, for example, is composed of anatomically segregated groups of cells with highly specialized functions, and even damage limited to a relatively small, discrete population of cells may upset the functional interrelationships between diverse cell groups and produce a generalized loss of functional renal capacity. The fetal kidney, in contrast, is both anatomically and functionally immature and insult occurring during this period generally manifests as structural anomalies such as renal agenesis, renal cysts or hydronephrosis (Gibson, 1976). The relative amount of tissue damage initially produced may also determine the overall renal response to chemical insult because the normal kidney contains a significant functional reserve capacity that allows for the maintenance of normal kidney function despite the loss of functional tissue. The kidney, furthermore, is capable of limited regeneration of damaged tubular epithelium (Foulkes and Hammond, 1975). The effects of these factors on renal response to molecular lesions greatly complicate detection of renal injury and elucidation of the pathophysiology of toxic nephropathies.

3. Aspects of renal structure and function predisposing to kidney injury

Several factors contribute to the overall sensitivity of the mammalian kidney to chemical injury. These include a high rate of perfusion, high oxygen demand, active and passive transport of chemicals across tubular epithelium and the serial arrangement of the

nephrons. Although human kidneys comprise only 4% of body weight, they receive nearly 25% of resting cardiac output, most in the cortical region, enhancing the possibility that renal cells will be exposed to large amounts of blood-borne toxicants. In addition, the kidney has a high rate of oxygen consumption and is very sensitive to cellular anoxia (Berndt, 1976; Venkatachalam et al., 1978). Equally important in determining sensitivity to toxicants is the anatomical and functional segmentation of the nephron, the basic unit of the kidney. In brief, each nephron is composed of a vascular, a glomerular, and a tubular component. The vascular component regulates the initial flow of blood to the nephron but also remains closely associated with the tubular component with which it exchanges fluids and solutes (Tischer, 1976). Filtration of fluids and solutes from capillary blood into the tubular lumen occurs in the glomerular component and absorption, secretion and excretion of fluid and solute occurs largely in the tubular component. Because of the serial arrangement of the nephron components direct toxicant-induced damage and loss of normal function in one nephron component may indirectly alter function in the other components and produce a generalized loss of functional renal capacity.

The tubule appears to be the part of the nephron that is most sensitive to chemically induced injury (Schreiner and Maher, 1965). One reason for this sensitivity may be that renal tubular cells are exposed to much higher concentrations of certain toxicants than are other cells. For example, nearly 99% of the fluid volume filtered at the glomerulus is reabsorbed across tubular epithelium, most in the

proximal portion of the tubule. Thus, proximal tubular cells are exposed to large quantities of filtered, reabsorbed toxicants and cells of the pars recta as well as cells of the distal tubule are exposed to high concentrations of filtered, non-reabsorbed toxicants. Furthermore, the countercurrent multiplier system, dependent on high medullary tissue oncotic pressure and low medullary blood flow, may lead to the development of prolonged, high concentrations of toxicants in the renal medullary interstitium. Additionally, high intracellular concentrations of toxicants may be produced by the active transport of toxicants from blood or tubular fluid into tubular cells. Even toxicants tightly bound to plasma proteins may be accumulated intracellularly by active transport mechanisms (Foulkes and Hammond, 1975). Finally, cells of the proximal tubule contain cytochrome P-450 and several mixed-function oxidases (MFOs) that are potentially capable of metabolizing xenobiotic chemicals to reactive, toxic products (Uehleke and Greim, 1968; Fowler et al., 1977; Kluwe et al., 1978). These properties, therefore, may be largely responsible for the high sensitivity of renal tubular cells to chemical toxicity.

4. Nephrotoxic chemicals

A great number of chemicals with diverse structural and physical characteristics have been identified as nephrotoxicants. Many of these compounds are of great importance to medicine, agriculture and the manufacturing industries (Schreiner and Maher, 1965; Hook et al., 1978a). In addition, some food additives and food contaminants, many naturally occurring fungus-derived food contaminants and recognized pollutants of air and water produce renal injury when

administered to experimental animals (Suzuki et al., 1975; Krogh et al., 1976; Thacker and Carlton, 1977; Ross et al., 1978). However, the lack of sensitive, non-invasive diagnostic techniques for detecting functional renal damage and the absence of a centralized system for the reception and assimilation of reports concerning suspected cases of chemically induced nephropathy may delay or prevent the recognition of the nephrotoxic effects of many such chemicals in man. For these reasons it is difficult to assess the importance of nephrotoxic chemicals in the etiology of human kidney disease.

Therapeutic agents recognized as nephrotoxicants include many non-narcotic analgesics, general anesthetics, x-ray contrast materials, and numerous antibiotics (Mazze et al., 1976; Appel and Neu, 1977; Hook et al., 1978a). Except for non-narcotic analgesics, access to nephrotoxic drugs generally is restricted to persons under medical care and the hazard of renal injury can be minimized by judicious drug use and careful monitoring for signs of renal dysfunction.

Nephrotoxic agents to which exposure generally occurs in the absence of medical supervision, that is, the non-therapeutic chemicals, include many metals (As, Bi, Cd, Cr, Hg, Pb, Pt, Ur), organic solvents, glycols, monomeric chemicals (e.g., vinyl chloride), chemical flame retardants, pesticides and fungal toxins (Plaa and Larson, 1965; Schreiner and Maher, 1965; Berndt and Hayes, 1977; Kociba et al., 1977; Osterberg et al., 1977; Hook et al., 1978a; Ross et al., 1978). Although attempts have been made to identify chemical properties and structures common to nephrotoxicants, such endeavors to date have not been fruitful.

B. Metabolic Activation of Toxicants

1. Reactive metabolites and tissue injury

In 1947 Miller and Miller reported that the administration of several chemical carcinogens to rats resulted in covalent binding of the carcinogens to hepatic proteins. The initial studies documenting the covalent binding of carcinogens to genetic molecules were published ten years later (Wheeler and Skipper, 1957). Subsequent studies have demonstrated that nearly all chemical carcinogens bind covalently to macromolecules; either in their native form (alkylating agents) or after metabolism to electrophilic products (Miller, 1970; Cavalieri et al., 1978). Current evidence indicates that the non-specific alkylation or arylation of critical informational macromolecules may be an initiating event in the neoplastic transformation of mammalian cells by carcinogenic chemicals (Miller and Miller, 1974; Miller and Miller, 1977). Enzymatic metabolism within mammalian cells, furthermore, may largely determine the carcinogenic potential of certain classes of chemicals. Qualitative and quantitative differences in the metabolism of foreign compounds in various tissues and species appear to contribute greatly to the tissue-specificities and species-specificities of chemical carcinogens (Bartsch et al., 1977).

More recently, the metabolism of xenobiotic chemicals to toxic, reactive intermediates has been implicated in chemically induced mutagenesis, teratogenesis and necrogenesis and in the development of certain blood dyscrasias and immunological disorders (Brodie, 1967; Ames et al., 1973; Gillette et al., 1974). It has been proposed, for example, that strong electrophilic products of xenobiotic

metabolism covalently bind to nucleophilic sites on cellular macromolecules in a nonspecific manner (Gillette, 1974). Sufficient alkylation of essential macromolecules may lead to cell dysfunction and cell death. Relationships between the generation of reactive metabolites and the development of acute tissue injury have been studied most extensively in the rodent (primarily rat and mouse) liver, where positive correlations have been demonstrated between the binding of reactive metabolites to hepatic proteins and lipids and the disruption of membrane structures, loss of hepatocyte function and hepatocellular necrosis (Gillette, 1977). Few positive correlations between alkylation in non-hepatic organs and acute tissue injury, however, have been reported (Mitchell et al., 1977). Although direct cause and effect relationships between covalent binding and acute hepatic injuries have not been unequivocally demonstrated, many investigators have proposed that covalent binding may be a mechanism of toxicity and that the generation of reactive intermediates is the critical step in the activation of many hepatotoxicants (Gillette, 1974; Mitchell and Jollow, 1975; Thorgeirsson and Wirth, 1977).

2. Locations and functions of drug-metabolizing enzyme (DME) systems

Xenobiotic chemicals undergo at least four basic types of metabolism in mammals; oxidation, reduction, hydrolysis and conjugation. The metabolic activation of many toxicants is believed to be mediated primarily by cytochrome P-450-dependent MFOs, a heterogenous group of membrane-bound enzymes that catalyze the oxidation of numerous endogenous and exogenous compounds. MFO-mediated oxidations include: aliphatic and aromatic hydroxylations, N and O

dealkylations, N oxidations and hydroxylations, S demethylation, sulfoxidation, desulfuration and the deamination of primary and secondary amines (Goldstein et al., 1974). It is evident, therefore, that MFOs can accommodate a great variety of substrates.

Hepatic MFOs have been studied extensively in vitro. Homogenates of liver can be fractionated to obtain endoplasmic reticulum (ER) membrane fragments (microsomes) containing high specific activities of MFOs. By such investigations it has been determined that MFO reactions consume molecular oxygen and reduced pyridine nucleotides (NADPH) and require a specific hemoprotein (or a group of related hemoproteins) that acts as a terminal oxidase in the enzymatic reaction, and an unidentified lipid component (Goldstein et al., 1974). Cytochrome P-450 (P-450), the hemoprotein, is so-named because the difference spectrum of the reduced CO:hemoprotein complex displays an absorption maximum at 450 nm. Several forms of P-450 have been isolated from rat liver (Grasdalen et al., 1975; Ryan et al., 1975; Guengerich, 1977; Mailman et al., 1977; Ullrich and Kremers, 1977), and each appears to have different affinities for MFO substrates. This has led to speculation that quantitative and qualitative MFO activities within a single tissue, as determined by in vitro investigations with microsomes, may be regulated in part by the relative concentrations of the different forms of P-450 present in the tissue (Ullrich and Kremers, 1977).

P-450-dependent, MFO-mediated oxidations, as studied in vitro, consume one mole of molecular oxygen and two reducing equivalents (2 electrons) for each mole of substrate oxidized. The products

of the reaction are an oxidized substrate molecule and a mole of H_2O. In brief, the reaction is thought to occur in the following manner: the substrate binds to oxidized P-450, this complex is reduced by an electron from a reduced flavoprotein molecule (the electron is originally from NADPH), molecular oxygen "binds" with the reduced P-450-substrate complex and another electron (from NADPH or NADH) is introduced, whereupon the complex decomposes and releases oxidized substrate (containing an atom of oxygen from molecular oxygen), oxidized P-450 and H_2O (from the reduction of an atom of oxygen) (Goldstein et al., 1974). The wide substrate specificity of the P-450-dependent MFO system allows for the oxidative metabolism of a great variety of chemicals, both endogenous and exogenous, by a single enzyme system.

P-450-dependent MFOs are generally located in cellular membranes, primarily in the ER but also in the mitochondria and the nuclear envelope (Ghazarian and DeLuca, 1974; Kashnig and Kasper, 1969). Mitochondrial MFO activities are particularly high in the adrenal cortex where extensive steroid hydroxylation occurs (Zampaglione and Mannering, 1973). Although MFO systems have been identified in many extrahepatic tissues (lung, kidney, adrenal, intestinal epithelium, skin, gonads, placenta) the specific activities of MFOs in hepatic ER, in general, are much greater than those in extrahepatic organs (Litterst et al., 1975, 1977; Fry et al., 1978). Many substrates for MFOs are lipid soluble, a property which may facilitate diffusion across the plasma membrane and dissolution into the ER membrane prior to binding to P-450.

Non-microsomal oxidations and microsomal reductions have been less well-characterized. Their relationships to the metabolic activation of toxicants is not presently clear, though the metabolism of carbon tetrachloride to a reactive species appears to be mediated by microsomal reduction (Uehleke and Werner, 1975; Sipes et al., 1977). Enzymatic hydrolysis, on the other hand, is generally restricted to esters and amides (Goldstein et al., 1974) though the presence of an enzyme in the ER membrane that catalyzes the hydrolysis of epoxides (epoxide hydratase) has been reported recently and appears to be of great toxicological significance (Brooks, 1977; Oesch et al., 1977). Synthetic reactions include glucuronidation (a microsomal reaction), acetylation (a non-microsomal reaction), mercapturic acid formation (non-microsomal) and sulfotransferase reactions (non-microsomal).

DMEs were originally described as "detoxification" systems, largely because the pharmacological activities of many therapeutic drugs were reduced by enzymatic degradation and the excretion of lipophilic toxicants was frequently hastened by enzymatic biotransformation (Goldstein et al., 1974). More specifically, however, DMEs appear to convert lipophilic substrates into more polar products that can be readily excreted in the urine or feces (Goldstein et al., 1974). In certain instances, however, the products of microsomal metabolism, though more polar than the parent compound, may be chemically unstable species that rapidly react with appropriate, proximate, cellular molecules in a nonspecific manner (Gillette, 1977; Follow and Smith, 1977). Examples of such reactions include oxidative

dehalogenations of chlorinated aliphatic hydrocarbons and epoxidations of polycyclic aromatic hydrocarbons (Docks and Krishna, 1976; Pohl et al., 1977; Cavalieri et al., 1978). The metabolites produced by the above reactions are generally strong electrophiles that attack nucleophilic sites on proximate macromolecules in a nonspecific manner (Miller and Miller, 1970, 1974; Gillette, 1974, 1977).

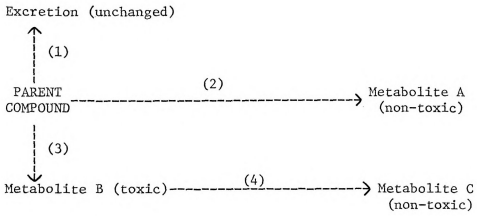
3. Balance between enzymatic toxification and detoxification

The extent of damage produced in a specific tissue by a highly reactive intermediate is probably proportional to the innate sensitivity of the affected tissue to the presence of the reactive species and proportional to the concentration of reactive metabolites in the tissue. Thus, tissues possessing mechanisms to protect against electrophile injury or to repair electrophile damage will probably be less sensitive than tissues without such protective mechanisms. In addition, the molecular lesion produced may be of greater functional consequence in some tissues than in others. Where these factors are equal, however, relative tissue injury may be directly proportional to the amount of reactive metabolite formed within the tissue.

Most P-450-dependent MFO activities appear to be first-order reactions under in vitro conditions (and, presumably, under in vivo conditions) (Goldstein et al., 1974; Jollow and Smith, 1977). The rate of generation of reactive metabolites, therefore, is dependent upon the affinity of the enzyme for the substrate and the amount of substrate available (Gillette, 1977). Availability of substrate (generally the parent compound) in biological systems can be directly

affected by the presence of competing pathways of metabolism and by the excretion of substrate in an unchanged form. In addition, toxic, reactive metabolites can frequently be enzymatically transformed to more stable, non-toxic products (Brooks, 1977; Jollow and Smith, 1977). These principles are illustrated in Figure 1 and have been reviewed recently by Jollow and Smith (1977). The relative activities of enzymatic metabolism to toxic products and to non-toxic products within a specific tissue may determine, to a large extent, the sensitivity of that particular tissue to chemical injury. That is, the balance between enzymatic toxification and detoxification reactions determines the concentration of reactive species present in the tissue at any specific time. Modifications of the relative activities of pathways 1, 2 or 3 in Figure 1 may change the percentage of parent compound metabolized to a toxic product via pathway 3 and,

Figure 1



thereby, cause an increase or decrease in the net amount of metabolite B formed. Similarly, changes in the activities of pathways 3 or

4 may alter the concentration of metabolite B, the proximate toxicant, within the tissue. Therefore, if tissue damage occurs as a result of the actions of a toxic, reactive intermediate on sensitive, cellular macromolecules then the extent of injury produced may be dependent on a set of interrelated pharmacokinetic parameters.

Reactions depicted by pathway 3 in Figure 1 are generally catalyzed by P-450-dependent microsomal MFOs and those of pathway 2 by MFOs or non-oxidative cytosolic enzymes (Jollow and Smith, 1977). Pathway 4 enzymes generally catalyze conjugation reactions (UDP-glucuronyl transferase, glutathione-S-transferases, 3'-phosphoadenosine-5'-phosphosulfate transferase), but may also catalyze hydrations of electrophilic epoxides (Goldstein et al., 1974; Brooks, 1977; Jerina and Bend, 1977). Conjugations with glutathione (glutathione-S-transferases) and sulfate (sulfotransferases) are catalyzed by several different forms of cytosolic enzymes. The fact that many reactive metabolites thought to be generated at the ER are conjugated with glutathione and sulfate by cytosolic enzymes suggests that even reactive metabolites may possess limited mobility within the cell. UDP-glucuronyl transferase and epoxide hydratase, though, are microsomal enzymes and may metabolize reactive intermediates to more stable products prior to relocation from the ER membrane.

Recent investigations have demonstrated that complex aromatic molecules may undergo oxidative metabolism at multiple sites. 3,4-Benzo(a)pyrene, for example, can be oxidized to the 4,5-, 7,8-, or 9,10-epoxides (Conney et al., 1977). Benzo(a)pyrene-7,8-epoxide can then be hydrated to benzo(a)pyrene-7,8-dihydrodiol and this

compound may be subsequently oxidized to benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide, a very potent mutagen and cytotoxicant (Borgen et al., 1973; Sims et al., 1974). Sequential metabolism of chemicals may enable relatively stable metabolites (e.g., benzo(a)pyrene-7,8-dihydrodiol) to travel from the site of formation to other tissues where activation to the proximate toxicant (e.g., benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide) may occur. For such chemicals the reaction sequences illustrated in Figure 1 may oversimplify the relationship of metabolism to toxicity.

4. Tissue-specific metabolic activation

The toxic effects of reactive intermediates are thought by many investigators to result from their chemical instability (Gillette, 1977; Jollow and Smith, 1977). Since biological membranes resist the passage of polar and ionic species, and since reactive chemical moieties exhibit short half-lives, it is unlikely that a truly reactive intermediate formed in one tissue would travel to another tissue and there produce direct injury. Bartsch et al. (1975, 1977), for example, reported a strong correlation between the abilities of various tissues to metabolize N-nitrosamine derivatives to reactive products and the sensitivities of the various tissues to nitrosamine injury. That is, N-nitrosamines appear to be metabolized to proximate toxicants and tissues most active in this biotransformation reaction are, accordingly, most susceptible to nitrosamine injury, implying that a cause and effect relationship exists between metabolic activation and tissue injury. Toxic metabolites with greater chemical stability, however, may produce injury in tissues distant from the site of

›lite formation, especially if distant tissues are more sensitive ›roximate tissues to the molecular effects of the toxic metabo-

The neurotropic carcinogen 3,3-dimethyl-1-phenyltriazene, for le, is metabolized in the liver to a weak alkylating agent, 3- l-1-phenyltriazene, but produces brain tumors rather than hepatic s (Preussmann et al., 1969a,b). Brain tissue does not appear to ate (demethylate) 3,3-dimethyl-1-phenyltriazene (Preussmann, ,b), but exhibits a relative inability, in comparison to the , to excise O^6-methylguanine, a methylated DNA base produced by hyl-1-phenyltriazene in brain and liver DNA that is likely to base mispairing during nucleotide synthesis. The estimated ·life of 3-methyl-1-phenyltriazene, the toxic metabolite, in ›us medium (pH 7.40, 37°C) is slightly greater than one minute. time appears sufficient to allow for transport from the in vivo of formation (liver) to the target site (brain) since 3-methyl- nyltriazene injected i.p. methylated DNA bases in both liver rain (Bartsch et al., 1977). For relatively stable toxic olites such as 3-methyl-1-phenyltriazene there may be little lation between organ-specific biotransformation and organ- fic injury. That is, damage appears to be dependent largely on esponse of the tissue to the molecular lesion.

Biotransformation Capacity of the Kidney

1. Characteristics

DMEs, including P-450-dependent MFOs, are present in kidney ther extrahepatic organs. Many substrates for hepatic microsomal

lation appear to undergo the same types of reactions when incubated
n renal microsomes, though the specific activities of the enzymes
olved are generally much lower in the kidney than in the liver
tterst et al., 1975, 1977; Fry et al., 1978). The subcellular
ations and actions of renal DMEs are generally similar to those
ribed for the liver.

The renal P-450-dependent MFO system in the rat has been
acterized by Jakobsson et al. (1970) and Orrenius et al. (1973).
greement with earlier investigations (Kato, 1966; Ichihara et al.,
), they reported that renal MFOs, like hepatic MFOs, required
ced pyridine nucleotides as cofactors, molecular oxygen, and a
rane-bound hemoprotein. The hemoprotein has been variously
rred to as cytochrome P-450, cytochrome P-450K, and cytochrome
(henceforth referred to as P-450) because of the absorption
ium at 454 nm in the reduced CO:hemoprotein difference spectrum.
ost apparent difference between renal and hepatic MFOs is that
pecific activities of most substrate oxidations and the concen-
ons of P-450 in renal microsomes appear to be only 10-30% of
in hepatic microsomes (Jakobsson et al., 1970; Orrenius et al.,
Litterst et al., 1975, 1977; Fry et al., 1978). In contrast,
ecific activities of ω and ω-1 hydroxylations of fatty acids
arly equal in renal and hepatic microsomes, prompting specula-
hat renal MFOs may primarily oxidize endogenous fatty acids
than lipophilic xenobiotic chemicals (Jakobsson et al., 1970;
et al., 1972; Jakobsson and Cinti, 1973). The current metho-
for assessing renal microsomal MFO activities in vitro is

asically that methodology developed to maximize the specific activi-
ies of hepatic microsomal MFOs. Relatively little has been reported
oncerning the optimization of renal MFO activities in vitro (e.g.,
, temperature, substrates, cofactor concentrations, antioxidant
oncentrations). Thus, renal MFO activities, as currently measured
vitro, may underestimate the biotransformation capacity of the
dney.

Additional differences between renal and hepatic MFOs in
dents (largely mice and rats) include dichotomies in sex-related
fferences in enzyme activities, differential responses to stress
.g., fasting, altered diet), and a resistance of renal MFOs to the
ductive effects of phenobarbital (Litterst et al., 1975, 1977;
abra and Fouts, 1974). Such differences suggest that control of
) activities is organ specific; for example, renal MFO activities
intrarenally controlled.

An alternate explanation for the low activities of MFOs in
al microsomal preparations in vitro may be that such enzymes are
fined to a relatively small population of renal cells. MFOs
ear to be components of the membrane of the smooth ER, an organelle
d in highest renal concentrations in the S_3 cells of the proximal
les. The concentration of S_3 cells is greatest in the pars recta
katachalam et al., 1978), that portion of the proximal tubule with
greatest apparent susceptibility to toxicant damage and the
est activity of active organic ion transport. If P-450-dependent
are largely confined to this renal cell type, then homogenates of
kidney or kidney cortex, either of which contain greatly diluted

ncentrations of the contents of S_3 cells, understandably exhibit low tivities toward most substrates for hepatic microsomal oxidation. support of the localization of high concentrations of P-450-pendent MFOs in S_3 cells, Fowler et al. (1977) have demonstrated at the anatomical distribution of MFO activities within the kidney ossly corresponds to the intrarenal distribution of S_3 cells. In dition, Zenser and Davis (1978) reported that renal MFO activities d P-450 concentration exhibited a cortical-papillary gradient ighest in the cortex and outer medulla and lowest in the papilla), pattern similar to the distribution of S_3 cells in the kidney. yler et al. (1977) also reported that treatment of rats with ,7,8-tetrachlorodibenzo-p-dioxin greatly increased renal MFO acti-ies and increased the amount of smooth ER in S_3 cells, though tomically adjacent S_2 cells and distal tubular cells were not ected. These studies suggest that P-450-dependent microsomal MFO ivities in S_3 cells may be quantitatively similar to those in atic parenchymal cells. Fractionation of microsomes from whole ney homogenates, however, results in a dilution of microsomes from cells and may be responsible for low specific activities of renal .

2. Physiological and toxicological significance of renal biotransformation

The urinary excretion of lipophilic agents is known to be nced by conjugation reactions that increase the polarity of genous and exogenous compounds and retard their passive reabsorp- (Goldstein et al., 1974). The activities of many conjugating

enzymes, as measured by in vitro techniques, are nearly as high in the kidney as in the liver (Chhabra and Fouts, 1974; Litterst et al., 1975; Fry et al., 1978), suggesting that renal enzymes may be important in the excretion of some lipophilic substances. For example, renal biotransformation has been demonstrated to increase the urinary excretion of certain chemicals (Quebbeman and Anders, 1973; Acara et al., 1977) and to alter the activities of some hormonal agents (Blackwell et al., 1975). In addition, the oxidation of 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol, the form of vitamin D most active in promoting intestinal absorption of calcium and bone mineral mobilization (Omdahl and DeLuca, 1971; Tanaka and DeLuca, 1971), appears to occur primarily in the kidney and is mediated by a P-450-dependent mitochondrial MFO (Omdahl and DeLuca, 1973; Ghazarian and DeLuca, 1974, 1977). Thus, renal MFOs may be important in maintaining mineral homeostasis. The kidney also appears to be very active in synthesizing and degrading prostaglandins (Blackwell et al., 1975; Zenser et al., 1977), derivatives of arachidonic acid believed to participate in control of renal function. Thus, renal drug metabolism may be important in maintaining renal function.

The overwhelming capacity of the liver for xenobiotic metabolism clearly makes it the dominant organ in the quantitative biotransformation of toxicants. For reasons specified earlier, however, the kidney may largely be responsible for the generation of chemically reactive metabolites that produce renal injury. Hill et al. (1975), for example, have reported great strain differences in the nephrotoxicity of $CHCl_3$ in male mice, an apparent genetic phenomenon,

though strain differences in the hepatotoxicity of $CHCl_3$ were slight. Similarly, male mice were markedly more sensitive to the nephrotoxicity of $CHCl_3$ than were female mice, though sex differences in the hepatotoxicity of $CHCl_3$ were not apparent (Klaassen and Plaa, 1967). These results may be due to inherent differences in the response of the kidneys in male and female mice and various strains of mice to $CHCl_3$ or to sex-related and strain-related differences in renal biotransformation of $CHCl_3$. Reports indicating that treatment with testosterone, a hormone that alters xenobiotic metabolism, enhanced the susceptibility of female mice to $CHCl_3$ nephrotoxicity without producing morphological changes in the kidney and that castration reduced the sensitivity of male mice to $CHCl_3$ nephrotoxicity suggest that the latter possibility, sex-related differences in renal biotransformation of $CHCl_3$, is the more likely reason for the insensitivity of female mice to $CHCl_3$ nephrotoxicity (Krus et al., 1974). In addition, Ilett et al. (1973) reported that more $CHCl_3$ metabolites were bound in kidneys of male mice than in kidneys of females following i.p. injection of $CHCl_3$ but no sex difference was observed in binding to liver. Thus, renal metabolism may be important in the activation of $CHCl_3$ to a reactive metabolite. Weekes (1975) has reported that a strong correlation exists between the abilities of renal microsomes from several strains of mice to metabolize dimethylnitrosamine to mutagenic products in vitro and the sensitivities of these same strains of mice to dimethylnitrosamine-induced renal tumorigenesis. Thus, renal MFO activities, though low when measured in whole kidney homogenates, may be of toxicological significance.

D. Nephrotoxicity of Halogenated Aliphatic Hydrocarbons

1. Incidence

The chemical and physical characteristics of nephrotoxic compounds are many and varied, as mentioned previously. One commonly used group of chemicals that has consistently proven to be nephrotoxic across a wide spectrum of mammalian species is the halogenated aliphatic hydrocarbons. A partial list of widely-used halogenated aliphatic hydrocarbons reported to be nephrotoxic in experimental animals is contained in Table 1. Although significant incidences of human intoxications resulting in renal failure have been reported for only 3 of these compounds ($CHCl_3$ and CCl_4, Von Oettingen, 1964; methoxyflurane, Mazze, 1976), the lack of extensive documentation of human renal damage produced by the other halogenated aliphatics should not be taken as an indication of their safety. Rather, human exposure to the rest of the compounds in Table 1 may currently be insufficient for the development of human nephropathies or for their detection by insensitive, non-invasive techniques.

Of recent concern is the increasing presence of halogenated aliphatic hydrocarbons, principally halomethanes and haloethanes, in surface waters used for municipal water supplies (Deinzer et al., 978) and the potential consequences to human and environmental ealth of such pollution (Kuzma et al., 1977; Cantor et al., 1978; raybill, 1978). Sources of halogenated aliphatic hydrocarbon contamination of the general environment include agricultural runoff d industrial effluents. Additionally, haloalkanes may be produced unintentional chlorination of organic materials during sewage

TABLE 1

Nephrotoxic Halogenated Aliphatic Chemicals

Chemical	Reference
allyl chloride	Ross et al., 1978
carbon tetrachloride	Ross et al., 1978
chloroform	Ross et al., 1978
dibromochloropropane	Torkelson et al., 1961
1,1-dichloroacetylene	Reichert et al., 1978
1,1-dichloroethylene	Jenkins and Andersen, 1978
1,3-dichloropropene	Torkelson and Oyen, 1977
ethylene dibromide	Ross et al., 1978
hexachlorobutadiene	Ross et al., 1978
methoxyflurane	Mazze, 1976
1,1,2-trichloroethane	Klaassen and Plaa, 1966
trichloroethylene	Klaassen and Plaa, 1966
tris(2,3-dibromopropyl) phosphate	Osterberg et al., 1977
trihalomethanes (most)	Von Oettingen, 1964

sterilization (Deinzer et al., 1978). Contamination of surface waters with trace quantities of chlorinated aliphatic hydrocarbons is of concern, in part, because at least three halogenated aliphatic chemicals of industrial importance, CCl_4, $CHCl_3$ and hexachlorobutadiene, have been reported to produce tumors in rodents ($CHCl_3$ and hexachlorobutadiene produced renal tumors, Kociba et al., 1977; Ross et al., 1978). Furthermore, a positive correlation has been found between the presence of $CHCl_3$ in drinking water and the incidence of renal tumors in males in certain industrialized regions of the United States (Cantor et al., 1978).

Very little, however, is known about the potential risk of neoplastic and non-neoplastic renal injury from long-term exposure to low concentrations of halogenated aliphatic hydrocarbons. Many of the compounds listed in Table 1 are used in chemical manufacturing processes and may be of special toxicological significance to occupational health. Although occupationally associated nephropathies do not appear to be common, two factors may obscure the relationship between occupation and kidney disease. First, there is an inability to detect renal damage with common clinical tests until functional reserve capacity has been overwhelmed (and the victim is near death). Second, there is frequently a lengthy latency period between chemical exposure and recognition of nephrotoxicity. Thus, the actual incidence of chemical nephropathy may be greater than is currently recognized.

In a recently published list, the National Science Foundation ranked organic chemicals according to their potential for producing harm to humans and the environment, based on parameters such as potencies as toxicants, yearly production volumes, rates of release into the environment, and biodegradation (Stephenson, 1977). Of the ten compounds perceived most hazardous, six were halogenated aliphatic hydrocarbons known to be nephrotoxic. Thus, it would seem that there should be concern for the possibility of renal injury resulting from industrial and occupational exposure to halogenated aliphatic hydrocarbons even though the incidence of recognized chemical nephropathy is currently low.

2. Toxicological manifestations

The nephrotoxic effects of CCl_4 were recognized as early as the turn of the century (cited in Smetana, 1939) and clinical descriptions of CCl_4 nephropathy from that time until the present abound in the literature (see Ross et al., 1978). In brief, human ingestion or inhalation of large quantities of CCl_4 (20-400 ml) is generally followed by the rapid onset of nonspecific symptoms such as gastrointestinal disturbances and intense abdominal pain (Smetana, 1939; Schreiner and Maher, 1965). Oliguria or anuria, depending on the severity of the intoxication, develops over the ensuing 3-9 day period. Should the patient survive this period, the anuria progresses to oliguria (which may last for as long as 60 days), then to a transient state of diuresis and finally to normal renal function. Renal biopsies from survivors of CCl_4 intoxications exhibited tubular swelling and nonspecific degenerative cellular changes that were most severe in the

area of the proximal tubules. Microscopic lesions were similar in appearance, but more severe, in renal tissue from CCl_4-induced fatalities examined at the time of autopsy. Frank renal cellular necrosis does not appear to be produced by even fatal amounts of CCl_4 (Smetana, 1939; Von Oettingen, 1964; Schreiner and Maher, 1965). The pathophysiology of CCl_4 intoxication in experimental animals (primarily rats, mice and rabbits) appears to be quite similar to that reported in humans except that the time sequence is shorter. That is, CCl_4-induced renal dysfunction occurs long after elimination of the parent CCl_4 from the body (2-3 days), damage is most severe in the area of the proximal tubules and either the animal dies or renal function slowly returns to normal (Striker et al., 1968).

The clinical manifestations of intoxication with $CHCl_3$ and other halogenated aliphatic hydrocarbons have not been characterized as well as those of CCl_4. Studies with experimental animals, however, suggest that most halogenated aliphatic compounds specifically damage the proximal tubules of the kidney (see references in Table 1). The lesion is evident histologically as a swelling of tubular epithelial cells that progresses, with increasing severity of intoxication, to necrosis and sloughing of tubular cells followed by tubular regeneration and a return to normal renal function or by tissue calcification and terminal renal failure. In contrast to the liver, where signs of intoxication are maximal within 24 hr, renal damage appears to be most severe within 2-7 days after exposure (see references in Table 1). The functional manifestations of halogenated aliphatic hydrocarbon

intoxication are basically those expected following damage to the proximal tubule; proteinuria, glucosuria, increased fractional excretion of sodium and decreased clearance of p-aminohippuric acid (Sirota, 1949; Von Oettingen, 1964; Striker et al., 1968). Although the renal sequelae of intoxication with a great number of halogenated aliphatic compounds appear to be qualitatively similar, it is not known if halogenated aliphatic hydrocarbons produce renal injury by a common mechanism.

3. Mechanisms of toxicity

The strength of the carbon-halogen bond was originally believed sufficient to prevent substantial metabolic degradation of halogenated aliphatic hydrocarbons within mammalian cells. Early references to the pharmacokinetics of halogenated anesthetic gases, for example, implied that such compounds were excreted from the body wholly in an unchanged form. Zeller, however, had reported in 1883 (cited in Rubenstein and Kanics, 1964) that serum chloride concentrations in dogs chronically depleted of chloride were significantly elevated by anesthesia with $CHCl_3$, suggesting that extensive dechlorination had occurred in vivo. Subsequent investigations have clearly demonstrated that substantial dehalogenation of organic compounds can occur in humans and experimental animals and, in fact, indicate that the primary method of excretion of some chlorinated hydrocarbons involves hepatic oxidative dehalogenation (Rubinstein and Kanics, 1964; Leibman, 1965; Brown et al., 1974a). The nephrotoxicity of at least one volatile anesthetic, methoxyflurane, has been attributed to hepatic defluorination and the resulting increase in

erum concentrations of fluoride ion, a nephrotoxic agent (Mazze, 976). The relationships between metabolism of halogenated aliphatic ydrocarbons and their nephrotoxicity, however, have not been ddressed extensively. In contrast, considerable efforts have been evoted to studying the relationship of metabolism to the hepato- oxicity of halogenated aliphatic chemicals. It appears, in many ases, that these compounds are metabolically activated to hepatotoxi- ants. That is, the parent compound, which is relatively innocuous o the liver, is metabolized by hepatic enzymes to toxic products that roduce hepatocellular necrosis (Mitchell and Jollow, 1975). Sensi- ivity of the liver to the damaging effects of many halogenated ali- hatic hydrocarbons can be greatly altered by modulation of the enzyme ystems involved in xenobiotic metabolism (Suarez et al., 1972; arlson, 1975).

Further support for the involvement of metabolites in halo- enated aliphatic hydrocarbon hepatotoxicity is provided by correla- ions between the chemical reactivity of such compounds and their ossibilities to produce liver damage. The hepatotoxicities of equimolar mounts of a series of trihalomethanes ($CHBr_3$ > $CHCl_3$ > CHI_3), for xample, is inversely proportional to the carbon-halogen bond energy -I > C-Cl > C-Br), suggesting that metabolic activation occurs st readily on molecules with labile bonds. Accordingly, increased logenation destabilizes the molecular structures of haloalkanes d increases their hepatotoxicity, but stabilizes the molecular ructures of haloalkenes and decreases their hepatotoxicity (Bonse d Henschler, 1976). The reactive, hepatotoxic metabolites

generally appear to be strong electrophiles that react with nucleophilic sites on cellular macromolecules in a nonspecific manner and may thereby produce cellular dysfunction, as discussed previously.

Relatively little is known about the role of biotransformation in the development of halogenated aliphatic hydrocarbon nephrotoxicity. Ilett et al. (1973) and Reid (1973), however, reported that metabolites of chlorobenzene, bromobenzene and $CHCl_3$ became covalently bound to renal proteins upon i.p. injection of the parent chemicals into mice. Renal binding in intact mice was greatest in the area of the proximal tubules, the site of halobenzene and $CHCl_3$-induced renal damage. It seemed possible, therefore, that renal MFOs, whose activities are greatest in the proximal tubule, may have transformed the parent compounds into reactive, nephrotoxic products in a manner similar to that postulated to occur in the liver. Renal microsomal protein, however, exhibited little or no capacity to generate reactive metabolites in vitro (Ilett et al., 1973; Reid, 1973). The authors speculated that reactive metabolites formed in the liver might have travelled to the kidney as plasma protein-bound complexes where they were released again as reactive, toxic intermediates (Ilett et al., 1973; Reid, 1973). As discussed previously, however, quantitative, in vitro measurement of renal microsomal enzyme activities may underestimate the biotransformation capacities of segregated renal cell types (e.g., S_3 cells). In addition, the techniques of in vitro covalent binding used by Ilett et al. (1973) and Reid (1973) may be of questionable worth (when renal microsomes were used) since Sipes et al. (1977) using the same technique, was

nable to demonstrate a dependency of covalent binding of $CHCl_3$ to enal microsomal protein on the presence of molceular oxygen and -450 and inhibition of covalent binding by CO.

. Purpose

The primary purpose of this investigation was to elucidate the mechanisms by which halogenated aliphatic hydrocarbons produce acute enal injury. For this reason the roles of hepatic and renal biotransformation and covalent binding in the development of halogenated alihatic hydrocarbon nephrotoxicity were evaluated. This required, to certain extent, characterization of the biochemical and physiolgical responses of the liver and kidney to toxic halogenated aliphatic ompounds and determination of the capacities of kidney and liver for enobiotic metabolism, their relative responses to enzyme induction nd inhibition, and the manner in which such responses affected the ephrotoxicities and hepatotoxicities of selected chlorinated alihatic compounds. Attempts were also made to relate pharmacokinetics--sorption, distribution, excretion, and metabolism--to organ-specific xicities in intact animals and to determine the role of changes in armacokinetic behavior in the potentiation and inhibition of halocane toxicity. In addition, the roles of reactive and electrophilic ermediates in $CHCl_3$ nephrotoxicity were investigated.

Insights into the mechanisms of halogenated aliphatic hydrocarbon rotoxicity in rodents may aid in the development of experimental ls with which to study human response to such toxicants. Appropte experimental models are vital for extrapolation of toxicity

data from experimental animals to man. Such knowledge may also aid in predicting the toxicological consequences of chronic exposure to low concentrations of halogenated aliphatic chemicals, the potential for synergistic effects between organohalides, and the potential toxicity of newly-synthesized halogenated aliphatic compounds.

It is hoped that the results of this investigation will aid in understanding the roles of hepatic and renal biotransformation, reactive metabolites, and covalent binding of reactive metabolites in the development of halogenated aliphatic hydrocarbon nephrotoxicity. Additionally, it is hoped that these results can be applied to the assessment of such parameters in human response to nephrotoxicants.

F. Objectives

The specific objectives of this investigation are listed below.

1. To determine the time-dependency and dietary concentration-dependency of induction of drug-metabolizing enzyme systems by dietary ingestion of polybrominated biphenyls and polychlorinated biphenyls.

2. To determine the effects of treatment with sodium phenobarbital, 3-methylcholanthrene, polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin on renal and hepatic drug-metabolizing enzyme systems. These compounds were used to modify chlorinated aliphatic hydrocarbon toxicities.

3. To examine the types of cytochrome P-450 induced in the kidney by polybrominated biphenyls, polychlorinated biphenyls, 3-methylcholanthrene and sodium phenobarbital.

4. To determine the effects of treatment with SKF 525-A and iperonyl butoxide on renal drug-metabolizing enzyme systems. These ompounds were used to modify chloroform toxicity.

5. To evaluate the effects of several inducers of drug-etabolizing enzyme systems (polybrominated biphenyls, polychlorinated iphenyls, hexachlorobenzene) on carbon tetrachloride toxicity.

6. To determine the effects of inhibitors of drug-metabolizing nzyme systems (SKF 525-A, piperonyl butoxide) on chlorform toxicity.

7. To elucidate the relationship between glutathione concen-rations in target tissues and chloroform toxicity.

8. To examine the dependency of organ-specific chloroform oxicity on covalent binding of chloroform metabolites to kidney and iver.

MATERIALS AND METHODS

A. Animals and Experimental Diets

ICR mice and Sprague-Dawley rats were purchased from Spartan Research Animals (Haslett, MI) and Fischer 344 rats from Harlan Industries (Indianapolis, IN). All animals were maintained in sanitary, ventilated animal rooms under controlled humidity, temperature and light-cycle for the duration of the experiments. Unless indicated otherwise, all animals were young adults at the time of use.

Experimental diets were prepared by mixing polybrominated biphenyls (PBB, Firemaster BP-6, Velsicol Chemical Co., St. Louis, MI) or polychlorinated biphenyls (PCB, Aroclor 1254, Monsanto Chemical Co., St. Louis, MO) in acetone slowly and evenly into finely-ground rodent pellets (Wayne Lab Blox, Chicago, IL). Analyses showed that the actual concentrations of PBB and PCB in diets prepared in this manner were within 8% of calculated concentrations. Control diet was formulated by mixing an equivalent amount of acetone into the ground diet.

B. Toxicity Tests

1. Serum analyses

Freshly-drawn whole blood was allowed to clot for 90-120 min at room temperature then carefully centrifuged and the serum fraction withdrawn. Glutamic oxaloacetic transaminase (GOT) and glutamic

pyruvic transaminase (GPT) activities were determined in aliquots of serum (S) using Sigma reagent kits (Sigma Chemical Co., St. Louis, MO) and quantified as Sigma-Frankel units per ml serum. Urea nitrogen concentrations (BUN) were determined in aliquots of serum using a Sigma reagent kit.

2. Renal cortical slice accumulation of PAH and TEA

The abilities of renal cortical slices to accumulate the organic anion para-aminohippurate (PAH) and the organic cation tetraethylammonium (TEA) were determined in the following manner. The animals (rats or mice) were weighed, killed by cervical dislocation or by decapitation and the kidneys removed, decapsulated, weighed and placed in ice-cold saline (0.9% NaCl). Thin slices (approximately 0.5 mm) of renal cortex were cut with a razor blade and 100-150 g of tissue (wet weight) incubated in 2.0 ml of a phosphate-buffered medium (Cross and Taggart, 1950) containing $1x10^{-3}$M acetate, $1x10^{-4}$M TEA (Eastman Organic Chemicals, Rochester, NY), $5.8x10^{-6}$M PAH (Sigma) and trace quantities of (^{3}H)-PAH and (^{14}C)-TEA (New England Nuclear, Boston, MA). The slices were allowed to incubate for 90 min (to reach equilibrium) at 25°C under an atmosphere of 100% O_2. They were then removed, blotted, weighed and homogenized in 1.5 ml of 10% trichloroacetic acid (TCA), brought to a final volume of 5.0 ml with distilled water and mixed thoroughly. A 1.0 ml aliquot of medium was mixed with 1.5 ml of 10% TCA and 2.5 ml of distilled water (total volume of 5.0 ml). The TCA solutions were then centrifuged to separate the precipitated protein and 1.0 ml aliquots of the supernatant fraction added to 10.0 ml of Aqueous Counting Scintillant (ACS,

Amersham Corp., Arlington Heights, IL) and radioactivity determined by liquid scintillation spectrometry. Disintegrations per min (dpm) were determined using (^{14}C)-toluene and (^{3}H)-H_2O (New England Nuclear) as internal standards. The slice-to-medium ratios, or S/M, of PAH and TEA were calculated as dpm/g tissue (wet weight) divided by dpm/ml incubation medium.

3. LD_{50} determinations

LD_{50} values for chloroform ($CHCl_3$) and carbon tetrachloride (CCl_4) were determined in male mice by injecting solutions of $CHCl_3$ or CCl_4 in corn oil (total injection volume of 5 ml/kg) into the peritoneal cavity and determining the cumulative number of deaths every 24 hr thereafter. At least 10 animals were used at each dose. All surviving animals were observed for an additional 18 days after injection of $CHCl_3$ and CCl_4. LD_{50} values, 95% confidence intervals and potency ratios were calculated by the method of Litchfield and Wilcoxin (1949).

4. Histology

Samples of liver and kidney were fixed in buffered formalin (3.7% formaldehyde in 0.3 M sodium phosphate buffer, pH 7.2), embedded in paraffin blocks, sectioned at 5 microns, mounted on glass slides and stained with hematoxylin and eosin under the direction of Dr. V.L. Sanger, Michigan State University, Department of Pathology, East Lansing, Michigan. Black and white photographs were taken at 400X magnification using Ektapan film (Eastman-Kodak Co., Rochester, NY). Histopathological examination was performed by Dr. V.L. Sanger.

For specific experiments, samples of liver and kidney were
lso stained with periodic acid-Schiff (PAS), with and without prior
lastase digestion, and evaluated for epithelial changes by Dr. J.
rnstein, William Beaumont Hospital, Department of Anatomic
thology, Royal Oak, MI, 48072. Formalin-fixed samples of liver were
so stained with Gomori's trichrome, Wilders reticulin, Perl's
rrocyanide for iron, and rubeanic acid for copper. Sections were
aluated for cellular necrosis and vacuolization, nuclear enlargement
olyploidy), inflammatory cell infiltration, and fibrosis or septa-
on on an arbitrary scale of 0-3+ (negative, mild, moderate, severe)
Dr. J. Bernstein. Regenerative activity was estimated by counting
totic figures per 40 random high-power fields.

5. Definitions of toxicity

Elevations of SGPT and SGOT activities were used as indica-
rs of CCl_4 and $CHCl_3$ hepatotoxicity. The hepatotoxic effects of
$_4$ were also defined histologically, in selected experiments, as
atocellular necrosis.

Decreases in PAH and TEA S/Ms were used as indicators of
$_4$ and $CHCl_3$ nephrotoxicity. Intoxication with $CHCl_3$ also in-
ased BUN concentrations and total kidney weight. Kidneys from
$_4$-intoxicated rats were examined by light microscopy but histolo-
al evidence of cellular injury was not detected.

Losses of body weight and death were also used as indicators
toxicity though the mechanism of such effects is unknown.

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C. Analyses of Components of the Drug-metabolizing Enzyme Systems

1. Tissue preparation

Samples of liver and kidney were weighed, minced, washed several times and homogenized in 3 volumes of ice-cold 20 mM Tris-1.15% KCl buffer, pH 7.40, using a motor-driven Potter-Elvehjem tissue grinder (0.10-0.15 mm clearance). The tunica albuginea of the testes were punctured and the extruded contents homogenized in 3 volumes of Tris-KCl buffer as described for livers and kidneys. The homogenates were then centrifuged at 600 x g for 5 min at 2°C to separate nuclei and unbroken cells, and the supernatant fractions centrifuged at 14,000 x g for 25 min at 2°C to separate mitochondria. An aliquot of the 14,000 x g supernatant fraction was removed and refrigerated and the rest of the supernate decanted and centrifuged at 100,000 x g for 0 min at 2°C. The resulting pellets (microsomal fraction) were resuspended in the original volume of 20 mM Tris-1.15% KCl buffer or 20 mM Tris-1.15% KCl-1 mM EDTA, pH 7.40, if the pellet was to be stored for later determination of cytochrome P-450) and centrifuged again at 100,000 x g for 30 min at 2°C. The final pellet was then resuspended in Tris-KCl buffer for enzyme assays or stored at -70°C for 2-3 days until assayed for cytochrome P-450 content.

2. p-Chloro-N-methylaniline N-demethylase (PCNMA) assay

PCNMA activity was determined in the 14,000 x g supernatant fraction of renal, hepatic and testicular homogenates as described by Epfer and Bruggeman (1966). Final concentrations in the incubation mixtures were as follows: 0.4 mM NADP, 15 mM $MgCl_2$, 10 mM nicotinamide, 5 mM semicarbazide, 8 mM glucose-6-phosphate, 1 U/ml glucose-6-phosphate

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ehydrogenase and 3 mM p-chloro-N-methylaniline (Calbiochem, La Jolla, A). Protein concentrations, as determined by the method of Lowry t al. (1951), were 2.0-2.5 mg/ml (liver), 4-5 mg/ml (kidney) and -10 mg/ml (testis).

3. Aryl hydrocarbon hydroxylase (AHH) assay

AHH activity was determined in the 14,000 x g supernatant raction or the resuspended 100,000 x g pellet fraction of renal, epatic and testicular homogenates as described by Nebert and Gelboin 1968) and Oesch (1976). Final concentrations in the incubation ixtures were as follows: 0.4 mM NADP, 0.25 mM NAD, 3 mM $MgCl_2$, 60 mM ris, 5.8 mM glucose-6-phosphate, 1 U/ml glucose-6-phosphate dehydro-enase and 1.0 mM 3,4-benzo(a)pyrene (Sigma). Protein concentrations ere 0.25-0.65 mg/ml (liver), 1-4 mg/ml (kidney) and 3-4 mg/ml testis) when the 14,000 x g supernatant fraction was used, and 0.1-.3 mg/ml (liver) and 1-2 mg/ml (kidney and testis) when the 100,000 g pellet fraction was used. Product formation was estimated luorometrically using an Aminco spectrofluorimeter (quartz cuvettes, cm light path) and activity (formation of product) expressed as elative fluorescence units per mg protein per min. A 10 μM solu-on of quinine sulfate in 0.05 M H_2SO_4 produced $2x10^3$ relative uorescence units (excitation wavelength, 365 nm; emission wave-ngth, 460 nm).

The inhibitory effects of SKF 525-A (Smith, Kline and French rp., Philadelphia, PA), piperonyl butoxide (PB, Chem Service, stchester, PA), allyl-isopropylacetamide (AIA, Hoffman-LaRoche, ley, NJ), metyrapone (MET, Aldrich Chemical Co., Milwaukee, WI)

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α-napthoflavone (ANF, Aldrich) on renal and hepatic AHH activities vitro were determined using the procedure outlined above for AHH ept that the concentration of 3,4-benzo(a)pyrene in the incubation ture was $1x10^{-4}$M. Stock solutions of the various inhibitors were ed to achieve concentrations of 0 M, $1x10^{-6}$M, $1x10^{-5}$M, $1x10^{-4}$M or 0^{-3}M. Stock solutions of SKF 525-A and MET were formulated in tilled water, stock solutions of AIA and PB in 1% poloxyethylene bitan mono-oleate (Tween 80, Sigma) and stock solutions of ANF in tone. The various stock solutions of inhibitors were added to the ubation mixtures in such a manner that the stock solution vehicle prised 1% (v/v) of the total incubation mixture. Control incuba- ns (no additions of inhibitors or inhibitor vehicles) were also ducted.

4. Biphenyl-2-hydroxylase (BP-2-OH) and biphenyl-4-hydroxylase (BP-4-OH) assays

BP-2-OH and BP-4-OH activities were determined in the 14,000 supernatant fraction of renal and hepatic homogenates according he method of Creaven et al. (1965). Final concentrations in the bation mixtures were as follows: 0.3 mM NADP, 2.5 mM $MgCl_2$, 3.8 lucose-6-phosphate, 0.5 U/ml glucose-6-phosphate dehydrogenase 10.0 mM biphenyl (Eastman Organic). Protein concentrations were 0.4 mg/ml (liver) and 0.8-1.2 mg/ml (kidney).

5. Cytochrome P-450 assays

Cytochrome P-450 concentrations in the microsomal pellets 000 x g pellet fraction) of hepatic, renal and testicular enates were determined spectrally using a dual-beam spectro- meter (Beckman UV5260). The microsomal pellets were suspended in

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00 mM sodium phosphate buffer, pH 7.20, and an extinction coefficient
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450 from the differences in soret maxima near 450 nm and the 490 nm
selines of the sodium dithionite-reduced CO:hemoprotein-complex
fference spectra (Omura and Sato, 1964).

Individual Experiments

1. Time-dependency and organ-specificity of induction and inhibition of renal and hepatic drug-metabolizing enzyme systems

   a. Effects of length of exposure and dietary concentrations of polybrominated biphenyls (PBB) and polychlorinated biphenyls (PCB) on renal and hepatic AHH activities:

   ICR, male mice (15-20 g) were maintained on diets con-
ning 25, 100, or 200 ppm of PBB or 25, 200 or 400 ppm of PCB for 14
s. Another group of mice received diets containing 100 ppm of PBB
200 ppm of PCB for 21 days. Control mice received diet formulated
n an equivalent amount of acetone. The mice were sacrificed, the
ers and kidneys removed and AHH activities determined in the 14,000
supernatant fraction of renal and hepatic homogenates.

   b. Effects of sodium phenobarbital (NaPb), 3-methylcholanthrene (3MC), PCB and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on enzyme activities in kidney and liver in mice

   ICR, male mice (15-20 g) were maintained on diets con-
ing 0 or 200 ppm of PCB for 28 days or were given a single i.p.
ction of TCDD (Dow Chemical Co., Midland, MI) in corn oil:acetone,
, 0 or 16 μg/kg (total injection volume of 5 ml/kg), 72 hr before
ifice. A separate set of mice received i.p. injections of NaPb
ma), 50 mg/kg in distilled water, once daily for 4 consecutive days,

or 3MC (Eastma

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or 3MC (Eastman Organic), 25 mg/kg in corn oil, once daily for 3 consecutive days. The total injection volumes for NaPb and 3MC were 5 ml/kg and the final doses were administered 24 hr before sacrifice. The mice were killed, livers and kidneys removed, and the activities of AHH, PCNMA, BP-2-OH and BP-4-OH determined in the 14,000 x g supernatant fraction of renal and hepatic homogenates.

c. Effects of single and multiple doses of PBB, PCB, NaPb, and 3MC on renal, hepatic and testicular enzymes and cytochrome P-450 in rats

PBB, PCB and 3MC were dissolved in corn oil to achieve oncentrations of 18, 18, and 8 mg/ml, respectively. NaPb was dissolved n distilled water at a concentration of 33.5 mg/ml. For single expoure studies male, Fischer 344 rats (125-150 g) received a single oral ose of PBB (90 mg/kg), PCB (90 mg/kg) or 3MC (40 mg/kg) by gavage, r a single i.p. injection of NaPb (75 mg/kg) and were sacrificed 9, 4, 72, or 216 hr later. Control rats received an appropriate volume corn oil. For multiple-dose studies male, Fischer 344 rats reeived successive oral doses of PBB or PCB (90 mg/kg) 24, 48, and 72 before sacrifice, or i.p. injections of NaPb (75 mg/kg) 24, 48, and 96 hr before sacrifice. Control rats received appropriate lumes of corn oil. Administrations were timed so that all animals re killed at 8 a.m. Kidneys, livers and testes were removed upon crifice and AHH and PCNMA activities and cytochrome P-450 concentraons determined. In selected cases, the sensitivities of renal and patic AHH to inhibition by MET and ANF were evaluated in vitro.

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d. Time-dependent effects of single doses of SKF 525-A and piperonyl butoxide (PB) on renal and hepatic enzyme activities in mice and rats

ICR, male mice (15-20 g) and Fischer 344, male rats 25-150 g) received single i.p. injections of PB (80% pure, ICN rmaceuticals, Plainview, NY), 600 mg/kg in corn oil, or SKF 525-A, mg/kg in water (total injection volumes of 5 ml/kg). The animals re killed 1, 2, 4, or 12 hr later and the activities of AHH and MA determined in the 14,000 x g supernatant fractions of renal hepatic homogenates.

2. Effects of stimulation and inhibition of renal and hepatic drug-metabolizing enzyme systems on $CHCl_3$ and CCl_4 toxicity

a. Effects of PBB and PCB on CCl_4 toxicity in mice

ICR, male mice (15-20 g) were maintained on diets con- ning 100 ppm of PBB or 200 ppm of PCB for 28 days. Control mice eived diet formulated with an equivalent amount of acetone. The e were then challenged with a single i.p. injection of CCl_4, 0.000, 05, 0.025, 0.125, or 0.625 ml/kg in corn oil, total injection ume of 5 ml/kg, and sacrificed 48 hr later by decapitation. od was collected, the serum fractions separated, and SGOT, SGPT BUN determined. Livers and kidneys were removed, weighed and the ities of renal cortical slices to accumulate PAH and TEA deter- d.

Another group of mice was maintained on diets con- ing 0, 20, or 100 ppm of PBB for 20 days and then challenged with ngle i.p. injection of one of several doses of CCl_4 in corn oil, l injection volume of 5 ml/kg. LD_{50} values for CCl_4 were deter- l in all three groups.

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b. Effects of PBB, PCB and hexachlorobenzene (HCB) on CCl_4 toxicity in rats

Sprague-Dawley, male rats (125-150 g) were maintained
n diets containing 100 ppm of PBB or 200 ppm of PCB for 20 days. A
hird group of rats received HCB (98% purity, Aldrich), 30 mg/kg in
orn oil, by gavage every 72 hr for a period of 20 days (total of 7
oses). A fourth group (controls) was maintained on diet formulated
th an equivalent amount of acetone and received corn oil by gavage
very 72 hr for a 20 day period. All rats were then challenged (day
) with a single i.p. injection of CCl_4 in corn oil, 0.00, 0.03, 0.25
2.00 ml/kg, total injection volume of 5 ml/kg, and were killed 48
later by a blow to the head. Blood was collected and the activi-
es of SGOT, SGPT, and BUN determined. Urine samples were collected
rectly from the bladder and analyzed for protein and glucose concen-
ations and for pH with reagent sticks (Hema-Combistix, Miles Labs.,
khart, IN). Kidneys and livers were removed, weighed and the
lities of renal cortical slices to accumulate PAH and TEA deter-
ed. Thin slices (approximately 0.5 mm thick) of renal cortex were
ubated in a phosphate-buffered medium (Cross and Taggart, 1950)
urated with O_2 at 30°C in an air-tight, magnetically-stirred
eptacle and the rate of oxygen consumption (QO_2) determined using
oxygen-sensitive electrode (Yellow Springs Instrument Co.,
low Springs, OH). Renal QO_2 values were expressed as microliters
O_2 consumed per g tissue per minute. Total lipids were extracted
n samples of liver and kidney by the method of Folch et al. (1957).
residues were dried to a constant weight and lipid content ex-
ssed as mg lipid (dry weight) per g of tissue (wet weight).

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c. Effects of PBB and PCB on hepatocellular GPT and GOT activities

Sprague-Dawley, male rats (125-150 g) were maintained
diets containing 100 ppm of PBB or 200 ppm of PCB for 20 days.
ntrol rats received diet formulated with an equivalent amount of
etone. The rats were killed on day 21 by a blow to the head and
mples of liver removed, weighed, washed several times in ice-cold
mM Tris-1.15% KCl buffer (pH 7.40) and homogenized in 3 volumes
Tris-KCl buffer in a Potter-Elvehjem tissue grinder, as described
rlier. The homogenates were then centrifuged at 600 x g for 5 min at
C to separate nuclei and unbroken cells and the supernatant fraction
ntrifuged at 14,000 x g for 25 min at 2°C to separate mitochondria.
e resulting supernatant fraction was decanted and centrifuged at
,000 x g for 60 min at 2°C and the activities of GOT and GPT
sured in the 100,000 x g supernatant fraction using Sigma reagent
s. Protein concentrations in the 100,000 x g supernatant (cyto-
ic) fractions were determined by the method of Lowry et al. (1951).
and GPT activities were expressed as Sigma-Frankel units per ml
100,000 x g supernate, per mg of 100,000 x g supernatant protein,
per 100 g of total body weight.

d. Effects of maternal consumption of PBB on the toxicities of $CHCl_3$ and CCl_4 in developing male rats

Timed-pregnant, Sprague-Dawley rats were placed on
s containing 0 or 100 ppm of PBB on day 8 of gestation. Litters
normalized to 10 pups each at birth. The pups were separated
the dams at 26 days of age (the approximate time of weaning),
omized, separated by sex and placed on diets containing either 0

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pm of PBB (all of the male pups from dams consuming 0 ppm PBB diet
nd half of the male pups from dams consuming 100 ppm PBB diet) or
00 ppm of PBB (half of the male pups from dams consuming 100 ppm
BB diet). Only male pups were used for the remainder of this study.
ody weights were determined at 52 days of age and the rats then
hallenged with a single i.p. injection of $CHCl_3$ or CCl_4 in corn oil,
00, 0.03, 0.25, or 2.00 ml/kg, total injection volume of 5 ml/kg.
e rats were killed 48 hr later, blood collected and the activities
SGOT and SGPT and the concentrations of BUN determined. The
dneys and livers were removed and weighed and the abilities of
nal cortical slices to accumulate PAH and TEA determined.

e. Effects of dietary PBB on $CHCl_3$ toxicity in mice

ICR, male mice (15-20 g) were maintained on diets con-
ining 0, 1, 25, or 100 ppm of PBB for 14 days and then challenged
h a single i.p. injection of $CHCl_3$, 0.0, 0.5, 2.5, 5.0, 25.0, or
0 µl/kg in corn oil, total injection volume of 5 ml/kg. The mice
e sacrificed 24 hr later by decapitation, blood collected and
ers and kidneys removed and weighed. SGPT, SGOT and BUN values
the abilities of renal cortical slices to accumulate PAH and TEA
e determined.

f. Effects of NaPb, 3MC, PCB and TCDD on $CHCl_3$ toxicity in mice

ICR, male mice (15-20 g) were treated with PCB, TCDD,
or NaPb as described previously (Methods, Section DIb). The
were challenged with a single i.p. injection of $CHCl_3$, 0.00, 0.05,
or 0.75 ml/kg in corn oil, total injection volume of 5 ml/kg, 24

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after the final dose of NaPb or 3MC, 72 hr after a single dose of
DD, or after 28 days on diet containing 200 ppm of PCB. The
imals were sacrificed 24 hr later, blood collected, and SGPT, SGOT
d BUN and the abilities of renal cortical slices to accumulate PAH
d TEA determined.

g. Effects of SKF 525-A and PB on $CHCl_3$ toxicity in mice

ICR, male mice (15-20 g) received i.p. injections of
, 600 mg/kg, or SKF 525-A, 75 mg/kg, either 120 min before or 60
n after a single i.p. injection of $CHCl_3$, 0.00, 0.25, or 0.75 ml/kg
corn oil, in a total injection volume of 5 ml/kg. The mice were
lled 24 hr after $CHCl_3$ administration, blood collected and SGPT,
OT and BUN and the abilities of renal cortical slices to accumulate
and TEA determined.

3. Interactions of $CHCl_3$ with renal and hepatic glutathione (GSH)

a. Effects of PBB and PCB on $CHCl_3$-induced GSH depletion in mice

ICR, male mice (15-20 g) were maintained on diets con-
ning 100 ppm of PBB or 200 ppm of PCB or on a control diet formu-
ed with an equivalent amount of acetone for 20 days. The mice were
n challenged with a single i.p. injection of $CHCl_3$ in corn oil,
), 0.05, 0.25, or 0.75 ml/kg, total injection volume of 5 ml/kg,
sacrificed 2 hr later by cervical dislocation. Livers and
eys were homogenized in 20 volumes of ice-cold 6% TCA and analyzed
reduced non-protein thiol content using Ellman's reagent (Ellman,
). Selected samples (from naive and treated animals) were also

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nalyzed specifically for reduced GSH content using the method of
ohn and Lyle (1966) and it was determined that at least 90% of the
duced non-protein thiols in the supernatant fractions of liver and
dney homogenates were reduced GSH. The concentrations of GSH in
e kidneys and livers were calculated using reduced GSH (Sigma) as
analytical standard and expressed as μg GSH/g tissue (wet weight).

b. Depletion of renal and hepatic GSH by diethyl maleate

ICR, male mice (15-20 g) received a single i.p. injec-
on of diethyl maleate (80% pure, Aldrich), 0 or 600 mg/kg in corn
, total injection volume of 5 ml/kg, and were sacrificed 2 hr later
cervical dislocation. Kidneys were removed, cut lengthwise and
tical, medullary and papillary sections dissected. Samples of
er and the various sections of the kidney were homogenized in 20
umes of ice-cold 6% TCA. The homogenates were centrifuged to
arate TCA-denatured protein and the concentrations of reduced,
-protein thiols in the supernatant fractions determined using
man's reagent. Analyses of random samples using a fluorimetric
ay specific for reduced GSH (Cohn and Lyle, 1966) demonstrated
at least 90% of the reduced, non-protein thiol in the super-
nt fraction was reduced GSH. The concentrations of GSH in the
r and the various sections of the kidney were calculated as
ribed previously.

c. Effects of diethyl maleate on GSH concentrations and $CHCl_3$ toxicity in mice

ICR, male mice (15-20 g) were given a single i.p.
ction of diethyl maleate, 0 or 600 mg/kg, followed in 90 min by

a single i.p.

The mice were

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single i.p. injection of $CHCl_3$, 0.000, 0.033, 0.100 or 0.300 ml/kg. e mice were killed 24 hr after $CHCl_3$ administration and blood was llected. SGPT, SGOT and BUN and the abilities of renal cortical ices to accumulate PAH and TEA were determined.

4. Covalent binding of $CHCl_3$ metabolites in mice

a. Covalent binding of $CHCl_3$ metabolites to renal and hepatic microsomal protein in vitro

ICR, male mice (15-20 g) were maintained on diets con- ning 100 ppm of PBB or 200 ppm of PCB or on a diet formulated with equivalent amount of acetone for 20 days. The mice were then led by cervical dislocation and kidneys and livers removed, homo- ized in 3 volumes of ice-cold 20 mM Tris-1.15% KCl buffer (pH 7.40) renal and hepatic microsomes isolated as described previously thods, Section C). Renal and hepatic microsomes were incubated n (^{14}C)-$CHCl_3$[1] (New England Nuclear) and a regenerating system ightly-stoppered 25 ml Erlenmeyer flasks as described by Ilett al. (1973). Final concentrations in the incubation mixtures were follows: 0.12 mM NADH, 0.20 mM NADPH, 2.0 mM nicotinamide, 2.0 mM ose-6-phosphate, 1 U/ml glucose-6-phosphate dehydrogenase and 0 mM $CHCl_3$ (1 μCi/ml), all dissolved in 20 mM Tris-1.15% KCl er, pH 7.40. Protein concnentrations, as determined by the method owry et al. (1951), were 1.0 mg/ml (liver) and 2.0 mg/ml (kidney).

[1] 4.9 mCi/mmole, radiochemical purity, 99%. The material, as nased from New England Nuclear, was dissolved in dimethylforma- to achieve a concentration of 20 mM and stored at -70°C. Radio- vity in the stock solution was determined each time it was used orrect for loss due to volatilization during handling.

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he reactions were stopped after 5 min by the addition of ice-cold 0% TCA. The denatured protein was separated by centrifugation and xtracted several times with hot (50°C) chloroform:methanol (2:1), s described by Ilett et al. (1973), to remove non-covalently bound adioactivity. (The final extracts contained no radioactivity.) The rotein samples were then dissolved in 1 N NaOH and aliquots used for etermination of protein and radioactivity using (^{14}C)-toluene as an nternal standard.

b. Clearance of $CHCl_3$ and metabolites from blood and covalent binding to total renal and hepatic protein

ICR, male mice (15-20 g) were maintained on diets con-aining 100 ppm of PBB, 200 ppm of PCB or on diet formulated with an quivalent amount of acetone for 14 days and then challenged with a ngle i.p. injection of (^{14}C)-$CHCl_3$, 1.75 mmoles/kg (0.10 ml/kg), 0 μCi/kg. Samples of whole venous blood (10 μl) were withdrawn om the retro-orbital sinus into heparinized glass capillary tubes , 30, 45, 60, 90, 120 and 180 min after administration of (^{14}C)-Cl_3. Blood samples were dissolved in Soluene-350 (Packard Instru-nt Co., Downer's Grove, IL) and radioactivity determined by liquid intillation spectrometry using (^{14}C)-toluene as an internal standard. e mice were sacrificed by cervical dislocation 3, 6 or 12 hr after Cl_3 administration. Livers and kidneys were removed and homo-nized in 3 volumes of ice-cold distilled water. Aliquots of the nogenates were mixed with an equal volume of ice-cold 10% TCA, the tures centrifuged to separate the denatured proteins, and the ulting pellets extracted 5 times with hot (50°C) chloroform:

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ts of resuspended protein were then dissolved in 1 N NaOH and ali-
ts of whole homogenates dissolved in Soluene-350. Radioactivity
determined by liquid scintillation spectrometry.

c. Covalent binding of $CHCl_3$ metabolites to subcellular fractions in vivo

Mice were sacrificed 3 hr after an i.p. injection of
C)-$CHCl_3$ (1.75 mmoles/kg, 110 μCi/kg). Kidneys and livers were
ised, minced, washed several times with ice-cold 0.3 M sucrose-
05 M Hepes (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid,
ma) buffer, pH 7.40. The tissues were then homogenized in 5
umes of 0.3 M sucrose-0.005 M Hepes buffer with a Potter-Elvehjem
sue grinder (0.10-0.15 mm clearance) and centrifuged at 1,000 x g
10 min at 2°C. The supernatant fractions were decanted and
ed, the pellets rehomogenized in the original volume of 0.3 M
rose-0.005 M Hepes-0.2% Triton X-100 buffer, pH 7.40, and centri-
ed again at 1,000 x g for 10 min at 2°C. Nucleotides (RNA and DNA)
e extracted from this pellet by the method of Goodman and Potter
'2). RNA concentrations were determined spectrophotometrically
'60 nm, pH 1.0 (Pomerai et al., 1974) and DNA concentrations deter-
d by the method of Ceriotti (1958).

The supernatant fractions saved from the initial 1,000
centrifugation were centrifuged at 8,000 x g for 10 min at 2°C,
supernatant fractions decanted and saved, and the pellets re-
ended in 0.3 M sucrose-0.005 M Hepes buffer and centrifuged as
re. This process was repeated again (total of 3 centrifugations

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: 8,000 x g) and the final pellet, termed the mitochondrial fraction, ›mogenized in 2 ml of distilled water.

The supernatant fractions from the initial 8,000 x g ntrifugation were centrifuged at 14,000 x g for 25 min at 2°C and e supernatant fractions decanted and centrifuged at 100,000 x g for min at 2°C. The resulting pellets were resuspended in 0.3 M crose-0.005 M Hepes buffer, centrifuged again at 100,000 x g for 60 n at 2°C, and the final pellet, termed the microsomal or endoplasmic ticulum fraction, homogenized in 2 ml of distilled water.

One ml aliquots of the microsomal and mitochondrial actions were mixed with 1 ml of ice-cold 10% TCA, the denatured otein separated by centrifugation, and the pellets extracted 5 nes with hot (50°C) chloroform:methanol, 2:1. The protein pellet s then dissolved in 1 N NaOH. Lipids were extracted from 1 ml iquots of microsomal and mitochondrial fractions by the method of ch et al. (1957), the extracts allowed to dry under a gentle eam of N_2, and the residues dissolved in 1.5 ml $CHCl_3$ and allowed dry under N_2, twice, to remove non-bound, residual radioactivity. ioactivity in the lipid residues, protein solutions and nucleo- e solutions was determined by liquid scintillation spectrometry ng (^{14}C)-toluene as an internal standard.

Statistics

Unless stated otherwise, all data were analyzed by analysis of iance (completely randomized design or blocked design) and treat- means were compared using the Least Significant Difference or

the Student-N

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the Student-Newman-Keuls tests (Sokal and Rohlf, 1969). The criterion of significance in all cases was $p<0.05$.

The linearity of the curves relating the log concentrations of ^{14}C-activity in the blood to the time after administration of (^{14}C)-$CHCl_3$ was confirmed by regression analysis. Data from the three curves (control, PBB and PCB) were then analyzed by analysis of covariance and the slopes compared using Student's *t*-test with $p<0.05$ as the criterion of significance (Sokal and Rohlf, 1969).

A. Enzyme In

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RESULTS

A. Enzyme Induction and Inhibition

1. Effects of length of exposure and dietary concentration of PBB and PCB on renal and hepatic AHH activities

The magnitude of increase in renal and hepatic AHH activities was proportional to the concentration of PBB and PCB in the diet when ICR, male mice were fed diets containing 25, 100 or 200 ppm of PBB or 25, 200 or 400 ppm of PCB for 14 days (Table 2). Both renal and hepatic AHH activities were increased in mice consuming diets containing 25 ppm of PBB for 14 days, though 25 ppm of PCB was insufficient to produce an increase in AHH activities. In general, PBB appeared to be a more potent inducer of both renal and hepatic AHH activities than was PCB, and hepatic AHH appeared to be more sensitive than renal AHH to the inductive effects of dietary PBB and PCB. Increasing the length of dietary exposure to PBB and PCB from 14 days to 21 days did not change the magnitudes of the increases in specific activities of renal and hepatic AHH (activities per mg of protein, Table 3). The liver weight-to-body weight ratio, however, was slightly greater in mice fed PBB and PCB for 21 days than in mice fed the same diets for 14 days, suggesting that continued ingestion of PBB and PCB may increase total hepatic biotransformation capacity (data not shown).

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$p<0.05$.

TABLE 2

Dietary Concentration-Dependent Induction of Aryl Hydrocarbon Hydroxylase (AHH) Activities by Polybrominated Biphenyls (PBBs) and Polychlorinated Biphenyls (PCBs)

Treatment	ppm	AHH Activities[a] Liver	Kidney
PBB	25	216±19[b]	150±14[b]
PBB	100	438±37[b,c]	310±13[b,c]
PBB	200	942±41[b,c,d]	466±26[b,c,d]
PCB	25	139±14[b,c]	85± 6[b,c]
PCB	200	339±47[b,c,d]	292±12[b,c,d]
PCB	400	598±56[b,c,d]	478±13[b,c,d]

[a] Relative fluorescence units/mg protein/min, mean % of control ± 1 S.E., N=6 animals. Control values: 12.10±2.10 (liver), 0.08±0.01 (kidney).

[b] Significantly greater than control, $p<0.05$.

[c] Significantly greater than 25 ppm, $p<0.05$.

[d] Significantly greater than 100 ppm (PBB) or 200 ppm (PCB), $p<0.05$.

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TABLE 3

Time-Dependent Induction of Aryl Hydrocarbon Hydroxylase (AHH) Activities by Polybrominated Biphenyls (PBB) and Polychlorinated Biphenyls (PCB)

Treatment	Time[b]	AHH Activities[a]	
		Liver	Kidney
PBB, 100 ppm	14 d	438±37	310±13
PBB, 100 ppm	21 d	413±26	302±21
PCB, 200 ppm	14 d	339±47	292±12
PCB, 200 ppm	21 d	267±17	300±15

[a]Relative fluorescence units/mg protein/min, mean % of control ± 1 S.E., N=6 animals. Control values: 12.10±2.10 (liver), 0.08±0.01 (kidney).

[b]Number of days (d) on designated diet.

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2. Effects of NaPb, 3MC, PCB and TCDD on renal and hepatic enzymes in mice

The effects of multiple i.p. injections of NaPb and 3MC, a ingle i.p. injection of TCDD and dietary consumption of PCB on renal nd hepatic MFO activities are summarized in Table 4. In general, reatment with 3MC, TCDD and PCB increased the activities of both enal and hepatic MFOs while treatment with NaPb increased hepatic FO activities, only.

3. Effects of single and multiple administrations of PBB, PCB, NaPb and 3MC on renal, hepatic and testicular enzyme activities and cytochrome P-450 concentrations in rats

Cytochrome P-450 (P-450) concentrations and AHH and PCNMA tivities in kidney, liver and testis from naive rats are summarized Table 5. Enzyme activities and P-450 concentrations, in general, re greater in liver than in kidney, and greater in kidney than in stis.

Hepatic P-450 content was increased by single oral doses of and PCB but not by single doses of NaPb or 3MC (Figure 2). Renal testicular P-450 contents were not affected by single doses of PBB, , NaPb or 3MC. Multiple doses of PBB, PCB and 3MC increased both atic and renal P-450 contents. The magnitude of this effect, ever, was greater in the liver than it was in the kidney after atment with PBB and PCB (Figure 2). Multiple doses of NaPb inased P-450 content in the liver but not in the kidney. Testicular 50 content was not increased by any of the inducing chemicals used.

The induced P-450, in many cases, exhibited soret maxima soret maximum is the absorbance peak near 450 nm in the differspectrum of the dithionite-reduced CO:hemoprotein complex. See

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TABLE 4

Induction of Mixed-Function Oxidases (MFOs) in Liver and Kidney

Organ	Inducing Chemical	Enzyme Activities			
		AHH	PCNMA	BP-2-OH	BP-4-OH
		mean % control ± 1 S.E.			
Liver	NaPb[a]	125±22	201±14*	137±42	331±56*
Kidney	NaPb[a]	93±14	106±11	100±20	100±30
Liver	3MC[b]	346±53*	87±21	n.d.	n.d.
Kidney	3MC[b]	286±21*	113±19	n.d.	n.d.
Liver	TCDD[c]	945±186*	n.d.	118±10	141±20
Kidney	TCDD[c]	5560±1200*	n.d.	200±100	200±50
Liver	PCB[a]	356±49*	217±36*	n.d.	n.d.
Kidney	PCB[a]	211±36*	165±18*	n.d.	n.d.

PCNMA, AHH, BP-2-OH and BP-4-OH activities were measured in the 14,000 x g supernatant fraction of homogenates of livers and kidneys of mice treated with NaPb, 3MC, TCDD or PCB. Activities are presented as a percentage of control ± 1 S.E. n.d. = Not determined.

*Significant increase ($p<0.05$). [a]N=6 animals. [b]N=5 animals

[c]N=4 animals (data from Hook et al., 1978b).

Activities in control mice were as follows:

Liver: AHH, 11.20±1.50 fluorescence units per mg of protein per min; PCNMA, 0.139±0.012 O.D. units per mg of protein per hr; BP-2-OH, 0.11±0.01 nmol of 2-hydroxybiphenyl produced per mg of protein per min; BP-4-OH, 2.08±0.28 nmol of 4-hydroxybiphenyl produced per mg of protein per min.

Kidney: AHH, 0.05±0.01 fluorescence units per mg of protein per min; PCNMA, 0.080±0.009 O.D. units per mg of protein per hr; BP-2-OH, 0.01±0.01 nmole of 2-hydroxybiphenyl produced per mg of protein per min; BP-4-OH, 0.02±0.01 nmol of 4-hydroxybiphenyl produced per mg of protein per min.

Abbreviations: sodium phenobarbital (NaPb), 3-methylcholanthrene (3MC), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polychlorinated biphenyls (PCB), p-chloro-N-methylaniline N-demethylase (PCNMA), aryl hydrocarbon hydroxylase (AHH), biphenyl-2-hydroxylase (BP-2-OH), biphenyl-4-hydroxylase (BP-4-OH)

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[a] nmoles per

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TABLE 5

Measurements of Aryl Hydrocarbon Hydroxlyase (AHH) Activities, p-Chloro-N-methylaniline N-demethylase (PCNMA) Activities and Cytochrome P-450 Concentrations in Liver, Kidney and Testis of Naive Rats

Parameter	Organ: Liver	Kidney	Testis
Cytochrome P-450[a]	0.571±0.061	0.110±0.019	0.059±0.016
AHH[b]	331±16	6.20 ±0.66	1.83 ±0.37
PCNMA[c]	0.457±0.023	0.229±0.012	0.105±0.011

AHH and PCNMA activities were measured in the 14,000 x g supernatant fractions of hepatic, renal and testicular homogenates. Values are represented as the mean ± 1 S.E. of 6 animals.

[a] nmoles per mg microsomal protein.

[b] Relative fluorescence units per mg protein per min.

[c] nmoles product (p-chloro-N-methylaniline) formed per mg protein per min.

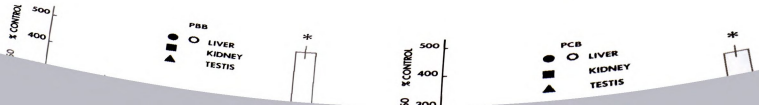
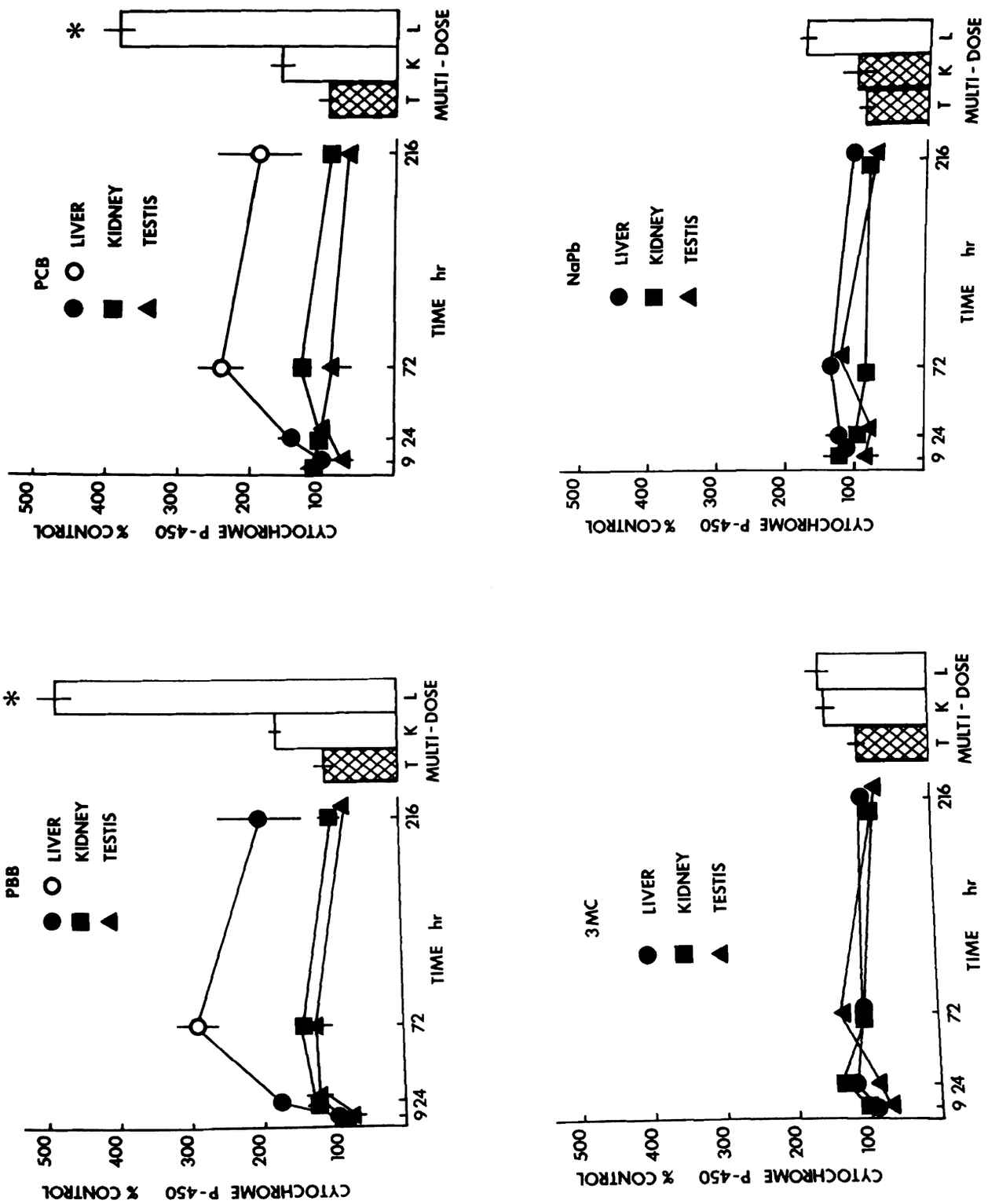


Figure 2. Time-dependent induction of cytochrome P-450 in liver, kidney and testis. Symbols represent the means ± 1 S.E. (N=4 rats) of the microsomal cytochrome P-450 content in animals sacrificed 9, 24, 72 or 216 hr after a single dose of polybrominated biphenyls (PBB), polychlorinated biphenyls (PCB), sodium phenobarbital (NaPb) or 3-methylcholanthrene (3MC). Bars represent the means ± 1 S.E. (N=4 rats) of the microsomal cytochrome P-450 content in animals sacrificed 24 hr after the final dose of a multiple-dose regimen of PBB, PCB, NaPb or 3MC. T, testis; K, kidney; L, liver. Open symbols or bars, significantly greater than in control rats; closed symbols or bars, not significantly greater than in control rats; $p<0.05$. *Significant difference between percent increases in liver and kidney, $p<0.05$.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Methods, Section C5) of shorter wavelengths than P-450 from naive rats. After multiple doses of PBB, PCB or 3MC, hepatic soret maxima were shifted from 450 nm to 448 nm, and renal soret maxima were shifted from 454 nm to 453 or 452 nm (Table 6). Treatment with NaPb did not produce shifts in soret maxima of renal or hepatic P-450. Though a single oral dose of PBB, PCB and 3MC produced shifts in hepatic soret maxima within 9 hr, maximum shifts appeared to occur 72 hr after a single dose and were still apparent at 216 hr (except for 3MC)(Table 6). Effects of PBB, PCB and 3MC on soret maxima of renal P-450 were similar to those on hepatic P-450 except that maximum shifts appeared to occur at 9-24 hr and had disappeared by 216 hr (72 hr for 3MC).

Multiple doses of PBB, PCB and 3MC greatly increased both hepatic and renal AHH activities while multiple doses of NaPb produced a relatively modest increase in hepatic AHH only (Figure 3). Induction of hepatic AHH activity, in general, was first evident at 24 hr and peaked at 72 hr after single doses of PBB, PCB and 3MC. Renal AHH activities, however, were greatly increased at 9 hr, maximally increased at 24 hr and had fallen considerably by 72 hr after single doses of PBB, PCB and 3MC (Figure 3). Testicular AHH activities were not increased by single or multiple doses of the inducing chemicals.

PCNMA activity in liver was increased (approximately 2X) by multiple doses of PBB, PCB and NaPb (data not shown). Renal and testicular PCNMA activities in rats, however, were not significantly increased by PBB, PCB, NaPb or 3MC (data not shown).

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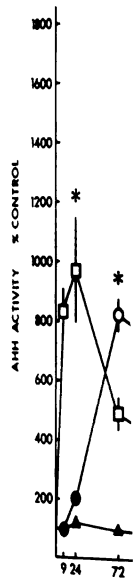
TABLE 6

Time-Dependent Effects of Polybrominated Biphenyls (PBB), Polychlorinated Biphenyls (PCB), Sodium Phenobarbital (NaPb), and 3-Methylcholanthrene (3MC) on the Location of Soret Maxima of Reduced Cytochrome P-450 Difference Spectra

Treatment	Organ	Time (hr):	Wavelength (nm) of the soret maximum 9	24	72	216	Multi-dose
Control	Liver		450	450	450	450	450
PBB	Liver		449	449	449	449	448
PCB	Liver		449	449	448	449	448
NaPb	Liver		450	450	450	450	450
3MC	Liver		449	449	449	450	448
Control	Kidney		454	454	454	454	454
PBB	Kidney		453	453	453	454	452
PCB	Kidney		453	453	453	454	453
NaPb	Kidney		454	454	454	454	454
3MC	Kidney		454	453	454	454	452

Rats were sacrificed 9, 24, 72 or 216 hr after a single dose of PBB, PCB, NaPb or 3MC, or 24 hr after the final dose of a multiple-dose regimen. Cytochrome P-450 was determined in the microsomal pellets and the soret maximum in the vicinity of 450 nm recorded to the nearest wavelength. Values are represented as the means of 4 animals rounded off to the nearest integer.



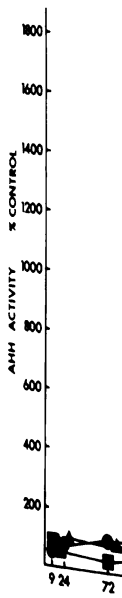


Figure 3. Time-dependent induction of aryl hydrocarbon hydroxylase (AHH) activity in liver, kidney and testis. Symbols represent the means ± 1 S.E. (N=4 rats) of the activities of AHH in animals sacrificed 9, 24, 72 or 216 hr after a single dose of polybrominated biphenyls (PBB), polychlorinated biphenyls (PCB), sodium phenobarbital (NaPb) or 3-methylcholanthrene (3MC). Bars represent the means ± 1 S.E. (N=4 rats) of the activities of AHH in animals sacrificed 24 hr after the final dose of a multiple-dose regimen of PBB, PCB, NaPb or 3MC. T, testis; K, kidney; L, liver. Open symbols or bars, significantly greater than in control rats; closed symbols or bars, not significantly greater than in control rats; $p<0.05$. *Significant difference between percent increases in liver and kidney, $p<0.05$.

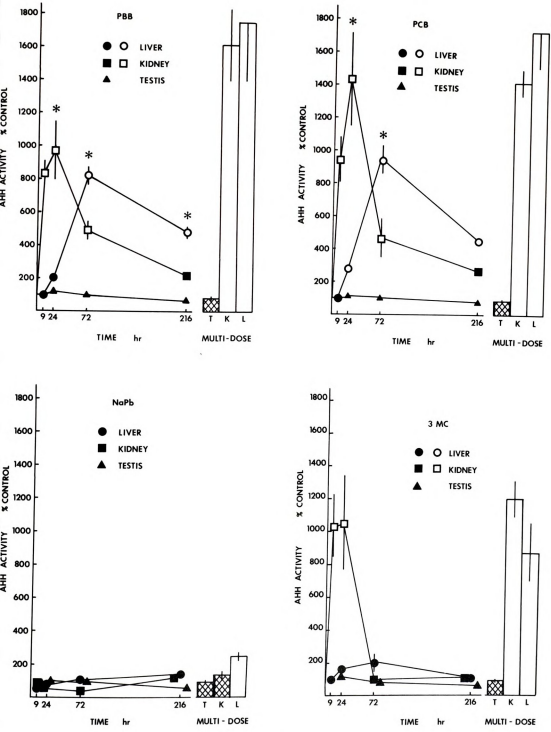

PBB
LIVER
KIDNEY
TESTIS
AHH ACTIVITY % CONTROL
1800
1600
1400
1200
1000
800
600
400
200
9 24
72
216
TIME hr
T K L
MULTI-DOSE
PCB
LIVER
KIDNEY
TESTIS
AHH ACTIVITY % CONTROL
TIME hr
MULTI-DOSE
NaPb
LIVER
KIDNEY
TESTIS
AHH ACTIVITY % CONTROL
TIME hr
MULTI-DOSE
3 MC
LIVER
KIDNEY
TESTIS
AHH ACTIVITY % CONTROL
TIME hr
MULTI-DOSE

Figure 3

The

AHH activitie

hepatic homog

using MET and

P-450-depende

Gelboin, 1975

hepatic AHH a

hepatic AHH a

testicular AH

values) while

nearly 30%, a

(testicular d

appeared to i

slight extent

concomitantly

inhibitory ef

opposite effe

comparison to

ANF but hepat

of MET (Figur

of PBB and PC

to the inhibi

rats, however

to that of re

AHH from PBB

to the inhibi

The inhibitory effects of ANF and MET ($1x10^{-4}M$) in vitro on AHH activities in the 14,000 x g supernatant fractions of renal and hepatic homogenates are illustrated in Figure 4. Inhibition studies using MET and ANF have previously been used to classify the type of P-450-dependent AHH activity induced by various agents (Wiebel and Gelboin, 1975; Goujon et al., 1972). MET preferentially inhibits hepatic AHH activity induced by NaPb while ANF preferentially inhibits hepatic AHH activity induced by 3MC. ANF reduced hepatic, renal and testicular AHH activities to approximately 30% of normal (uninhibited values) while MET reduced hepatic and testicular AHH activities to nearly 30%, and renal AHH activities to nearly 60% of normal values (testicular data not shown). Treatment with multiple doses of NaPb appeared to increase the susceptibility of hepatic AHH (and, to a slight extent, renal AHH) to the inhibitory effects of MET while concomitantly reducing the susceptibility of hepatic AHH to the inhibitory effects of ANF (Figure 4). Treatment with 3MC had the opposite effect; renal and hepatic AHH became more susceptible (in comparison to AHH from control animals) to the inhibitory effects of ANF but hepatic AHH became less susceptible to the inhibitory effects of MET (Figure 4). Hepatic AHH from rats receiving multiple doses of PBB and PCB did not exhibit net alterations in susceptibilities to the inhibitory effects of ANF and MET. Renal AHH from these same rats, however, responded to ANF and MET in a manner quite similar to that of renal AHH from rats treated with 3MC. That is, renal AHH from PBB and PCB-treated rats exhibited increased sensitivity to the inhibitory effects of ANF (Figure 4). Inhibition of

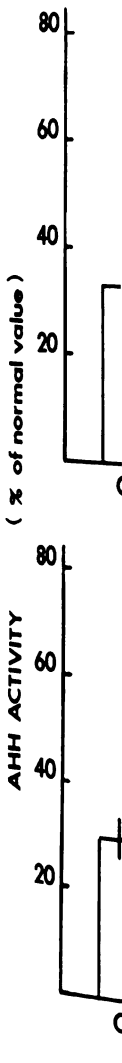


Figure 4. α-Napthoflavone (ANF) and metyrapone (MET) inhibition of aryl hydrocarbon hydroxylase (AHH) activities in vitro after multiple doses of polybrominated biphenyls (PBB), polychlorinated biphenyls (PCB), sodium phenobarbital (NaPb) or 3-methylcholanthrene (3MC). Bars represent the means ± 1 S.E. (N=4 rats) of the activities of AHH, in the presence of ANF ($1x10^{-4}M$) or MET ($1x10^{-4}M$), from rats sacrificed 24 hours after the final dose of a multiple-dose regimen of PBB, PCB, NaPb or 3MC. The data are expressed as percentages of normal (no inhibitor present) values. *Significantly different from control (C), $p<0.05$.

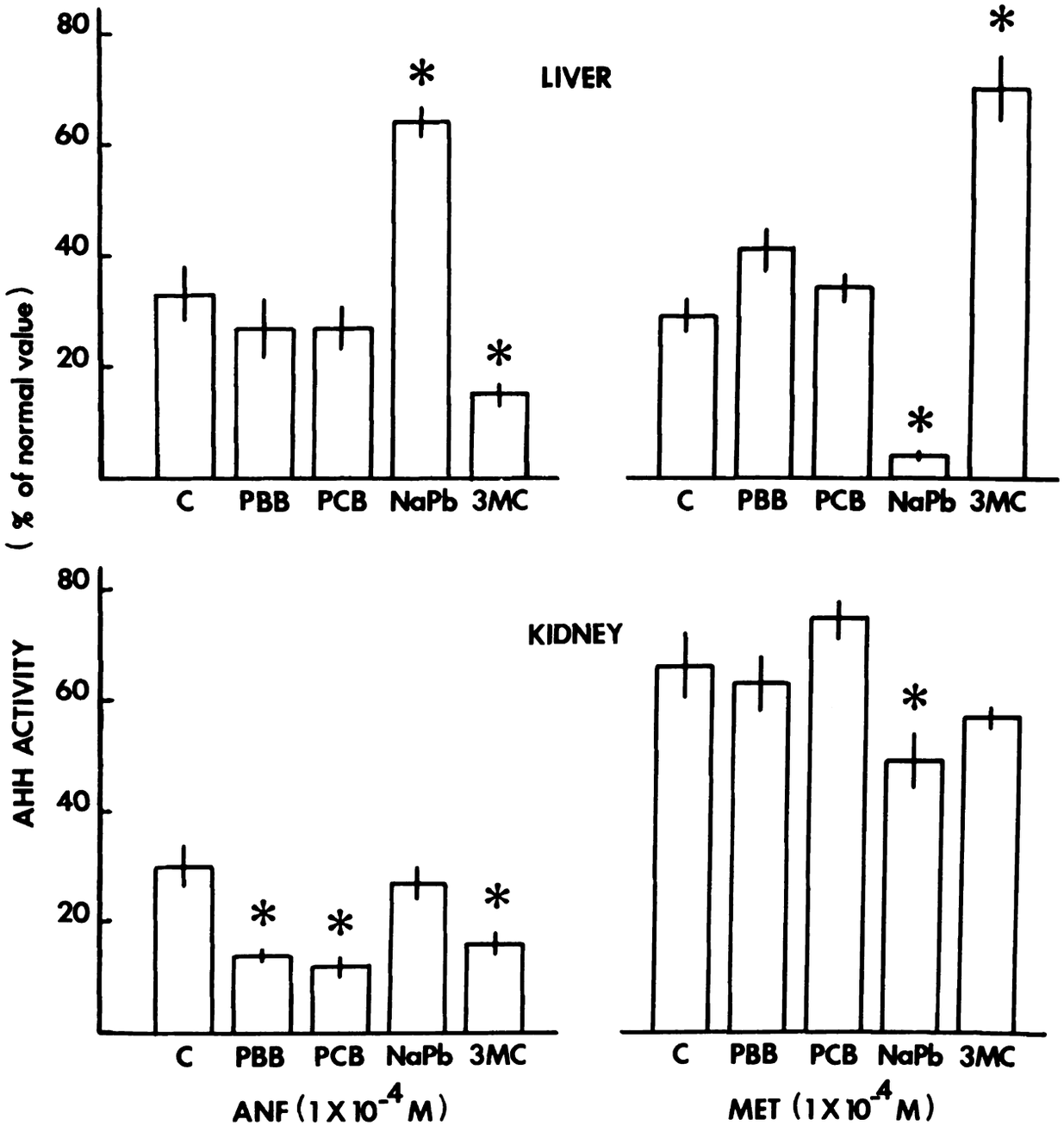

LIVER
KIDNEY
AHH ACTIVITY (% of normal value)
80
60
40
20
C
PBB
PCB
NaPb
3MC
ANF (1 X 10-4 M)
MET (1 X 10-4 M)

Figure 4

testicular AHH by ANF and MET was unaltered by the administration of multiple doses of PBB, PCB, NaPb and 3MC (data not shown).

Table 7 illustrates the time-dependencies of PBB and PCB-induced alterations in susceptibility of renal AHH to the inhibitory effects of ANF. Of the times evaluated (9, 72 and 216 hr after a single dose of PBB or PCB), increased susceptibility to the inhibitory effects of ANF appeared to be maximal at 9 hr. Although still more susceptible to ANF than renal AHH from control animals, renal AHH from PBB and PCB-treated rats was less susceptible to the inhibitory effects of ANF at 216 hr (72 hr for PCB-treated rats) than at 9 hr (Table 7). Thus, the time courses of the PBB and PCB-induced increases in the susceptibility of renal AHH to the inhibitory effects of ANF paralleled the time courses of the PBB and PCB-induced increases in renal AHH activity (Figure 3).

4. Inhibition of renal and hepatic AHH activity in vitro

The effects of several inhibitors of MFO activities on renal and hepatic microsomal AHH activities in vitro are illustrated in Figure 5. Tween 80, the detergent used for dissolution of AIA and PB, produced significant inhibition of microsomal AHH activity when present in the medium at a concentration of 0.01% (v/v). Hepatic AHH activity appeared more sensitive to inhibition by Tween 80 (activity reduced to 45% of control values) than did renal AHH activity (reduced to 65% of control values) (Figure 5). Renal and hepatic microsomal AHH activities, however, were not further reduced by the presence of AIA in the incubation mixtures (Figure 5). PB, on the other hand, decreased the activities of renal and hepatic AHH, and renal and

TABLE 7

α-Napthoflavone (ANF) Inhibition of Renal Aryl Hydrocarbon Hydroxylase (AHH) Activities In Vitro at Various Times After a Single Dose of Polybrominated Biphenyls (PBB) or Polychlorinated Biphenyls (PCB)

Treatment	AHH Activity (% of normal values) ± 1 S.E.		
Time (hr):	9	72	216
Control	33±1	30±4	33±3
PBB	9±1[a]	12±1[a]	20±2[a,b]
PCB	6±1[a]	20±1[a,b]	17±3[a,b]

Rats were sacrificed 9, 72 or 216 hr after a single dose of PBB or PCB. AHH activity in the 14,000 x g supernatant fraction of renal homogenates was determined in the presence of ANF ($1x10^{-4}$M). Values represent the means ± 1 S.E. of 4 animals. The data are expressed as percentages of normal (no inhibitor present) values.

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

[b] Significantly different from 9 hr, same treatment, $p<0.05$.

Figure 5. Inhibition of renal and hepatic microsomal aryl hydrocarbon hydroxylase (AHH) activities in vitro by allyl-isopropyl-acetamide (AIA), SKF 525-A, metyrapone (MET), piperonyl butoxide (PB) and α-napthoflavone (ANF). AHH activities were determined in the presence of AIA, SKF 525-A, MET, PB or ANF; 0, $1x10^{-6}$, $1x10^{-5}$, $1x10^{-4}$, or $1x10^{-3}$M, and with no inhibitors or vehicles present (control). Activities are expressed as percentages of control, mean ± 1 S.E. Circles represent renal microsomal activities and squares represent hepatic microsomal activities. Open symbols, significantly different from control (no inhibitor). *Significant difference between percent inhibition in renal and hepatic AHH even though both were significantly inhibited. N=5 independent experiments, $p<0.05$.

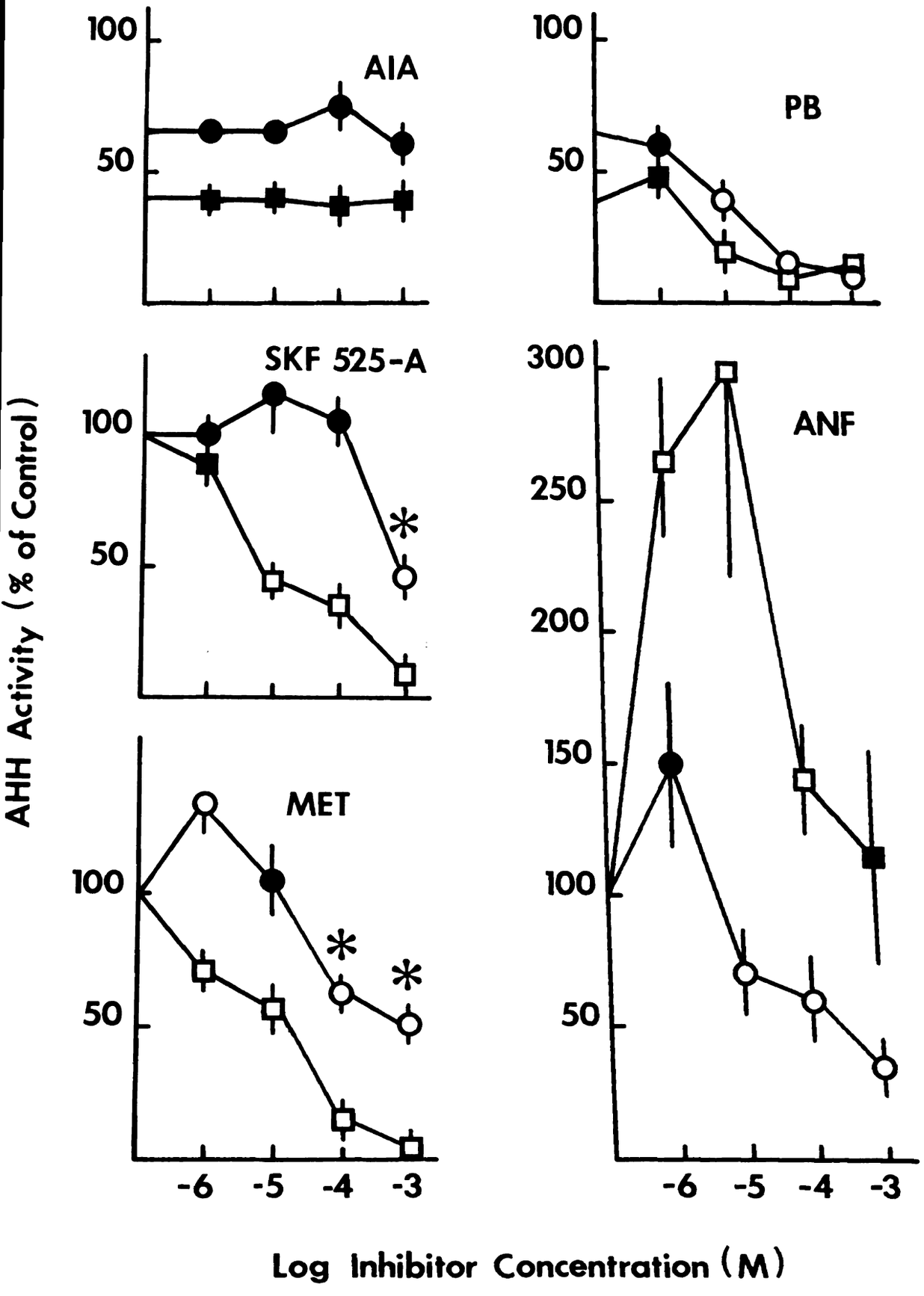

AIA
PB
SKF 525-A
ANF
MET
100
50
300
250
200
150
-6
-5
-4
-3
*
AHH Activity (% of Control)
Log Inhibitor Concentration (M)

Figure 5

patic AHH appeared to be equally susceptible to inhibition by in-
easing concentrations of PB in the incubation mixture. The presence
SKF 525-A and MET in the incubation mixtures produced concentration-
pendent decreases in the activity of hepatic AHH but increases, at
w concentrations, in the activity of renal AHH (Figure 5). Addition
ANF to the incubation mixtures produced large increases in hepatic
H activity that were maximal at a concentration of $1x10^{-5}$M and
clined as the concentration of ANF was increased. In contrast, ANF
concentrations of $1x10^{-5}$M and greater inhibited renal AHH acti-
y (Figure 5).

5. Time-dependent effects of single doses of SKF 525-A and PB on renal and hepatic PCNMA and AHH activities in rats and mice

Administration of a single i.p. injection of SKF 525-A, 75
kg, to Fischer 344 rats produced a transient decrease in hepatic
MA activity, an effect that was maximal 2 hr after dosing, and a
longed reduction in hepatic AHH activity (Figure 6). Renal PCNMA
ivity was not reduced by a single treatment with SKF 525-A; renal
activity was reduced by SKF 525-A, but not to the extent that
atic AHH activity was reduced. Administration of PB, 600 mg/kg,
rats produced only a slight reduction in hepatic PCNMA activity
a prolonged reduction in hepatic AHH activity (Figure 6). Renal
activity was also reduced by treatment with PB, though not to
extent that hepatic AHH activity was reduced. Treatment with PB
not affect the activity of renal PCNMA. Qualitatively similar
ults were obtained when these experiments were conducted on ICR,
e mice (W.M. Kluwe and J.B. Hook, unpublished observations).

Figure 6. Time-dependent inhibition of p-chloro-N-methylaniline N-demethylase (PCNMA) and aryl hydrocarbon hydroxylase (AHH) activities by SKF 525-A and piperonyl butoxide (PB). Rats were sacrificed 1, 2, 4 or 12 hr after administration of SKF 525-A (75 mg/kg), PB (600 mg/kg) or vehicle. Enzyme activities were measured in the 14,000 x g supernatant fraction of renal and hepatic homogenates and expressed as percentages of control (vehicle-treated). The data are represented as the mean ± 1 S.E. of 6 animals. *Significant difference between percent inhibition in kidney and liver, $p<0.05$.

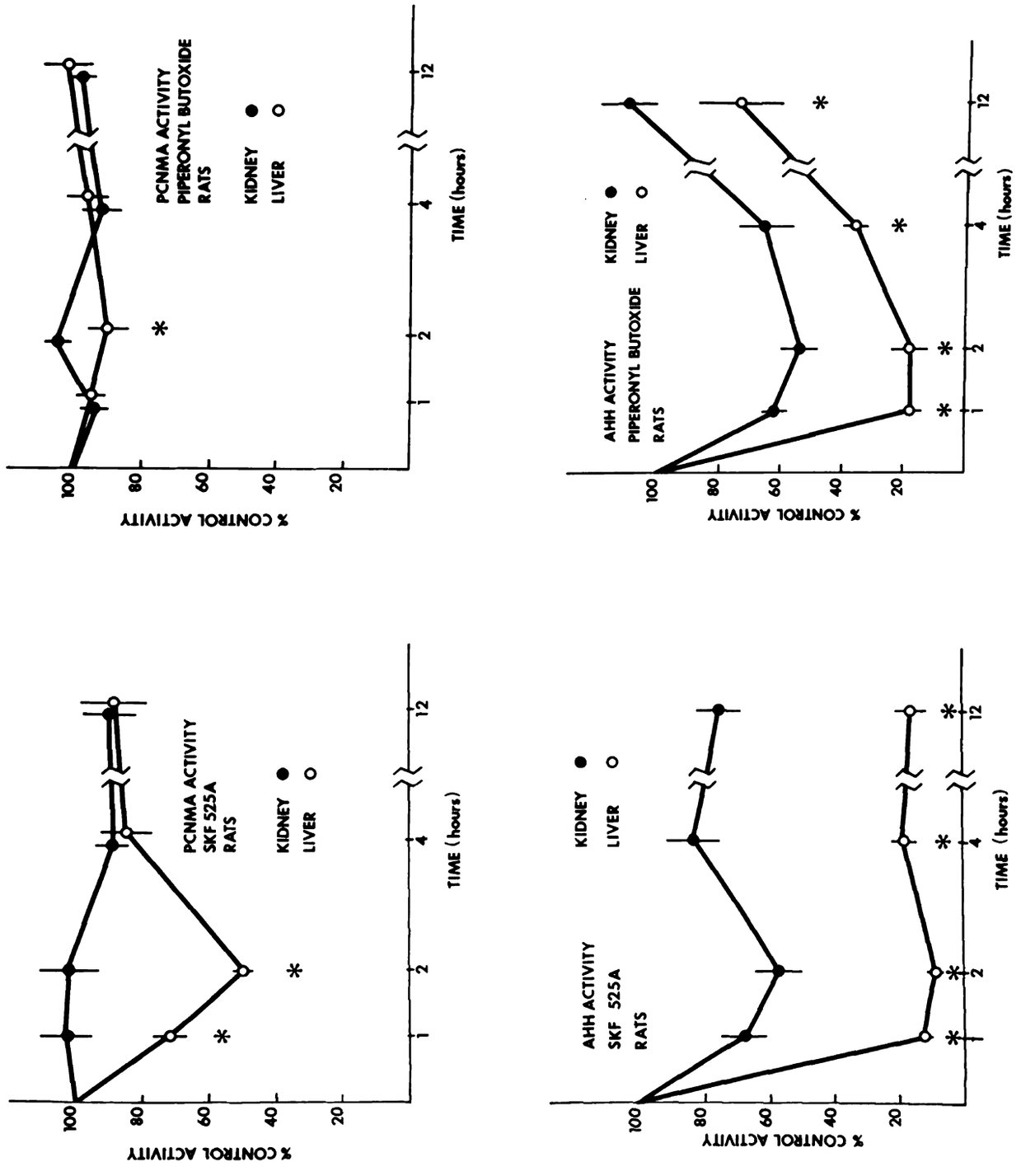

PCNMA ACTIVITY
PIPERONYL BUTOXIDE
RATS
KIDNEY ●
LIVER ○
% CONTROL ACTIVITY
TIME (hours)
PCNMA ACTIVITY
SKF 525A
RATS
KIDNEY ●
LIVER ○
% CONTROL ACTIVITY
TIME (hours)
AHH ACTIVITY
PIPERONYL BUTOXIDE
RATS
KIDNEY ●
LIVER ○
% CONTROL ACTIVITY
TIME (hours)
AHH ACTIVITY
SKF 525A
RATS
KIDNEY ●
LIVER ○
% CONTROL ACTIVITY
TIME (hours)

Figure 6

B. Enzyme Modulation and CCl_4 and $CHCl_3$ Toxicity

1. Effects of dietary PBB and PCB on CCl_4 toxicity in mice

Maintenance of ICR, male mice on diets containing 100 ppm of PBB or 200 ppm of PCB for 28 days markedly increased liver weight-to-body weight ratios (Table 8) without significantly affecting total body weight. The effect of PBB on liver weight was greater than that of PCB, even though PCB was present in the diet at a greater concentration than was PBB. Kidney weight-to-body weight ratios were not affected by ingestion of PBB or PCB (Table 8).

Ingestion of PBB and, to a lesser extent PCB, appeared to enhance the susceptibility of mice to the toxic effects of CCl_4. The CCl_4-induced rise in SGOT activity in PBB-treated mice was greater than that in control mice 24 hr after administration of 0.125 ml/kg CCl_4, and greater in PCB-treated mice than in control mice after administration of 0.625 ml/kg CCl_4 (Figure 7). The CCl_4-induced increases in SGPT activities were affected by PBB and PCB in manners similar to those of SGOT (data not shown). PBB appeared to increase the susceptibility of mice to CCl_4 nephrotoxicity. Although administration of up to 0.625 ml/kg CCl_4 to control and to PCB-treated mice failed to produce a decrease in the abilities of renal cortical slices to accumulate PAH and TEA (PAH and TEA S/Ms), as little as 0.125 ml/kg CCl_4 produced significant decreases in PAH and TEA S/Ms in PBB-treated mice (Figure 7, TEA data not shown).

The LD_{50} values for CCl_4 in mice were inversely related to the concentrations of PBB in the diet (Table 9). Ingestion of 20 ppm of PBB in the diet for 20 days increased the susceptibility of mice

TABLE 8

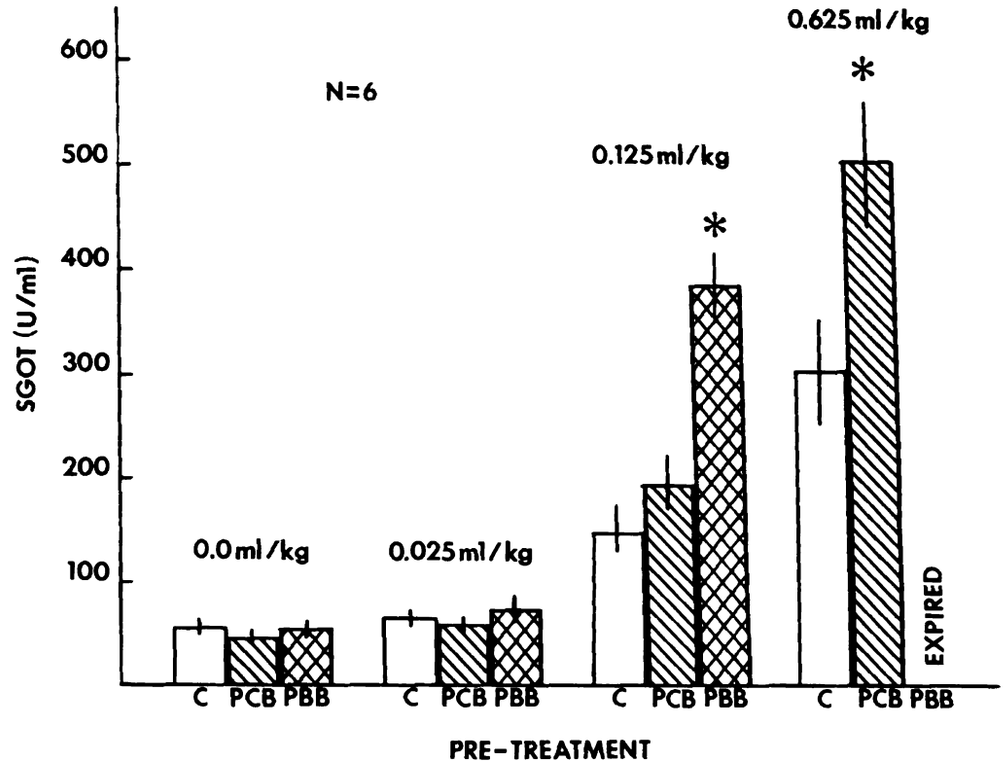
Effects of Dietary Polybrominated Biphenyls (PBB) and Polychlorinaed Biphenyls (PCB) on Liver Weight/Body Weight (LW/BW) and Kidney Weight/Body Weight (KW/BW)

Diet	LW/BW ± 1 S.E. (x 100)	KW/BW ± 1 S.E. (x 100)
C	5.93±0.09	1.51±0.03
PCB	8.29±0.20[a]	1.42±0.04
PBB	13.62±0.35[a]	1.42±0.04

Mice were maintained on control diet (C) or the same diet formulated to contain 100 ppm of PBB or 200 ppm of PCB for 28 days.

[a]Significantly greater than control (C), $p<0.05$.

Figure 7. Effects of polybrominated biphenyls (PBB) and polychlorinated biphenyls (PCB) and CCl_4 on SGOT and PAH S/M. Mice were maintained on control diet (C) or the same diet formulated to contain 100 ppm of PBB or 200 ppm of PCB for 28 days prior to a single i.p. injection of one of several doses of CCl_4. Mice were killed 48 hr later and SGOT and PAH S/Ms determined. *Significant difference in comparison to mice receiving the same dose of CCl_4 but ingesting control diet (C), $p<0.05$.


600
500
400
300
200
100
SGOT (U/ml)
N=6
0.625 ml/kg
*
0.125 ml/kg
*
0.0 ml/kg
0.025 ml/kg
EXPIRED
C PCB PBB
C PCB PBB
C PCB PBB
C PCB PBB
PRE-TREATMENT

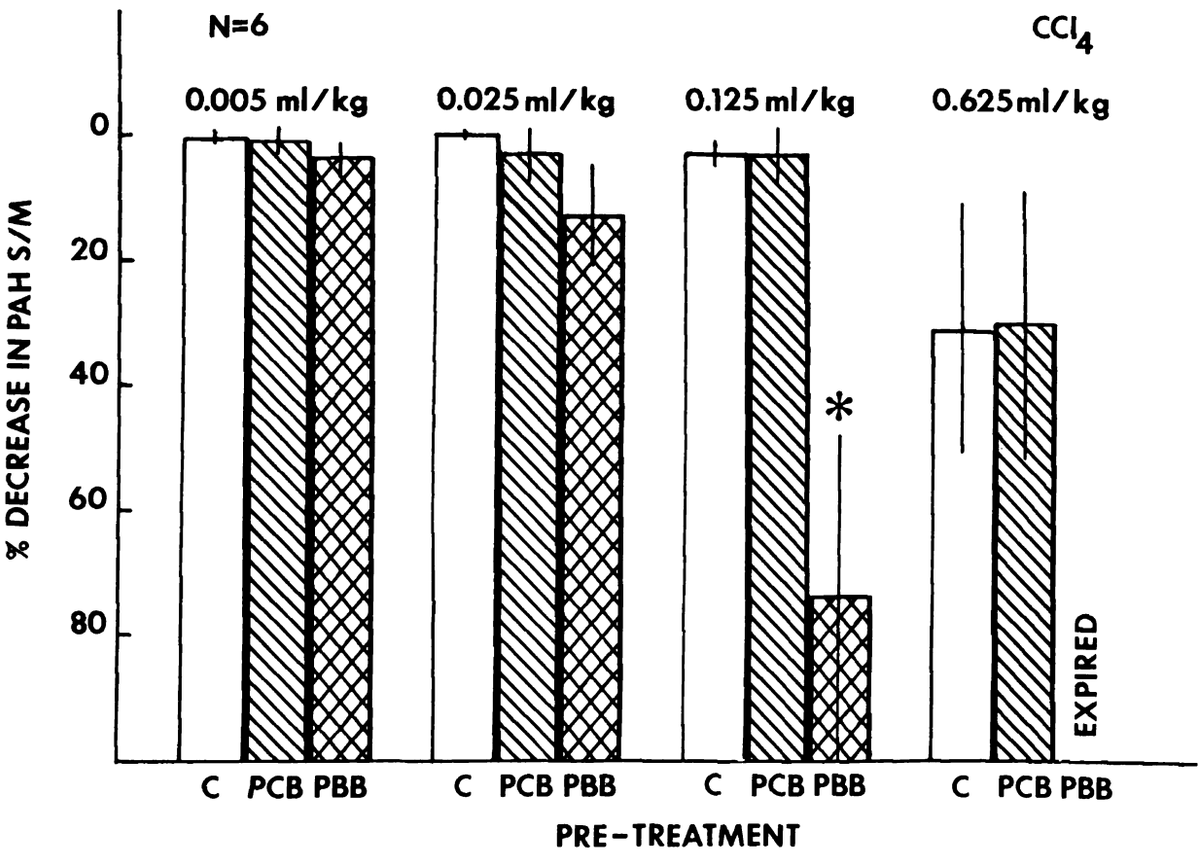

N=6
CCl_4
0.005 ml/kg
0.025 ml/kg
0.125 ml/kg
0.625 ml/kg
0
20
40
60
80
% DECREASE IN PAH S/M
*
EXPIRED
C PCB PBB
C PCB PBB
C PCB PBB
C PCB PBB
PRE-TREATMENT

Figure 7

TABLE 9

Effects of Polybrominated Biphenyls (PBB) on the Acute LD_{50} of CCl_4

Dietary PBB(ppm)	96-hour LD_{50} (ml/kg)	Potency Ratio
0	1.84 (1.55-2.18)	
20	1.00 (0.82-1.22)[a]	1.84 (1.46-2.32)[a]
100	0.28 (0.25-0.31)[a,b]	6.57 (5.48-7.88)[a,b]

Mice were fed diets formulated to contain 0, 20, or 100 ppm polybrominated biphenyls (PBB) for 20 days prior to a single injection of CCl_4, in corn oil, in a total volume of 5 ml/kg. Each dose of CCl_4 was administered to 10 animals. Deaths occurring within 96 hr after CCl_4 administration were recorded. The 95% confidence limits are in parentheses following LD_{50} and potency ratio values.

Potency ratio is defined as the LD_{50} in mice ingesting 0 ppm PBB divided by the LD_{50} in mice ingesting 20 or 100 ppm PBB.

[a]Significant decrease/increase in comparison to 0 ppm PBB.

[b]Significant decrease/increase in comparison to 20 ppm PBB.

to the lethal effect of CCl_4 by nearly a factor of 2 while ingestion of 100 ppm of PBB in the diet for 20 days increased susceptibility to the lethal effect of CCl_4 by a factor of more than 6. The maximum cumulative number of deaths produced by CCl_4 in both control mice and PBB-treated mice occurred 96 hr after administration of CCl_4. Mice surviving the initial 4 days after CCl_4 administration were observed for an additional 2 weeks during which time no deaths occurred. In addition, the dose-response (cumulative death) curves (plotted on log vs. probit scales) were parallel, suggesting that the mechanism of CCl_4-induced death was the same in the control and the PBB-treated mice.

2. Effects of PBB, PCB and HCB on CCl_4 toxicity in rats

Ingestion of the aromatic organohalides, HCB, PBB and PCB, by male, Sprague-Dawley rats over a 20 day period produced hepatomegaly; liver weight-to-body weight ratios were increased (Table 10) but total body weights were not affected by the aromatic organohalides (data not shown). Kidney weight-to-body weight ratios, in contrast, were unaffected by aromatic organohalide treatment. The increase in relative liver size was accompanied by an increase in hepatic lipid content, though renal lipid content was not affected (Table 11). Treatment of rats with single injections of CCl_4 (0.25 or 2.00 ml/kg) also increased hepatic lipid content, an effect that was not additive to that of PBB, PCB or HCB on hepatic lipid content (Table 11). Renal lipid content was not increased by treatment with CCl_4 nor by any combination of CCl_4 and aromatic organohalides.

TABLE 10

Effects of Aromatic Organohalides on Liver Weight/Body Weight (LW/BW) and Kidney Weight/Body Weight (KW/BW)

Treatment[a]	LW/BW ± 1 S.E. (x 100)	KW/BW ± 1 S.E. (x 100)
Control	4.54±0.08	0.745±0.011
HCB	6.08±0.06[b]	0.776±0.013
PBB	7.06±0.15[b,c]	0.753±0.013
PCB	5.77±0.06[b]	0.766±0.053

[a]Sprague-Dawley, male rats were treated over a 20 day period with hexachlorobenzene (HCB), polybrominated biphenyls (PBB) or polychlorinated biphenyls (PCB). Control rats received the appropriate vehicles.

[b]Significantly greater than control, $p<0.05$.

[c]Significantly greater than HCB or PCB, $p<0.05$.

N=6 except HCB, 2.00 ml/kg CCl_4, where N=4.

TABLE 11

Effects of Aromatic Organohalides and CCl_4 on Lipid Content of Liver (L) and Kidney (K)

Organ	Pretreatment[a]: CCl_4 (ml/kg)	Lipid Content (mg/gram) ± 1 S.E. Control	HCB	PBB	PCB
L	0.00 (vehicle)	39.11±2.00	55.30± 4.50[b]	59.24± 3.15[b]	64.80± 3.07[b]
L	0.03	43.37±3.33	57.40± 3.11	60.10±10.56	72.40±11.53[b]
L	0.25	62.08±2.16[c]	50.18± 1.30	61.80± 3.06	68.28±10.76
L	2.00	64.28±2.94[c]	83.91±20.37	----	----
K	0.00 (vehicle)	39.20±1.85	38.75± 1.21	41.57± 4.01	47.65± 9.96
K	0.25	33.53±3.03	40.98± 4.24	35.11± 5.04	41.48± 9.60
K	2.00	37.78±2.44	38.96± 7.02	----	----

[a] Sprague-Dawley, male rats were pretreated over a 20 day period with hexachlorobenzene (HCB), polybrominated biphenyls (PBB), polychlorinated biphenyls (PCB) or the appropriate vehicles (Control) and then challenged with one of several doses of CCl_4 (0.00-2.00 ml/kg).

[b] Significant increase in comparison to control, same amount of CCl_4, $p<0.05$.

[c] Significant increase in comparison to animals receiving the same pretreatment but 0.00 ml/kg CCl_4 (vehicle), $p<0.05$.

N=3 animals.

Ingestion of PBB, PCB and HCB increased the susceptibility of rats to the lethal effects of i.p.-administered CCl_4 (Table 12). Accordingly, losses in body weight induced by CCl_4 occurred following lower doses of CCl_4 in PBB, PCB and HCB-treated rats than in control rats (Table 13).

Treatment with HCB, PBB and PCB, alone, did not significantly affect the abilities of renal cortical slices to accumulate PAH and TEA (Figure 8, TEA data not shown). Rats treated with HCB, PBB and PCB, however, exhibited significant decreases in PAH and TEA S/Ms after receiving as little as 0.25 ml/kg CCl_4, though a decrease was not apparent in control rats until the dose of CCl_4 was increased to 2.00 ml/kg (Figure 8). The combined effects of CCl_4 and the aromatic organohalides on renal cortical slice respiration paralleled their effects on PAH and TEA S/Ms, though in an inverse manner. Increases in renal cortical slice QO_2 were evident in renal tissue from control rats only after administration of 2.00 ml/kg CCl_4 while QO_2 from PBB and PCB-treated rats was increased after as little as 0.25 ml/kg CCl_4 (Figure 8).

Treatment with HCB, PBB and PCB similarly increased the susceptibility of the liver to the toxic effects of CCl_4. Increases in SGPT activities were detected in PCB-treated rats after as little as 0.03 ml/kg CCl_4 (Figure 9). Furthermore, increased SGPT activities were detected in HCB, PBB and PCB-treated rats, but not in control rats, following administration of 0.25 ml/kg CCl_4. Control rats exhibited increased SGPT activities after receiving 2.00 ml/kg CCl_4 (Figure 9).

TABLE 12

Effects of Aromatic Organohalides and CCl_4 on 48-hour Survival[a]

Pretreatment[b]	Number of Survivors/Number Treated CCl_4 (ml/kg): 0.25	2.00
Control	6/6	6/6
HCB	6/6	4/8
PBB	6/6	0/6
PCB	6/9	0/6

[a] 48-hour survival refers to the ability of HCB, PBB and PCB-pretreated rats or control rats to survive for 48 hr after a single injection of CCl_4.

[b] Sprague-Dawley, male rats were pretreated over a 20 day period with hexachlorobenzene (HCB), polybrominated biphenyls (PBB), polychlorinated biphenyls (PCB) or the appropriate vehicles and then challenged with one of several doses of CCl_4 (0.00-2.00 ml/kg).

TABLE 13

Effects of Aromatic Organohalides and CCl_4 on 48-hour Body Weight Gain

Pretreatment[b]: CCl_4 (ml/kg)	Percent of Original Body Weight[a] ± 1 S.E. Control	HCB	PBB	PCB
0.00 (vehicle)	107±2	108±1	111±2	111±2
0.03	106±1	104±1[c,d]	102±1[c,d]	100±2[c,d]
0.25	100±1[d]	94±1[c,d]	86±1[c,d]	84±2[c,d]
2.00	87±2[d]	88±2[d]	---	---

[a]Determined as body weight at time of sacrifice ÷ body weight 48 hr prior to sacrifice (time of CCl_4 administration) x 100.

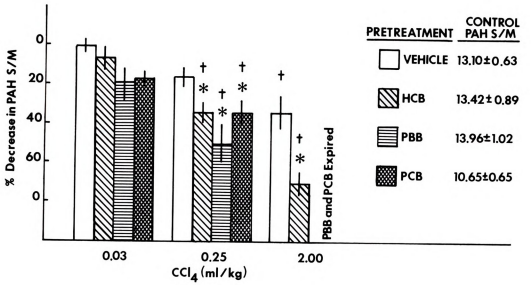
[b]Sprague-Dawley, male rats were pretreated over a 20 day period with hexachlorobenzene (HCB), polybrominated biphenyls (PBB), polychlorinated biphenyls (PCB) or the appropriate vehicles and then challenged with one of several doses of CCl_4 (0.00-2.00 ml/kg).

[c]Significantly lower than in control animals receiving the same amount of CCl_4, $p<0.05$.

[d]Significantly lower than in animals receiving the same pretreatment but 0.00 ml/kg CCl_4 (vehicle), $p<0.05$.

N=6 except HCB, 2.00 ml/kg CCl_4, where N=4.

Figure 8. Effects of aromatic organohalides and CCl_4 on renal QO_2 and PAH S/Ms. Rats treated subacutely (20 days) with hexachlorobenzene (HCB), polychlorinated biphenyls (PCB), polybrominated biphenyls (PBB) or vehicle were sacrificed 48 hr after i.p. injection of CCl_4 (0.00, 0.03, 0.25 or 2.00 ml/kg) and renal QO_2 and PAH S/Ms determined. QO_2 values are depicted as μl O_2 consumed/g tissue/min. Control values for PAH S/M and QO_2 are illustrated on the right portion of the figure. N=6 animals except HCB, 2.00 ml/kg, where N=4 animals. †Significant difference in comparison to animals receiving the same aromatic organohalide but 0.00 ml/kg CCl_4, $p<0.05$. *Significant difference in comparison to animals receiving the same dose of CCl_4 but no aromatic organohalide (vehicle), $p<0.05$.


% Decrease in PAH S/M
0
20
40
60
0
0.03
0.25
2.00
CCl4 (ml/kg)
PBB and PCB Expired
PRETREATMENT
CONTROL PAH S/M
VEHICLE 13.10±0.63
HCB 13.42± 0.89
PBB 13.96±1.02
PCB 10.65±0.65

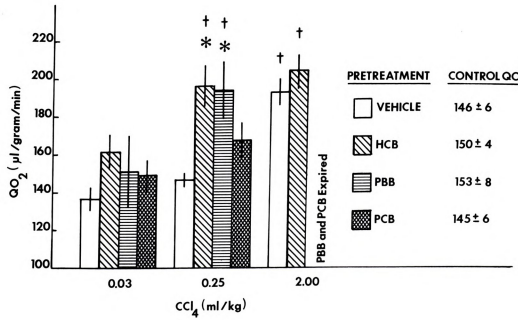

QO2 (µl/gram/min)
220
200
180
160
140
120
100
0.03
0.25
2.00
CCl4 (ml/kg)
PBB and PCB Expired
PRETREATMENT
CONTROL QO2
VEHICLE 146 ± 6
HCB 150 ± 4
PBB 153 ± 8
PCB 145 ± 6

Figure 8

Figure 9. Effects of aromatic organohalides and CCl_4 on SGPT activities. Rats treated subacutely with hexachlorobenzene (HCB), polybrominated biphenyls (PBB), polychlorinated biphenyls (PCB) or vehicle were sacrificed 48 hr after i.p. injection of CCl_4 (0.00 0.03, 0.25, or 2.00 ml/kg) and SGPT activities determined. N=6 animals except for HCB, 2.00 ml/kg, where N=4 animals. †Significant difference in comparison to animals receiving the same aromatic organohalide but 0.00 ml/kg CCl_4, $p<0.05$. *Significant difference in comparison to animals receiving the same dose of CCl_4 but no aromatic organohalide (vehicle), $p<0.05$.

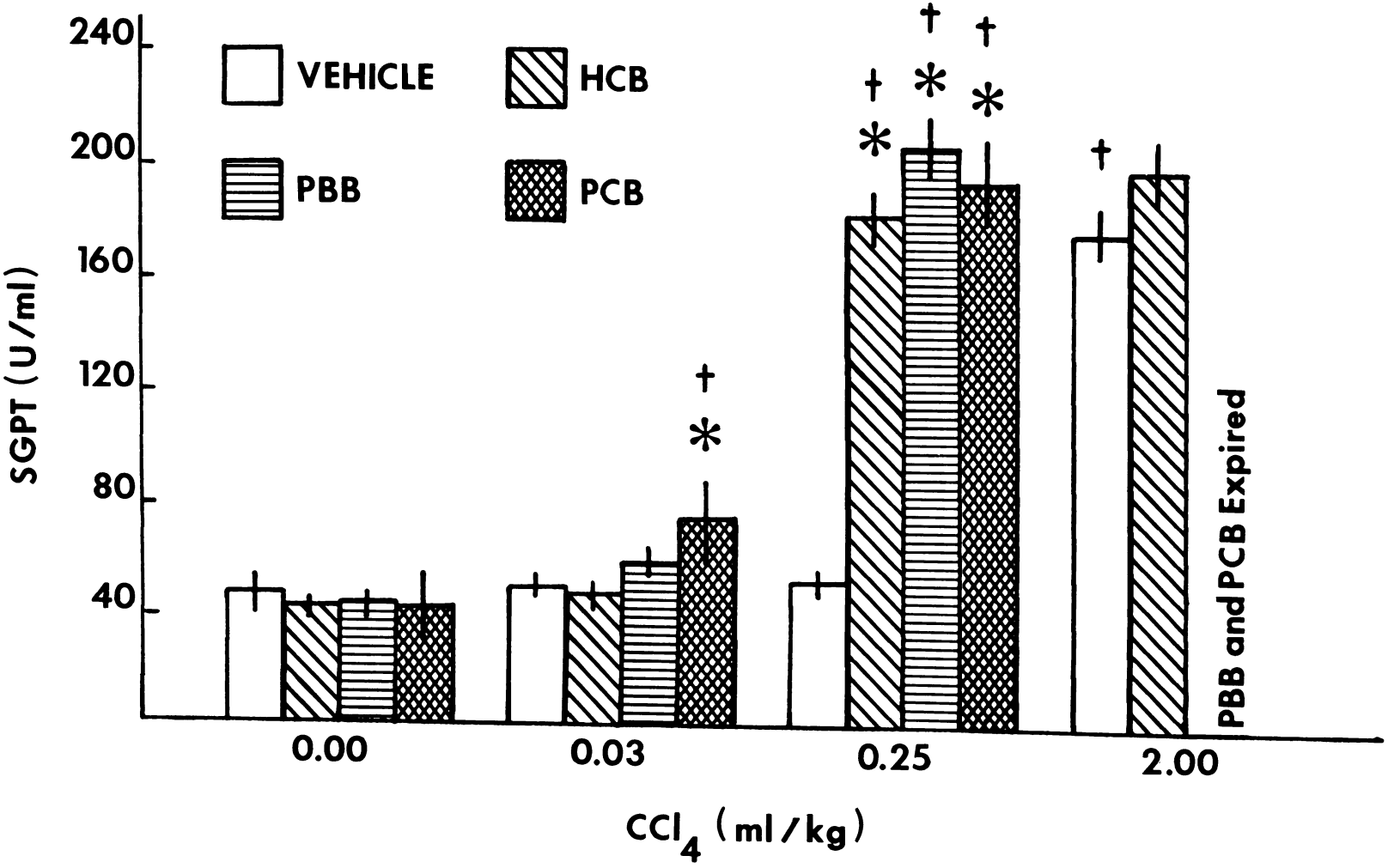

240
200
160
120
80
40
SGPT (U/ml)
VEHICLE
HCB
PBB
PCB
0.00
0.03
0.25
2.00
PBB and PCB Expired
CCl4 (ml/kg)

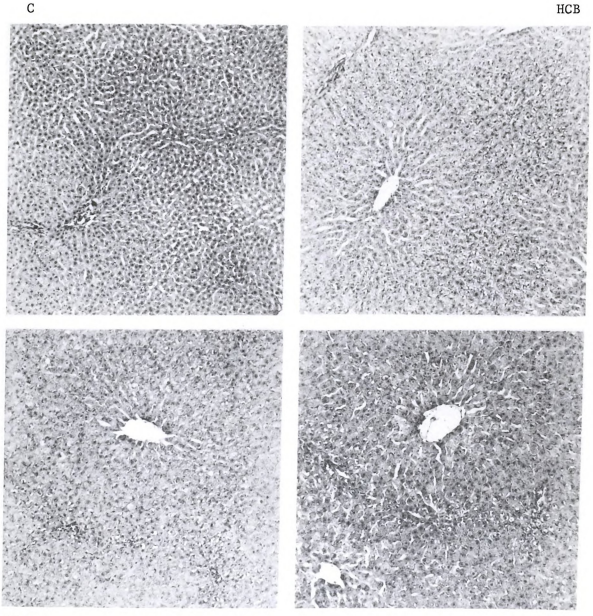
Figure 9

Sections of livers from control, HCB, PBB and PCB-treated rats injected with 0.00 (vehicle), 0.03, 0.25 or 2.00 ml/kg CCl_4 are illustrated in Figures 10, 11, 12 and 13, respectively. Normal liver histology was exhibited by tissue from control rats receiving no CCl_4 (Figure 10). Tissue from PBB, PCB and HCB-treated rats receiving no CCl_4 exhibited dilation of the hepatic vein, hepatocellular swelling and early degenerative changes (e.g., vacuolation) at the periphery of the lobules (Figure 10). With increasing doses of CCl_4 centrilobular necrosis (swelling and degeneration of the cells closest to the central vein) encompassed an increasing percentage of the liver in samples from control rats (Figures 11-13). Livers from PBB, PCB and HCB-treated rats exhibited similar progressions of centrilobular necrosis but the extent of necrosis in aromatic organohalide-treated rats appeared to be greater than that in control rats after doses of 0.03, and 0.25 ml/kg CCl_4 (Figures 11 and 12).

3. Effects of PBB and PCB on hepatocellular GPT and GOT activities in rats

The effects of dietary PBB and PCB, potent inducers of microsomal enzyme activities, on the activities of GPT and GOT within the liver were investigated to determine if PBB and PCB induced hepatic GPT and GOT activities and to determine if such effects might be responsible for potentiation of CCl_4-induced elevation of SGPT and SGOT activities. As shown in Table 14, the activities of hepatocellular GPT, per ml of 100,000 x g supernatant fraction or per mg of 100,000 x g supernatant protein, were not greater in PBB and PCB rats than in control rats. Relative liver weights, however, were significantly increased by ingestion of PBB and PCB (Table 11). If the

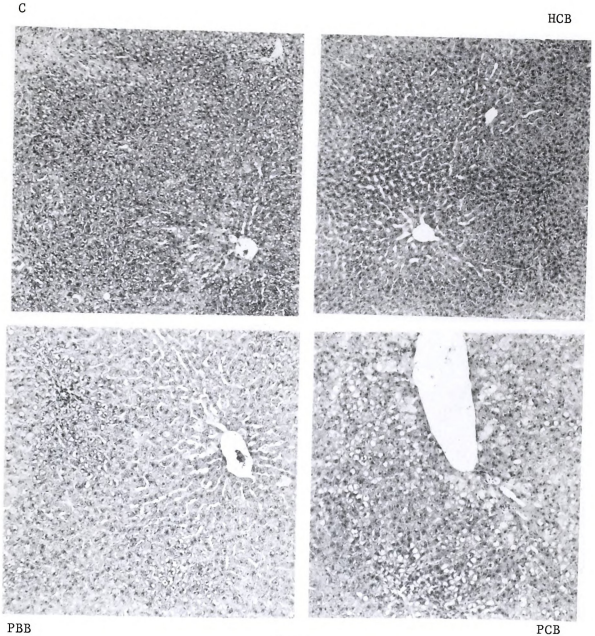
Figure 10. Sections of livers from rats receiving 0.00 ml/kg CCl_4. Hematoxylin and eosin stain, X40. A) No aromatic organohalide pretreatment. Parts of several lobules can be seen. Central veins are small, sinusoids are visible and hepatic cords present an orderly arrangement. Interlobular spaces are scarcely visible and all liver cells throughout the lobule present an identical appearance. B) Pretreated with hexachlorobenzene. Central veins are slightly dilated and hepatic cells at the periphery of the lobule are vacuolated. Swelling of cells has obliterated sinusoids at the periphery of the lobules. Hepatic cords remain regular in arrangement. C) Pretreated with polybrominated biphenyls. Appearance of the liver tissue is very similar to that described in 4B. D) Pretreated with polychlorinated biphenyls. The central vein is dilated, sinusoids are nearly obliterated by cellular swelling and light vacuolation of hepatic cells can be seen at the periphery of the lobule.



0.00

Figure 10

Figure 11. Hepatic tissue from rats receiving 0.03 ml/kg CCl_4. Hematoxylin and eosin stain, X40. A) No aromatic organohalide pretreatment. Hepatic cells are swollen; sinusoids are obliterated except those adjacent to the central vein and cells show vacuolation throughout the lobule. B) Pretreated with hexachlorobenzene. The central vein is symmetrical in outline, sinusoids are slightly visible and cellular vacuolation can be seen at the periphery of the lobule. C) Pretreated with polybrominated biphenyls. The central vein is dilated, adjacent sinusoids are open but peripheral hepatic cells are vacuolated. D) Pretreated with polychlorinated biphenyls. The central vein is markedly dilated and irregular in shape. Sinusoids are completely obliterated because of swelling of hepatic cells, many cells are necrotic and others are vacuolated.



0.03

Figure 11

Figure 12. Hepatic tissue from rats receiving 0.25 ml/kg CCl_4. Hematoxylin and eosin stain, X40. A) No aromatic organohalide pretreatment. The larger dose of CCl_4 caused a remarkable vacuolation of hepatic cells extending from the periphery to midway of the lobule. B) Pretreatment with hexachlorobenzene. Swollen hepatic cells throughout the lobule obliterate the sinusoids although the central vein remains readily visible. There is remarkable vacuolation of a few hepatic cells at the periphery of the lobule. C) Pretreatment with polybrominated biphenyls. The central vein is markedly dilated, hepatic cells are swollen and necrotic or vacuolated. Many nuclei are pyknotic. D) Pretreatment with polychlorinated biphenyls. Hepatic tissue is in a state of degeneration. Much necrosis has occurred, living cells are swollen, sinusoids are irregular, central veins are obliterated and hepatic architecture is deranged.

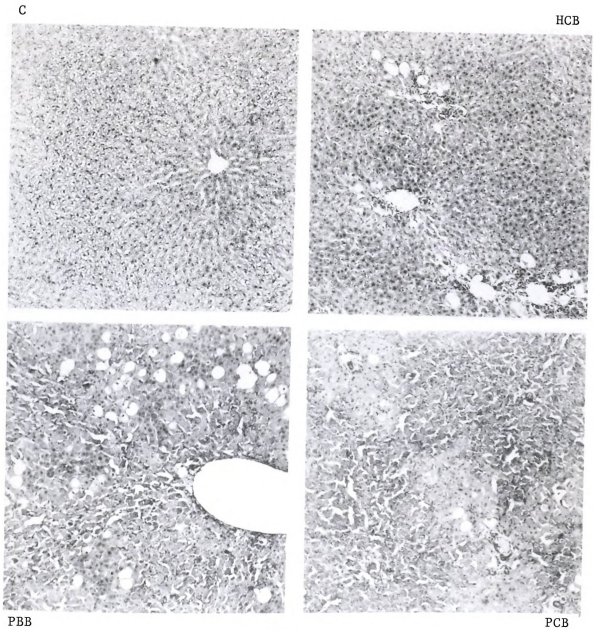
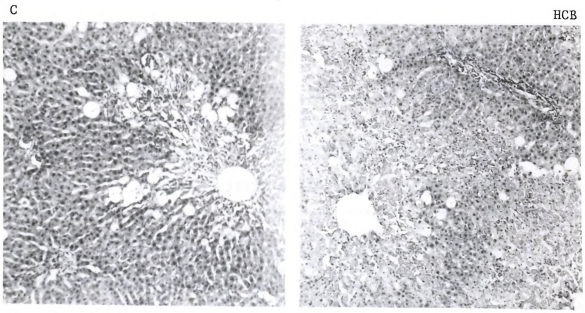


Figure 12

Figure 13. Hepatic tissue from rats receiving 2.00 ml/kg CCl_4. Hematoxylin and eosin stain, X40. A) No aromatic organohalide pretreatment. Liver cells are vacuolated and in the region of the central vein, cells have undergone necrosis with subsequent disruption of hepatic cords and sinusoids. The cells at the periphery of the lobule are less severely affected. B) Pretreatment with hexachlorobenzene. Some vacuolation of cells can be seen. Necrosis of cells extends from the central vein nearly to the periphery of the lobule. Sinusoids and liver cords are visible only near the periphery of the lobules.



2.00

Figure 13

TABLE 14

Effects of Dietary Polybrominated Biphenyls (PBB) and Polychlorinated Biphenyls (PCB) on Hepatic GPT Activity

Treatment	Mean GPT activity ± 1 S.E. (% of control)		
	U/ml x 10^{3}[a]	U/mg[b]	U/100g x 10^{3}[c]
Control	4.72±0.32	442±36	86±6
PBB	4.17±0.12 (87)	361±15 (82)	118±34 (138)[d]
PCB	5.34±0.48 (113)	423±42 (95)	123±11 (144)[d]

GPT activities were measured in the 100,000 x g supernatant fraction of hepatic homogenates from rats maintained for 20 days on diets containing 100 ppm of PBB or 200 ppm of PCB or diet formulated with an equivalent amount of acetone (control). Activities were expressed per ml of 100,000 x g supernatant fraction (*a*), per mg of 100,000 x g supernatant protein (*b*), and per 100 g of body weight (*c*).

[d]Significantly greater than control, $p<0.05$. N=5 rats.

increases in liver weight are taken into account, then GPT activities, per 100 g body weight, were increased by treatment with PBB and PCB (Table 14). Quantitatively similar effects were produced by PBB and PCB on hepatic GOT activities (data not shown).

4. Effects of maternal consumption of PBB on the toxicities of CCl_4 and $CHCl_3$ in developing male rats

Sprague-Dawley, male rats were exposed to PBB preweaning by the inclusion of 100 ppm of PBB in the dam's diet from day 8 of gestation, and to PBB postweaning by the inclusion of 100 ppm of PBB in the diet consumed by the pups postweaning. At 52 days of age rats exposed to PBB continuously (100-100) had lower body weights than rats exposed to PBB during preweaning only (100-0), and rats exposed to PBB preweaning had lower body weights that control rats (0-0) (Figure 14). Ingestion of PBB greatly increased the susceptibility of rats to the hepatotoxic and nephrotoxic effects of CCl_4. SGPT activities were elevated by as low a dose of CCl_4 as 0.03 ml/kg in rats exposed to PBB preweaning or continuously, but were increased in control rats only by a much larger dose of CCl_4 (2.00 ml/kg) (Figure 15). Similarly, PAH and TEA S/Ms were reduced in control rats by administration of 2.00 ml/kg CCl_4, but not lower doses, while as low a dose as 0.03 ml/kg CCl_4 reduced PAH and TEA S/Ms in rats exposed to PBB preweaning or continuously (Figure 15, TEA data not shown).

5. Effects of dietary PBB on $CHCl_3$ toxicity in mice

Ingestion of PBB by ICR, male mice produced dietary concentration-dependent increases in liver weight-to-body weight ratios (Table 15). Since body weights were not significantly affected by

Figure 14. Effects of exposure to polybrominated biphenyls (PBB) during development on body weight at 52 days of age. Control diet (0-0); 100 ppm PBB fed to dam, pups weaned onto control diet (100-0); 100 ppm PBB fed to dams, pups weaned onto 100 ppm PBB diet (100-100), N=16. *Significantly less than 0-0, $p<0.05$. †Significantly less than 100-0, $p<0.05$.

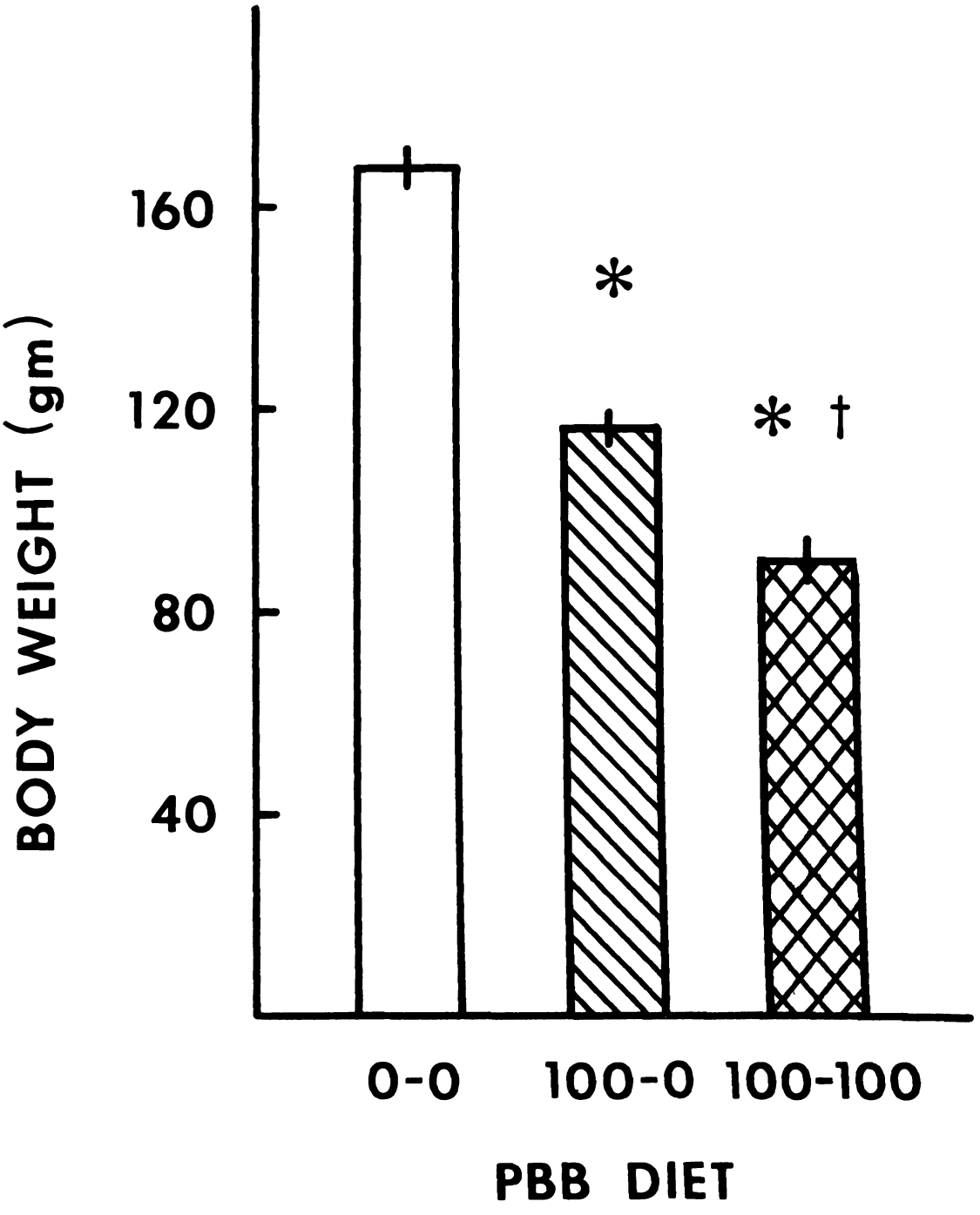
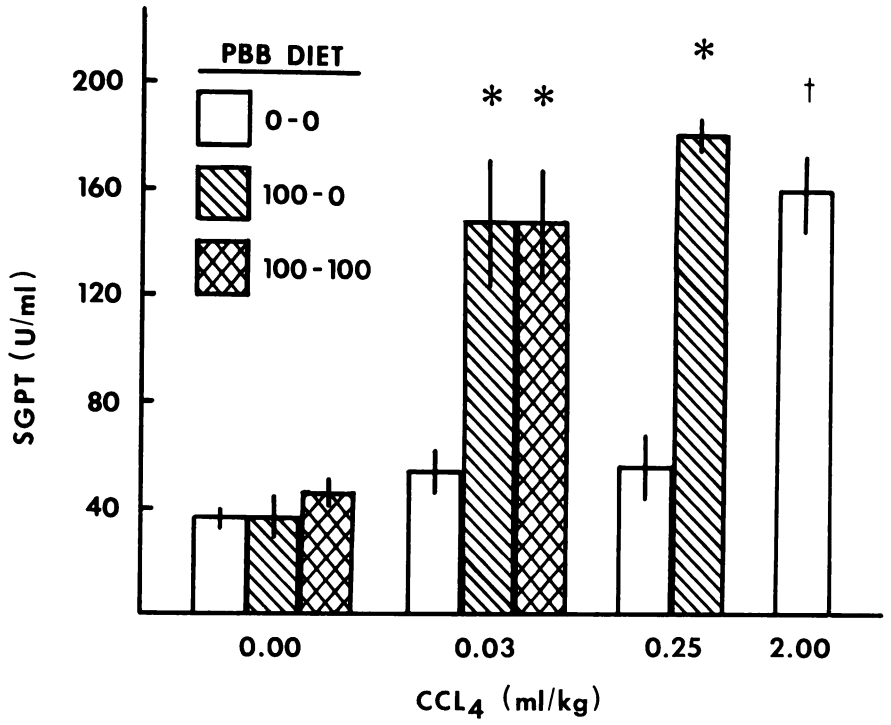


Figure 14

Figure 15. Effects of polybrominated biphenyls (PBB) and CCl_4 on SGPT and PAH S/M. Rats were sacrificed 48 hr after a single i.p. injection of CCl_4 (0.00, 0.03, 0.25 or 2.00 ml/kg) and SGPT and PAH S/Ms determined. Control diet (0-0); 100 ppm PBB fed to dam, pups weaned onto control diet (100-0); 100 ppm PBB fed to dams, pups weaned onto 100 ppm PBB diet (100-100). N=4 rats except 100-0, 0.25 ml/kg CCl_4, where N=2 rats. *Significantly different from 0-0 receiving the same amount of CCl_4, $p<0.05$. †Significantly different from 0-0 receiving 0.00 ml/kg CCl_4, $p<0.05$.


PBB DIET
0-0
100-0
100-100
200
160
120
80
40
SGPT (U/ml)
* *
*
†
0.00
0.03
0.25
2.00
CCL4 (ml/kg)

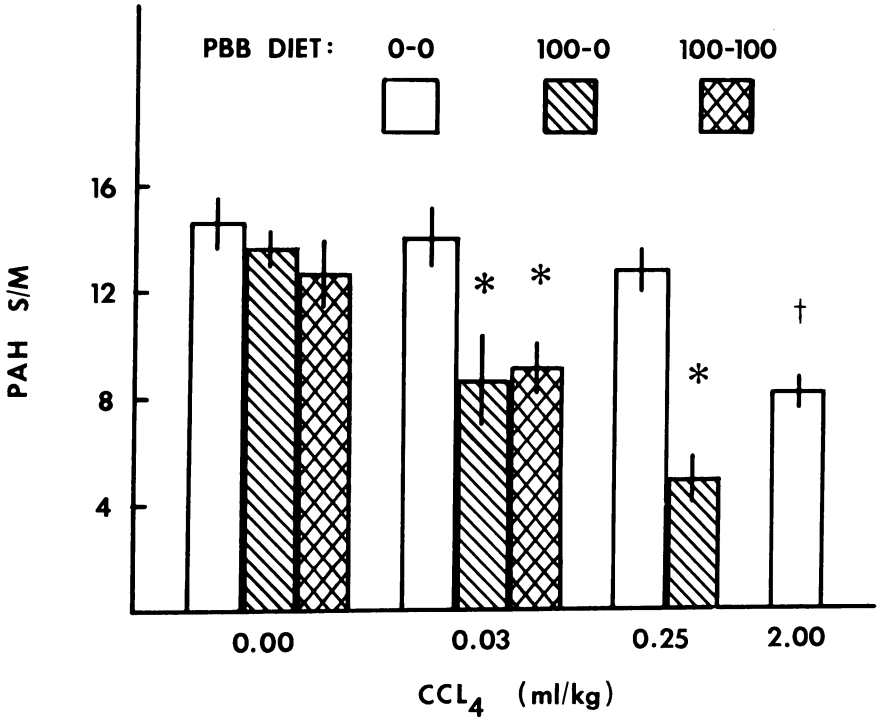

PBB DIET:
0-0
100-0
100-100
16
12
8
4
PAH S/M
* *
*
†
0.00
0.03
0.25
2.00
CCL4 (ml/kg)

Figure 15

TABLE 15

Effects of Dietary Polybrominated Biphenyls (PBB) on Liver Weight/Body Weight (LW/BW) and Kidney Weight/Body Weight (KW/BW)

PBB Diet[a]	LW/BW ± 1 S.E. (x 100)	KW/BW ± 1 S.E. (x 100)
Control	5.97±0.23	1.54±0.05
1 ppm	6.10±0.19[b]	1.49±0.04
25 ppm	6.91±0.31[b]	1.47±0.03
100 ppm	9.68±0.38[b]	1.58±0.04

[a]Animals were maintained on diets containing 0 (control), 1, 25 or 100 ppm of PBB for 14 days. Each value represents the mean ± 1 S.E. of 6 animals.

[b]Significantly greater than control, $p<0.05$.

dietary PBB the increases in liver weight-to-body weight ratios are indicative of PBB-induced increases in liver size. Kidney weight-to-body weight ratios, in contrast, were not affected by consumption of PBB (Table 15). The hepatotoxicity and nephrotoxicity of $CHCl_3$ were increased by ingestion of PBB. SGOT activities, already slightly elevated in mice ingesting 100 ppm of PBB for 14 days, were further increased by 25 µl/kg $CHCl_3$ in mice ingesting 100 ppm PBB diet but not in mice receiving lower dietary concentrations of PBB (Figure 16). PBB produced similar effects on $CHCl_3$-induced elevations of SGPT activities (data not shown). $CHCl_3$-induced increases in BUN and decreases in PAH and TEA S/Ms were also potentiated by PBB in a dietary concentration-dependent manner (Figure 17, TEA data not shown). Increases in BUN and decreases in PAH and TEA S/Ms were evident in control mice only after a dose of 50 µl/kg $CHCl_3$. Increases in BUN, however, were produced by 25 µl/kg of $CHCl_3$ in mice ingesting 25 and 100 ppm of PBB, and decreases in PAH and TEA S/Ms were produced by 25 µl/kg of $CHCl_3$ in mice ingesting 1 and 25 ppm of PBB and by 2.5 µl/kg of $CHCl_3$ in mice ingesting 100 ppm of PBB (Figure 17).

6. Effects of NaPb, 3MC, PCB and TCDD on $CHCl_3$ toxicity in mice

Treatment with NaPb appeared to increase the susceptibility of mice to the hepatotoxic effects of $CHCl_3$; 0.25 ml/kg of $CHCl_3$ increased SGPT activity in NaPb-treated mice but not in control mice (Figure 18). Though 3MC and PCB treatment had no marked effects on hepatic response to $CHCl_3$, treatment with TCDD may have protected the liver from the toxic effects of $CHCl_3$; 0.75 ml/kg of $CHCl_3$ increased SGPT activity in both control and TCDD-treated mice, but the magnitude

Figure 16. Effects of dietary polybrominated biphenyls (PBB) and i.p. $CHCl_3$ on SGOT activity. Animals were maintained on diets containing 0 (control), 1, 25 or 100 ppm of PBB for 14 days prior to a single administration of $CHCl_3$. SGOT was determined 24 hr after $CHCl_3$ administration. Each value represents the mean ± 1 S.E. of 6 animals. *Significant increase in comparison to C.

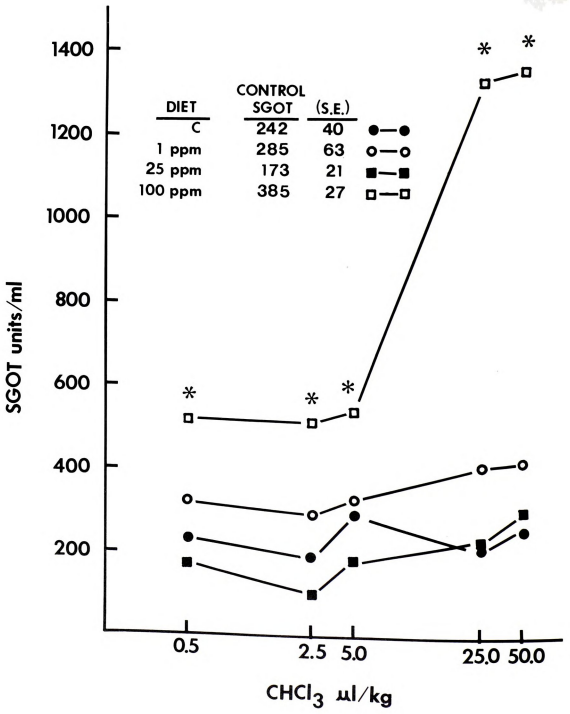
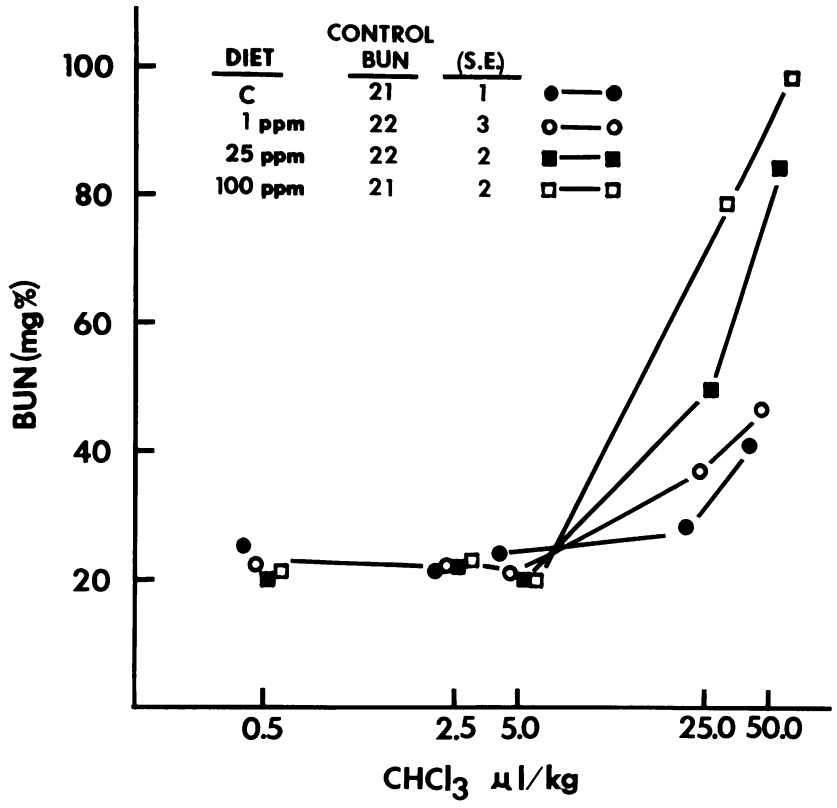

1400
1200
1000
800
600
400
200
SGOT units/ml
DIET
CONTROL SGOT
(S.E.)
C 242 40
1 ppm 285 63
25 ppm 173 21
100 ppm 385 27
*
*
*
*
*
0.5
2.5
5.0
25.0
50.0
CHCl3 µl/kg

Figure 16

Figure 17. Effects of dietary polybrominated biphenyls (PBB) and i.p. $CHCl_3$ on BUN concentration and PAH S/M. Animals were maintained on diets containing 0 (control), 1, 25 or 100 ppm of PBB for 14 days prior to a single administration of $CHCl_3$. BUN and PAH S/Ms were determined 24 hr after $CHCl_3$ administration. Each value represents the mean ± 1 S.E. of 6 animals.


CONTROL
DIET BUN (S.E.)
C 21 1
1 ppm 22 3
25 ppm 22 2
100 ppm 21 2
BUN (mg%)
100
80
60
40
20
0.5
2.5
5.0
25.0
50.0
CHCl3 µl/kg

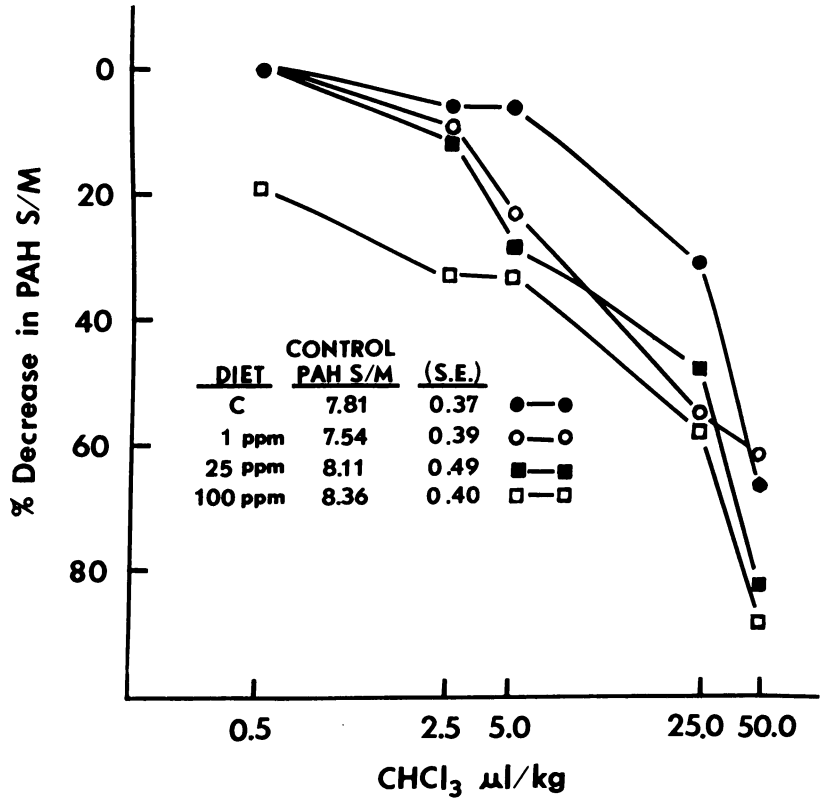
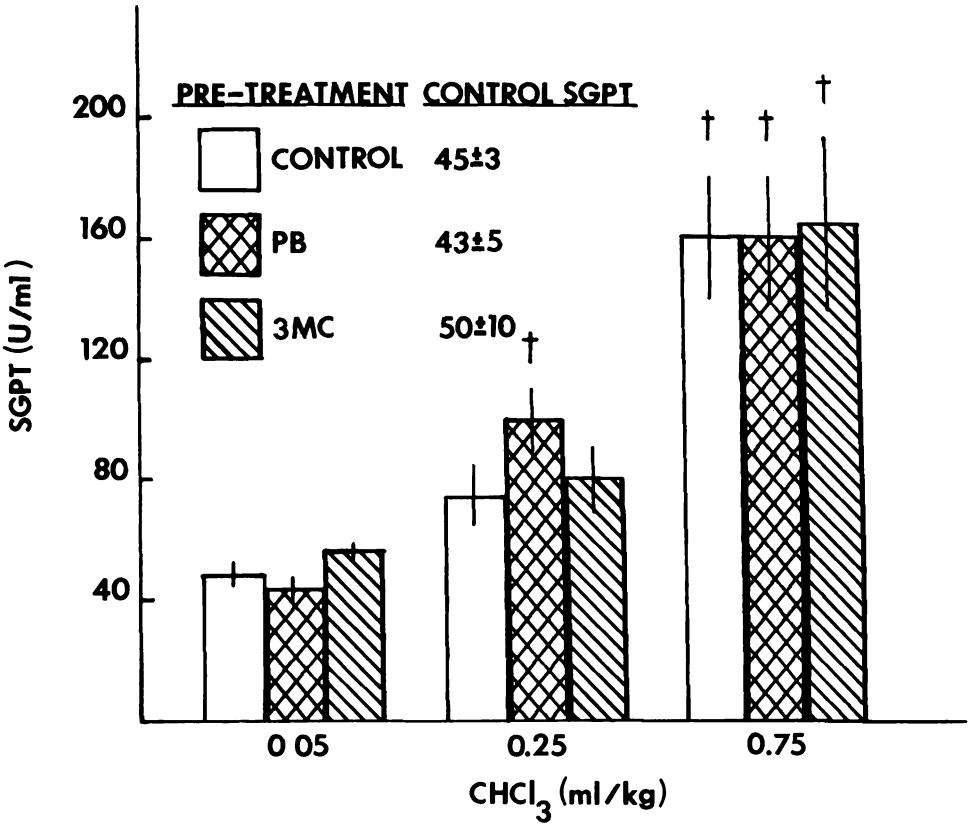

% Decrease in PAH S/M
0
20
40
60
80
CONTROL
DIET PAH S/M (S.E.)
C 7.81 0.37
1 ppm 7.54 0.39
25 ppm 8.11 0.49
100 ppm 8.36 0.40
0.5
2.5
5.0
25.0
50.0
CHCl3 µl/kg

Figure 17

Figure 18. Effects of chloroform and inducers of mixed-function oxidases (MFOs) on SGPT activity. Control mice or mice pretreated with phenobarbital (PB), 3-methylcholanthrene (3MC), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or polychlorinated biphenyls (PCB) were challenged with single i.p. injections of $CHCl_3$. Mice were sacrificed 24 hr later and SGPT determined. Each bar represents the mean ± 1 S.E. of 6 animals. *Significant difference in comparison to control mice receiving the same dose of $CHCl_3$ ($p<0.05$). †Significant increase in comparison to mice receiving the same pretreatment and 0.00 ml/kg of $CHCl_3$ (vehicle) ($p<0.05$).


PRE-TREATMENT CONTROL SGPT
CONTROL 45±3
PB 43±5
3MC 50±10
SGPT (U/ml)
200
160
120
80
40
0 05
0.25
0.75
CHCl3 (ml/kg)

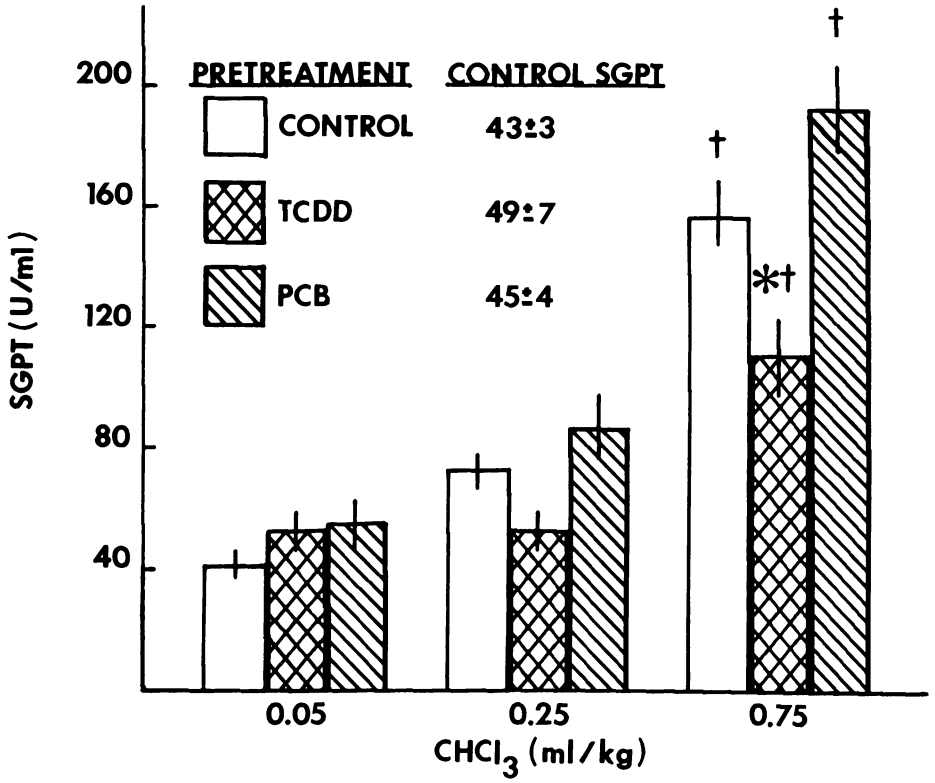

PRETREATMENT CONTROL SGPT
CONTROL 43±3
TCDD 49±7
PCB 45±4
SGPT (U/ml)
200
160
120
80
40
0.05
0.25
0.75
CHCl3 (ml/kg)

Figure 18

of the increase was significantly greater in control mice than in TCDD-treated mice (Figure 18). Treatment with 3MC, TCDD, and PCB prevented or reduced the magnitude of the $CHCl_3$-induced decrease in PAH and TEA S/Ms (Figure 19, TEA data not shown). NaPb, however, did not alter the sensitivity of mice to the nephrotoxic effects of $CHCl_3$.

7. Effects of SKF 525-A and PB on $CHCl_3$ toxicity in mice

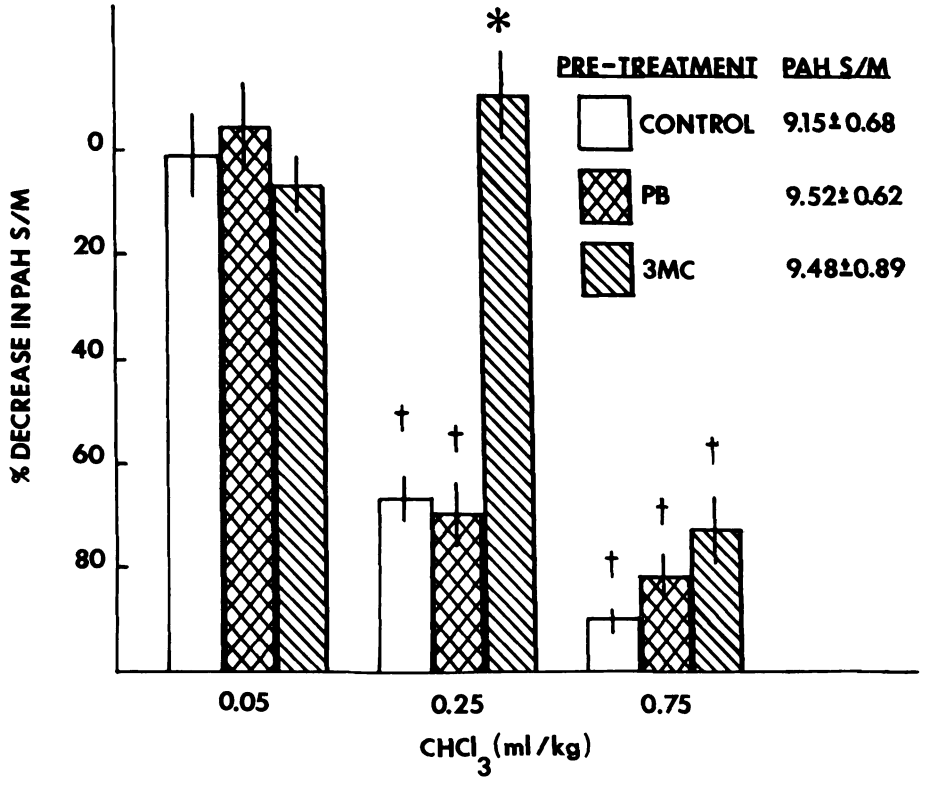
Mice treated with PB, 600 mg/kg, 2 hr before $CHCl_3$ administration exhibited no increase in SGPT activity following administration of 0.25 ml/kg $CHCl_3$, though this dose of $CHCl_3$ increased SGPT activity in control mice (Figure 20). Similarly, 0.75 ml/kg $CHCl_3$ produced less of an increase in SGPT activity in PB-pretreated mice than in control mice. Administration of PB 1 hr after $CHCl_3$ potentiated the $CHCl_3$-induced elevation of SGPT activity, as did administration of SKF 525-A, 75 mg/kg, either 2 hr before or 1 hr after $CHCl_3$. Treatments with SKF 525-A and PB had qualitatively similar effects on renal response to $CHCl_3$; pretreatment with PB partially reduced the magnitudes of $CHCl_3$-induced decreases in PAH and TEA S/Ms (TEA data not shown), but administration of PB after $CHCl_3$, or SKF 525-A either before or after $CHCl_3$, lacked this effect (Figure 20).

C. Interactions of $CHCl_3$ with GSH

1. Effects of PBB and PCB on $CHCl_3$-induced depletion of GSH

Intraperitoneal injection of $CHCl_3$ reduced the concentrations of hepatic and renal reduced, non-protein thiols (mostly reduced GSH; see Methods, Section D3) in a dose-dependent manner (Figure 21). Control mice exhibited a significant reduction of renal and hepatic

Figure 19. Effects of chloroform and inducers of mixed-function oxidases (MFOs) on PAH S/M. Control mice or mice pretreated with phenobarbital (PB), 3-methylcholanthrene (3MC), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or polychlorinated biphenyls (PCB) for varying periods of time were challenged with single i.p. injections of $CHCl_3$. Mice were sacrificed 24 hr later and PAH S/M was determined. Each bar represents mean ± 1 S.E. of 6 animals. *Significant difference in comparison to control mice receiving the same dose of $CHCl_3$ ($p<0.05$). †Significant decrease in comparison to mice receiving the same pretreatment and 0.00 ml/kg of $CHCl_3$ (vehicle) ($p<0.05$).


PRE-TREATMENT
PAH S/M
CONTROL
9.15±0.68
PB
9.52±0.62
3MC
9.48±0.89
% DECREASE IN PAH S/M
0
20
40
60
80
0.05
0.25
0.75
CHCl3 (ml/kg)

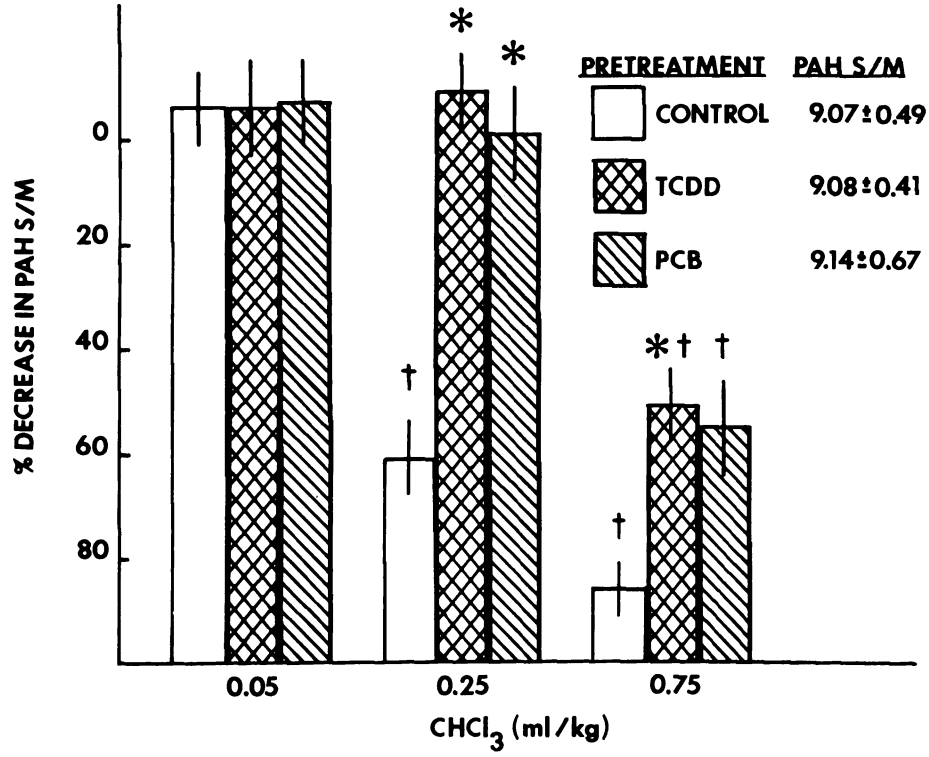
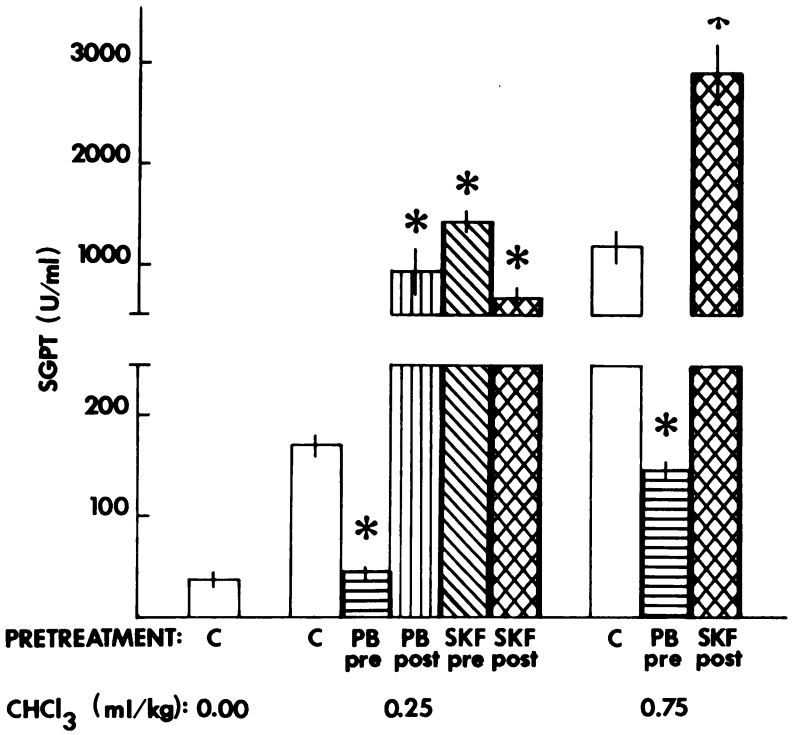

PRETREATMENT
PAH S/M
CONTROL
9.07±0.49
TCDD
9.08±0.41
PCB
9.14±0.67
% DECREASE IN PAH S/M
0
20
40
60
80
0.05
0.25
0.75
CHCl3 (ml/kg)

Figure 19

Figure 20. Effects of $CHCl_3$ and piperonyl butoxide (PB) or SKF 525-A (SKF) on SGPT and PAH S/M. Mice received a single i.p. injection of PB or SKF 525-A 120 min before (pre) or 60 min after (post) a single i.p. injection of $CHCl_3$. Bars represent the mean ± 1 S.E. of 6 animals. *Significantly different than in control mice (C) receiving the same amount of $CHCl_3$, $p<0.05$.


3000
2000
1000
200
100
SGPT (U/ml)
PRETREATMENT: C C PB pre PB post SKF pre SKF post C PB pre SKF post
CHCl3 (ml/kg): 0.00 0.25 0.75

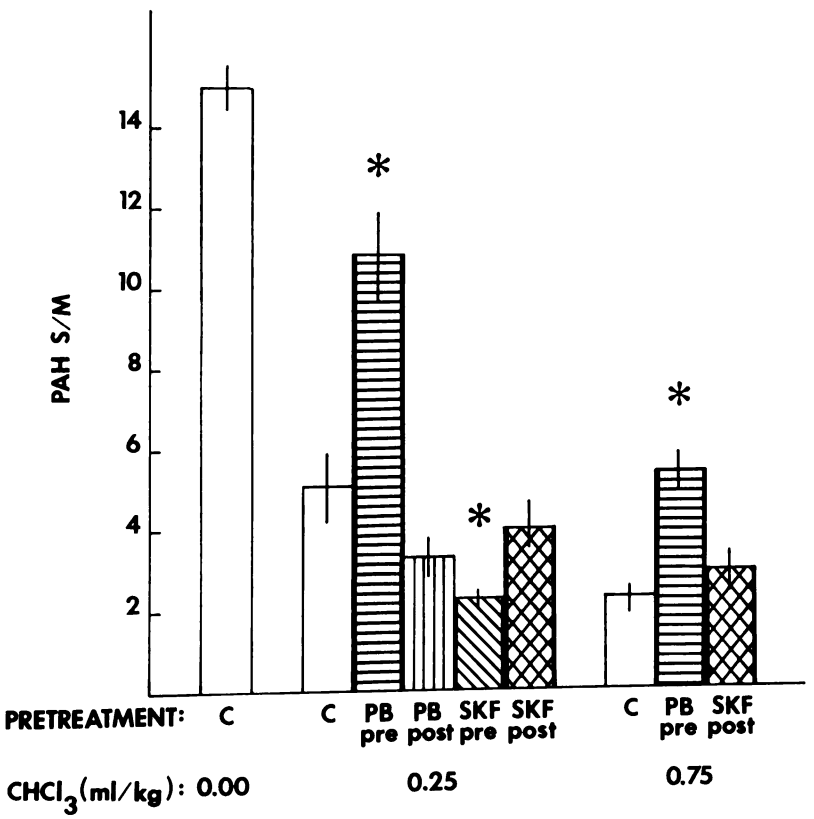

14
12
10
8
6
4
2
PAH S/M
PRETREATMENT: C C PB pre PB post SKF pre SKF post C PB pre SKF post
CHCl3(ml/kg): 0.00 0.25 0.75

Figure 20

Figure 21. Reduction of renal and hepatic reduced glutathione (GSH) content by $CHCl_3$: Effects of pretreatment with polybrominated biphenyls (PBB) and polychlorinated biphenyls (PCB). Mice were fed control diet or a similar diet formulated to contain 200 ppm of PCB or 100 ppm of PBB for 20 days prior to a single i.p. injection of $CHCl_3$. Bars represent the mean ± 1 S.E. of 6 animals. *Significantly different than in control mice receiving the same amount of $CHCl_3$, $p<0.05$. †Significantly different than in mice receiving the same diet but 0.00 ml/kg $CHCl_3$, $p<0.05$.

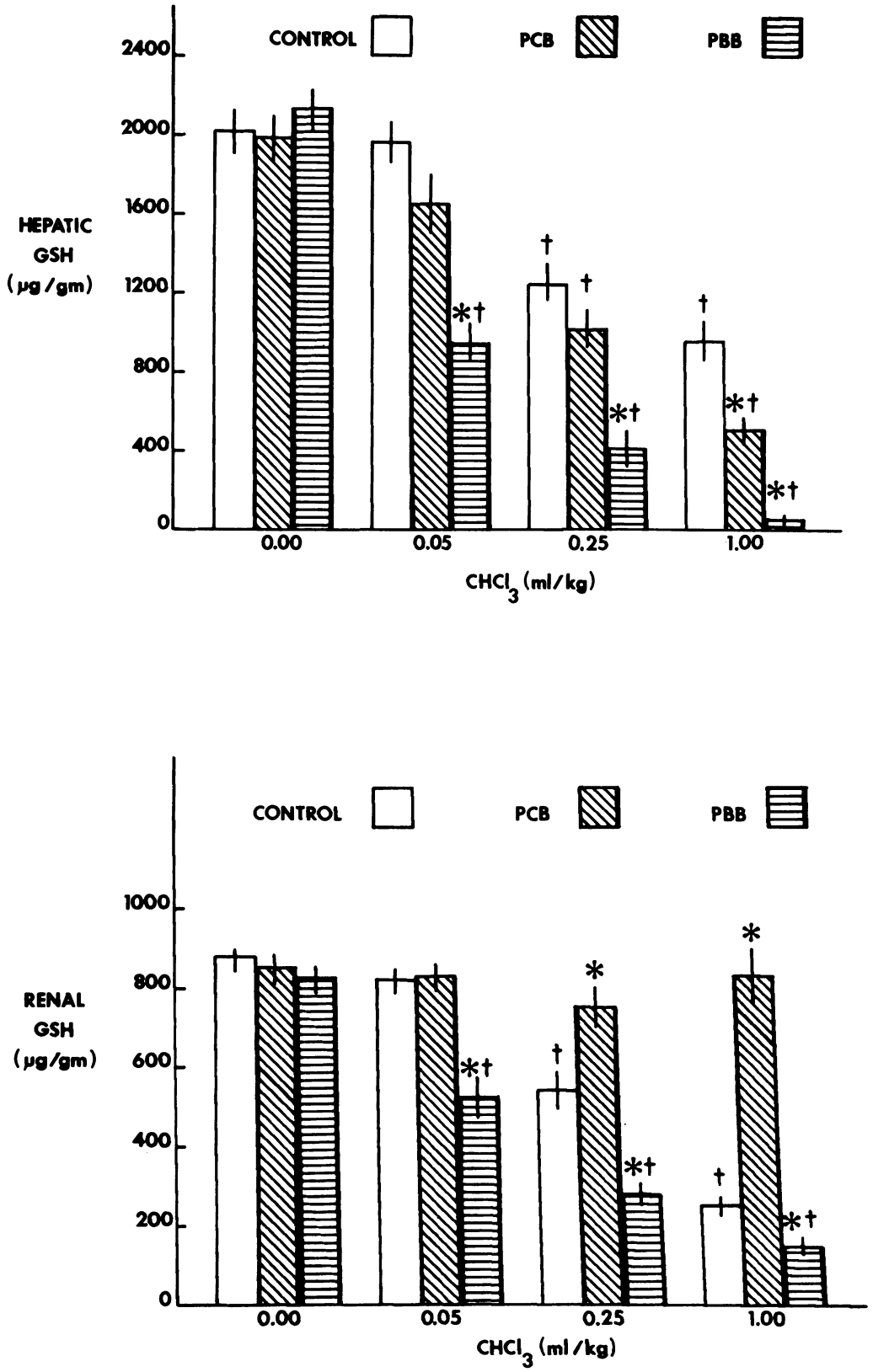

CONTROL
PCB
PBB
HEPATIC GSH (µg/gm)
2400
2000
1600
1200
800
400
0
0.00
0.05
0.25
1.00
CHCl3 (ml/kg)
RENAL GSH (µg/gm)
1000
800
600
400
200
0
0.00
0.05
0.25
1.00
CHCl3 (ml/kg)

Figure 21

reduced GSH content following administration of 0.25 ml/kg $CHCl_3$. As little as 0.05 ml/kg, however, produced decreases in renal and hepatic GSH contents in mice treated with PBB. Mice treated with PCB, in contrast to control mice, failed to exhibit decreases in renal GSH content following administration of 0.25 ml/kg and 1.00 ml/kg of $CHCl_3$ (Figure 21). Treatment with PCB did not alter the susceptibility of liver to $CHCl_3$-induced depletion of GSH except at the highest dose of $CHCl_3$ employed, 0.75 ml/kg, where depletion of hepatic GSH was slightly greater in PCB-treated mice than in control mice.

2. Effects of diethyl maleate on GSH depletion and $CHCl_3$ toxicity in mice

The concentrations of reduced non-protein thiols in liver and kidney (primarily reduced GSH) are shown in Figure 22. The concentration of GSH in renal cortex was approximately half of that in liver. Medullary GSH content was slightly less than that in cortex and papillary GSH content was approximately half that of cortex. Treatment with diethyl maleate, 600 mg/kg, decreased renal and hepatic GSH content. Maximum depletion occurred 2 hr after administration of diethyl maleate. As shown in Figure 22, the concentrations of GSH in both kidney and liver were reduced to approximately 15% of control values by diethyl maleate. Treatment of mice with diethyl maleate, 600 mg/kg, 90 min prior to $CHCl_3$ administration increased the susceptibility of mice to the toxic effects of $CHCl_3$. As illustrated in Figure 23, 0.033 and 0.100 ml/kg $CHCl_3$ increased SGPT activity in diethyl maleate-treated mice but not in control mice. 0.100 ml/kg $CHCl_3$ also decreased PAH and TEA S/Ms (TEA data not shown) in diethyl

Figure 22. Depletion of renal and hepatic reduced glutathione (GSH) by diethyl maleate. Mice were sacrificed 120 min after administration of diethyl maleate, 600 mg/kg. Bars represent the mean ± 1 S.E. of 5 animals. *Significantly lower than in control mice, $p<0.05$.

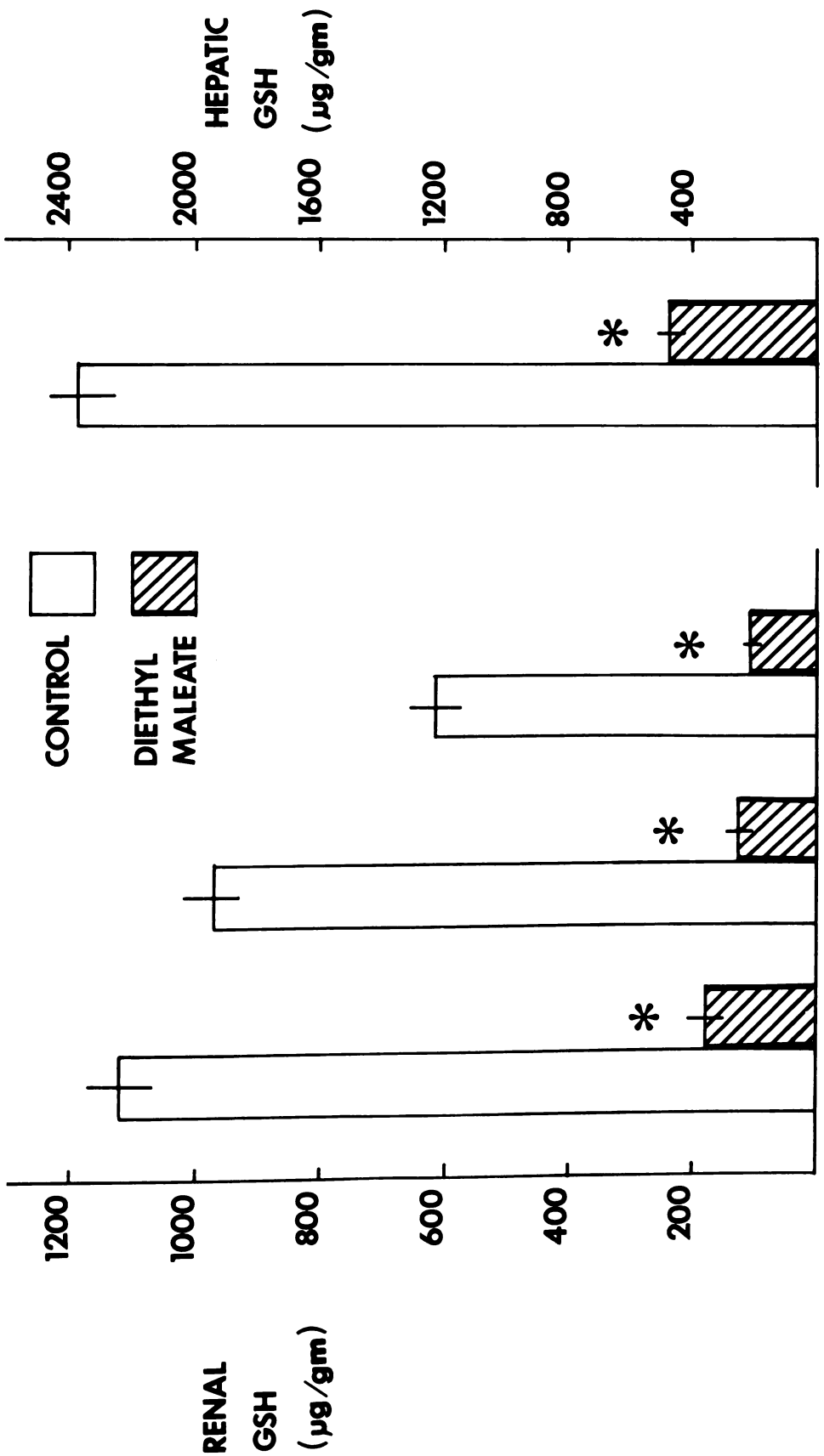
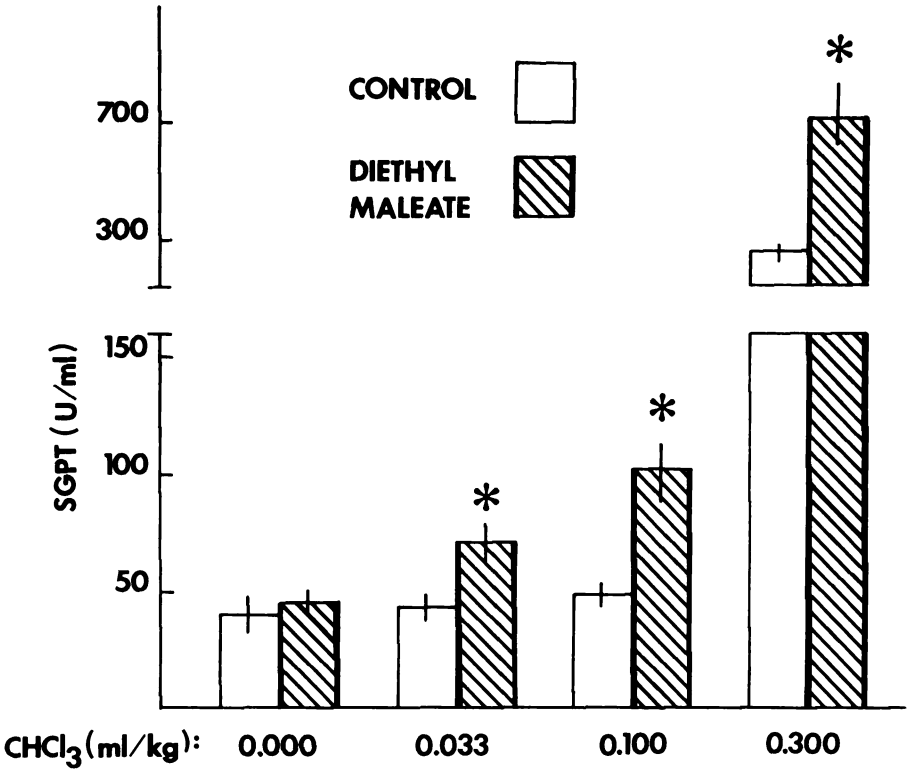

RENAL GSH (µg/gm)
1200
1000
800
600
400
200
CONTROL
DIETHYL MALEATE
CORTEX
MEDULLA
PAPILLA
*
*
*
HEPATIC GSH (µg/gm)
2400
2000
1600
1200
800
400
*

Figure 22

Figure 23. Effects of diethyl maleate and $CHCl_3$ on SGPT and PAH S/M. Mice received a single i.p. injection of diethyl maleate, 600 mg/kg, 90 min prior to $CHCl_3$ and were sacrificed 24 hr later. Bars represent the mean ± 1 S.E. of 6 animals. *Significantly different than in control mice receiving the same amount of $CHCl_3$, $p<0.05$.


CONTROL
DIETHYL MALEATE
700
300
150
100
50
SGPT (U/ml)
*
*
*
CHCl3 (ml/kg): 0.000 0.033 0.100 0.300

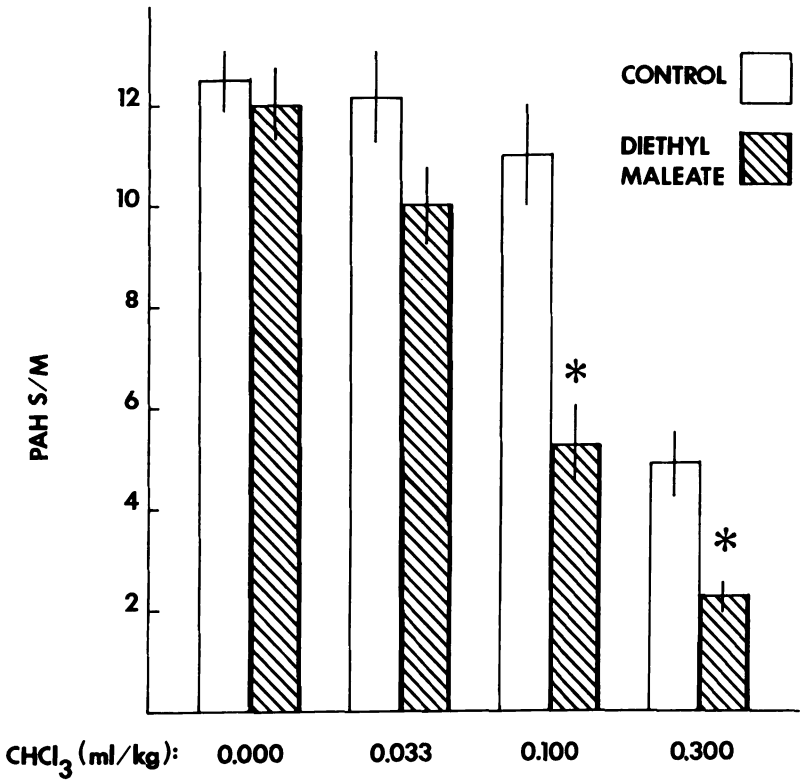

CONTROL
DIETHYL MALEATE
12
10
8
6
4
2
PAH S/M
*
*
CHCl3 (ml/kg): 0.000 0.033 0.100 0.300

Figure 23

maleate-treated mice though control mice were not affected at this dose (Figure 23). Although 0.300 ml/kg $CHCl_3$ increased SGPT activity and decreased PAH and TEA S/Ms in both control and diethyl maleate-treated mice, the magnitudes of the $CHCl_3$ effects were greater in diethyl maleate-treated mice than in control mice (Figure 23). The effects of treatments with $CHCl_3$ and diethyl maleate on SGOT activity were similar to their effects on SGPT activities (data not shown).

D. Covalent Binding of $CHCl_3$ Metabolites in Mice

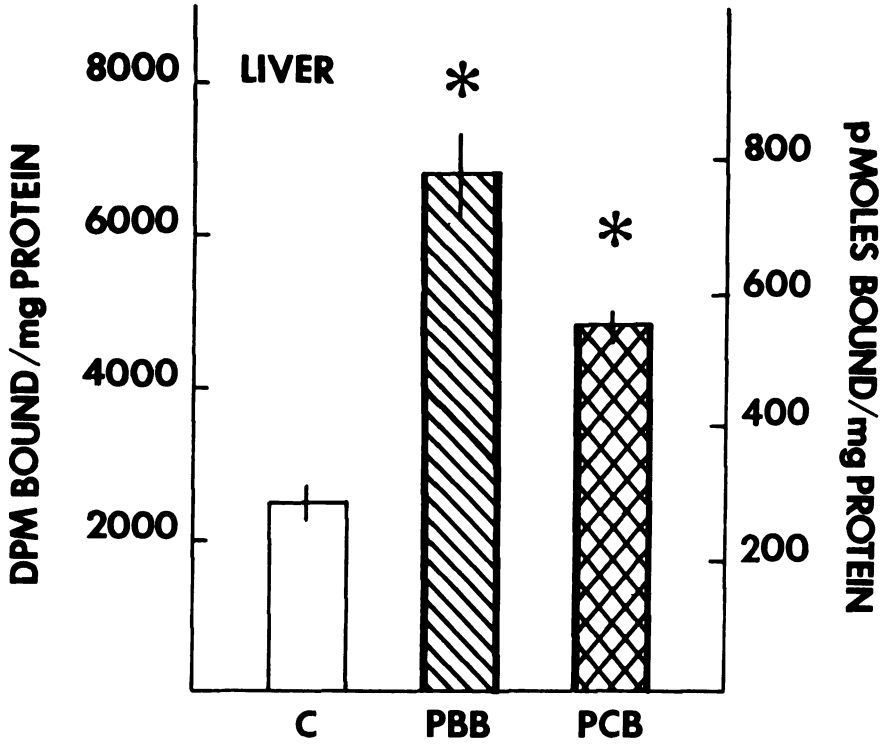
1. Covalent binding of $CHCl_3$ metabolites to renal and hepatic microsomal protein in vitro

Incubation of (^{14}C)-$CHCl_3$ with renal and hepatic microsomes in vitro resulted in the covalent binding of radioactivity to microsomal protein (Figure 24). The amount of radioactivity covalently bound per mg of protein per 5 min was nearly 15 times greater when hepatic microsomes were used than when renal microsomes were used. Hepatic microsomes from PBB and PCB-treated mice, furthermore, bound more radioactivity than hepatic microsomes from control mice. Covalent binding to renal microsomes from PCB-treated mice was quantitatively greater than covalent binding to renal microsomes from control mice (Figure 24). Covalent binding to renal microsomes from PBB-treated mice, however, was indistinguishable from that to renal microsomes from control mice.

2. Clearance of $CHCl_3$ and metabolites from blood and covalent binding to total renal and hepatic protein in vivo

Administration of (^{14}C)-$CHCl_3$ to intact mice (1.75 mmoles/kg) resulted in the covalent binding of radioactivity to renal and hepatic proteins (Figure 25). The amount of radioactivity bound, per mg of

Figure 24. Covalent binding of $CHCl_3$ metabolites to microsomal protein in vitro. (^{14}C)-$CHCl_3$ was incubated with renal or hepatic microsomal protein for 5 min and covalent binding of radioactivity to protein determined. Data are represented as the mean ± 1 S.E. of 6 independent experiments. *Significantly different than control, $p<0.05$.


LIVER
8000
6000
4000
2000
DPM BOUND/mg PROTEIN
800
600
400
200
pMOLES BOUND/mg PROTEIN
*
*
C
PBB
PCB

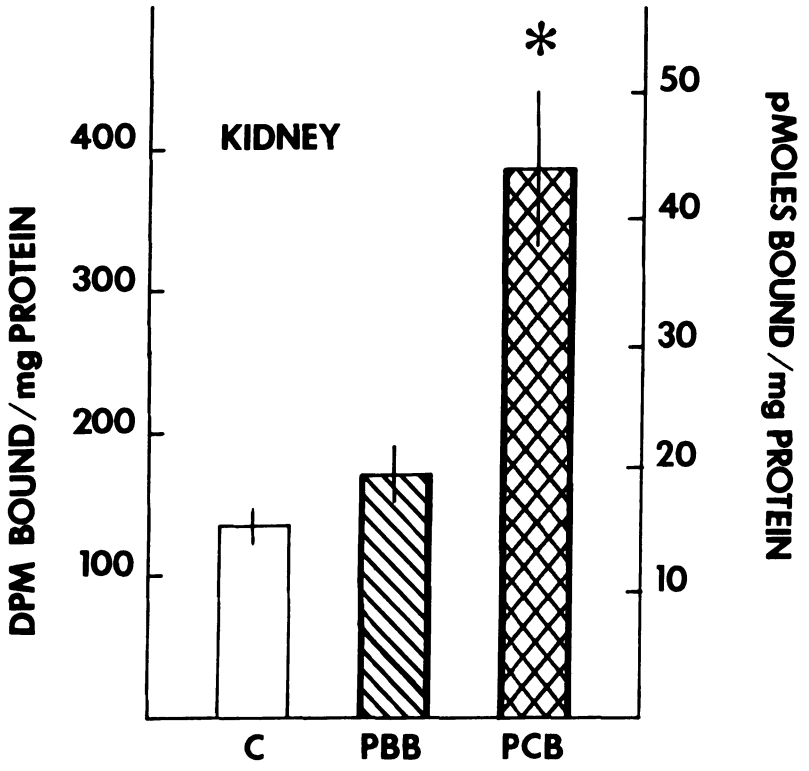

KIDNEY
400
300
200
100
DPM BOUND/mg PROTEIN
50
40
30
20
10
pMOLES BOUND/mg PROTEIN
*
C
PBB
PCB

Figure 24

Figure 25. Covalent binding of $CHCl_3$ metabolites to renal and hepatic protein in vivo and residual radioactivity in kidney and liver. Mice were sacrificed 3, 6 or 12 hr after i.p. administration of (^{14}C)-$CHCl_3$ (1.75 mmoles/kg) and residual radioactivity and protein-bound radioactivity determined in the kidneys and livers. Data are represented as the mean ± 1 S.E. of 4 animals. *Significantly different from control, $p<0.05$.

KIDNEY
C
PBB
PCB
dpm Bound/mg Protein
nmoles Bound / mg Protein
dpm / mg Tissue
nmoles/mg Tissue
TIME hours
LIVER
C
PBB
PCB
dpm Bound/mg Protein
nmoles Bound / mg Protein
dpm/mg Tissue
nmoles/mg Tissue
TIME hours

Figure 25

protein, appeared maximal in control mice 3 hr after $CHCl_3$ administration and was quantitatively greater in the kidney than in the liver. The patterns of total residual radioactivity in the liver and kidney 3, 6 and 12 hr after $CHCl_3$ administration closely resembled the patterns of covalent binding to renal and hepatic proteins (Figure 25). Mice pretreated with PBB and PCB bound more radioactivity to hepatic proteins and less radioactivity to renal proteins than did control mice (Figure 25). Residual radioactivity in the liver and kidney was affected by PBB and PCB in a similar manner.

The percentages of the total administered dose of (^{14})-$CHCl_3$ remaining in the liver and kidney and covalently bound to renal and hepatic protein 3, 6 and 12 hr after $CHCl_3$ administration are summarized in Table 16. Pretreatment with PBB and PCB led to increases (by a factor of approximately 2-4X) in the percentage of the administered dose covalently bound to hepatic protein and the percentage of the dose remaining in the liver, but decreases (by a factor of nearly 2) in the percentage of the administered dose covalently bound to renal protein and the percentage of the dose remaining in the kidney 3, 6 and 12 hr after $CHCl_3$ administration (Table 16).

The concentrations of total radioactivity ($CHCl_3$ and metabolites) in blood from control, PBB and PCB-treated mice appeared maximal 30 min after i.p. injection of (^{14}C)-$CHCl_3$, 1.75 mmoles/kg (Figure 26A). Maximum blood concentrations were lower in PBB and PCB-treated mice than in control mice and remained lower at each collection time thereafter. Plots of the log concentrations of blood radioactivity

TABLE 16

Percent of Administered Dose of $CHCl_3$ Remaining in Liver and Kidney 3, 6 and 12 hr After i.p. Administration

Time	Radioactivity	Liver			Kidney		
	Treatment:	C	PBB	PCB	C	PBB	PCB
3 hr	Total[a]	2.3	6.2	5.8	1.0	0.5	0.8
3 hr	Bound[b]	0.8	2.7	2.3	0.32	0.14	0.21
6 hr	Total	1.2	4.4	3.8	0.7	0.5	0.6
6 hr	Bound	0.7	3.0	2.4	0.25	0.15	0.16
12 hr	Total	0.9	3.3	2.7	0.4	0.2	0.3
12 hr	Bound	0.4	1.6	1.2	0.14	0.07	0.08

The total dose of $CHCl_3$ was 1.75 mmoles/kg (110 μCi/kg). Values were calculated using data from Figure 24, and mean liver weight-to-body weight ratios, mean kidney weight-to-body weight ratios, and mean protein concentrations determined for each treatment group at the time of sacrifice. The data are presented as means of 4 animals.

[a] Total radioactivity remaining in the tissue.

[b] Radioactivity covalently bound to protein.

Abbreviations: polybrominated biphenyls (PBB), polychlorinated biphenyls (PCB), control (C).

Figure 26. A) Blood concentrations of radioactivity versus time. B) Log blood concentrations of radioactivity versus time. Venous blood was withdrawn from the retro-orbital plexus 15, 30, 45, 60, 90, 120 and 180 min after an i.p. injection of (^{14}C)-$CHCl_3$ (1.75 mmoles/kg, 110 μCi/kg). The data are plotted as means ± 1 S.E. of 4 animals. The curves in B are linear and the slopes of the lines (upper right hand corner) are significantly different in PBB and PCB-treated mice from that in control mice (*), $p<0.05$.

Abbreviations: polybrominated biphenyls (PBB), polychlorinated biphenyls (PCB), control (C).

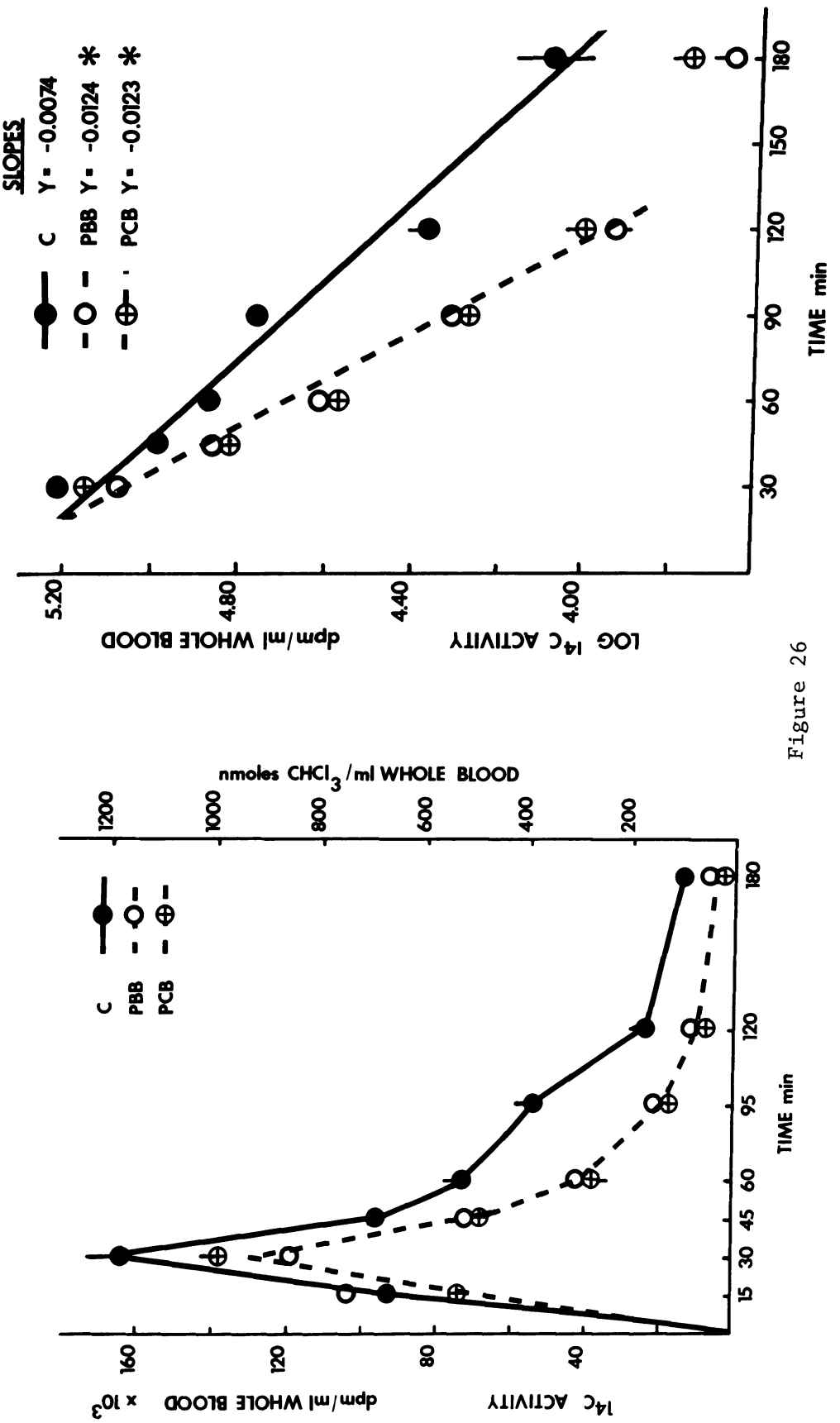

SLOPES
C Y= -0.0074
PBB Y= -0.0124 *
PCB Y= -0.0123 *
LOG 14C ACTIVITY
dpm/ml WHOLE BLOOD
TIME min
5.20
4.80
4.40
4.00
30
60
90
120
150
180
nmoles CHCl3/ml WHOLE BLOOD
1200
1000
800
600
400
200
C
PBB
PCB
14C ACTIVITY
dpm/ml WHOLE BLOOD x 10³
160
120
80
40
15
30
45
60
95
120
180
TIME min

Figure 26

versus time after administration of $CHCl_3$ produced straight lines, indicating that the disappearance of radioactivity from blood was a first-order process (Figure 26B). By analysis of co-variance it was determined that the slopes of the lines relating log blood concentrations of radioactivity to time after $CHCl_3$ administration from PBB and PCB-treated mice were significantly different from that of control mice. That is, mice pretreated with PBB and PCB removed $CHCl_3$ from the blood more rapidly than did control mice.

3. Covalent binding to subcellular fractions in vivo

Subcellular fractionation of samples of kidney and liver removed 3 hr after i.p. injection of (^{14}C)-$CHCl_3$ (1.75 mmoles/kg) to control, PBB and PCB-treated mice revealed that covalent binding of radioactivity had occurred to proteins and lipids in mitochondria (mitochondrial fraction), endoplasmic reticulum (microsomal fraction) and to cytosolic proteins (100,000 x g supernatant protein)(Table 17). The magnitude of covalent binding in control mice, per mg of protein or lipid, was nearly equal in renal and hepatic mitochondria and endoplasmic reticulum. In contrast, the magnitude of covalent binding to hepatic cytosolic protein was much greater than that to renal cytosolic protein. Mice treated with PBB and PCB bound more radioactivity (per mg of protein or lipid) than did control mice to hepatic mitochondria and cytosolic protein but not to hepatic endoplasmic reticulum. In contrast, less radioactivity was bound to renal endoplasmic reticulum, mitochondria and cytosolic protein in PBB and PCB-treated mice than in control mice (Table 17).

TABLE 17

Covalent Binding of $CHCl_3$ Metabolites to Subcellular Fractions In Vivo

Organ	Subcellular Fraction	Protein			Lipid		
Treatment:		C	PBB	PCB	C	PBB	PCB
Liver	Microsomal	730± 70	720± 40	670± 40	933± 50	1210±80	1030± 60
Kidney	Microsomal	846± 87	400± 20[a]	330± 30[a]	1530±180	817±50[a]	926±140[a]
Liver	Mitochondrial	832± 60	1300± 70[a]	1260± 72[a]	1110± 20	1360±40[a]	1450± 94[a]
Kidney	Mitochondrial	1380± 80	610± 20[a]	610± 72[a]	1190± 20	600±30[a]	570± 40[a]
Liver	Cytosolic	1690±110	2910±200[a]	3270±260[a]			
Kidney	Cytosolic	540± 60	330± 10[a]	370± 30[a]			

Mice were sacrificed 3 hr after i.p. administration of [^{14}C]-$CHCl_3$ (1.75 mmoles/kg) and radioactivity covalently bound to protein and lipid determined. Data are presented as pmoles bound per mg of protein or lipid ($\bar{X}$ ± 1 S.E., N=4 animals).

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

Abbreviations: polybrominated biphenyls (PBB), polychlorinated biphenyls (PCB), control (C).

Radioactivity was also bound to renal and hepatic RNA and DNA following i.p. injection of (^{14}C)-$CHCl_3$ (Table 18). In general, covalent binding (per mg of RNA or DNA) was greater to RNA than to DNA, and greater to renal nucleotides than to hepatic nucleotides. Treatments with PBB and PCB did not alter the amount of radioactivity bound (per mg of nucleotide) to hepatic RNA and DNA but covalent binding to renal nucleotides was reduced by treatments with PBB and PCB (Table 18).

TABLE 18

Covalent Binding of $CHCl_3$ Metabolites to RNA and DNA In Vivo

Organ	Nucleotides	Treatment: C	PBB	PCB
Liver	RNA	260±30	310±20[a]	290±30[a]
Kidney	RNA	1050±70	540±20[a]	470±30[a]
Liver	DNA	16± 1	20± 3	17± 3
Kidney	DNA	33± 2	20± 1[a]	20± 1[a]

Mice were sacrificed 3 hr after i.p. administration of [^{14}C]-$CHCl_3$ (1.75 mmoles/kg) and radioactivity covalently bound to nuclear RNA and DNA determined. Data are represented as nmoles bound per mg of RNA or DNA ($\bar{X}$ ± 1 S.E., N=4 animals).

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

Abbreviations: polybrominated biphenyls (PBB), polychlorinated biphenyls (PCB), control (C).

DISCUSSION

A. Chemical Modulation of Renal Drug Metabolism

Several chemicals are known to modulate hepatic MFO activities and, thereby, the metabolism and excretion of many endogenous and exogenous compounds. It is now apparent that MFO activities in several non-hepatic tissues can also be modified by administration of certain chemicals. Although such non-hepatic effects may not greatly alter the distribution or half-lives of xenobiotic compounds, they may alter the response of the affected tissues to the presence of chemicals enzymatically transformed to proximate toxicants. Alterations in extrahepatic biotransformation, therefore, may alter the toxicities of specific compounds.

The mechanisms by which chemicals induce microsomal MFO activities have been studied in the liver but not in the kidney (Gelboin, 1971; Venkatesan et al., 1971). However, since the molecular mechanisms of enzyme induction in these two organs are probably similar, the following discussion, though based on data obtained in the liver, is probably valid also for the kidney.

Theoretically, a change in enzyme activity may result from changes in the kinetic characteristics (Km, Vmax) of an enzyme reaction, or from an actual alteration in the amount of enzyme present. Gurtoo et al. (1968) have reported that some inducing agents appear to

alter the apparent affinity of hepatic AHH for selected substrates. Other investigators, however, believe that the common inducers of hepatic MFO activities increase enzyme activity primarily by increasing the actual amount of enzyme present (Venkatesan et al., 1971; Gelboin, 1971). Increased enzyme content could result from an increase in enzyme synthesis or from a decrease in active enzyme degradation. The former possibility appears to be the more likely possibility with most hepatic inducers because enzyme induction is blocked by concomitant and subsequent administration of cycloheximide, an inhibitor of protein synthesis, and because the half-lives of the enzymes involved do not appear to be significantly prolonged by inducer treatments (Gelboin, 1971; Schimke et al., 1968). A point of considerable controversy, however, is whether the initiating event leading to increased enzyme synthesis occurs at the level of transcription (RNA synthesis) or translation (protein synthesis). Nebert and Gelboin (1968, 1970), using cultured hamster fetal cells, reported that benz(α)anthracene-induced AHH activity was initially sensitive to inhibition by actinomycin D, an inhibitor of RNA synthesis. This suggests that benz(α)-anthracene increased AHH activity by an effect at the transcriptional level. Indeed, after 3 hr of incubation in the presence of inducer, the cells could be washed free of benz(α)anthracene and enzyme synthesis proceeded in the presence as well as the absence of actinomycin D, indicating that the protein synthetic phase, which was sensitive to inhibition by cycloheximide, was dependent on the effect of benz(α)-anthracene on RNA synthesis. Many other compounds (e.g., phenobarbital, 3-methylcholanthrene) also appear to exert their effects on enzyme

synthesis at the level of transcription; hepatic chromatin from rats treated with either of these two agents appeared to be better templates for DNA-dependent RNA synthesis *in vitro* (Madix and Bresnick, 1967; Piper and Bousquet, 1968) and hepatic microsomes isolated from phenobarbital-treated rats appeared to have greater amounts of mRNA bound than did hepatic microsomes isolated from control rats (Gelboin, 1971). In addition, the number of mRNA binding sites per mg of hepatic microsomes was increased by treatment with phenobarbital (Gelboin, 1971).

Other inducing agents may act at the level of translation. Conaway *et al*. (1977), for example, reported that induction of hepatic microsomal MFOs by p,p'-DDT was not dependent on RNA synthesis (not inhibited by actinomycin D). Rather, increased enzyme synthesis appeared to result from an effect of DDT on the initiation and elongation phases of polypeptide synthesis. That is, DDT appeared to affect the activities of cytosolic factors that modulate polypeptide synthesis within cells (Conaway *et al*., 1977).

In summary, the molecular mechanisms by which specific chemicals induce hepatic MFO activities are not precisely known but appear to involve interactions between the inducing agents and discrete portions of the protein synthetic machinery. The end result is a selective increase in enzyme synthesis within the affected cells. Although enzyme synthesis in kidney and liver are probably regulated by similar mechanisms it remains to be determined whether induction of renal MFOs occurs by the same or dissimilar mechanisms as induction of hepatic MFOs.

Differences in renal and hepatic response to exposure to inducing agents may also be a function of inducer distribution. That is, differences may exist between renal and hepatic cells in the intracellular concentrations of the inducing agents achieved. Access of the inducer to the effector site may be affected by the presence of endogenous regulators, assuming that the inducer acts on a normal effector site, which may differ in form and content between kidney and liver. In addition, the actual enzymes involved in specific MFO reactions in kidney and liver have not, for the most part, been isolated, characterized and compared. The possibility exists, therefore, that the enzymes may actually be different molecular species that respond to the presence of specific enzyme inducers in dissimilar manners.

The commercial brands of PBB and PCB used in these experiments were complex mixtures of non-polar, lipophilic compounds (mostly halobiphenyls) that would be expected to encounter little difficulty in passage from the gastrointestinal tract into the body. Matthews et al. (1977), for example, have reported that the absorption of 2,4,5, 2',4',5'-hexabromobiphenyl, the major component of PBB, was 90-100% complete following successive oral doses in rodents. Thus, the amounts of PBB and PCB absorbed by animals ingesting diets containing various concentrations of PBB or PCB would be expected to be proportional to the concentrations of PBB or PCB in the diet (25 ppm ≃ 3 mg/kg/day, 100 ppm ≃ 12 mg/kg/day, 200 ppm ≃ 24 mg/kg/day, 400 ppm ≃ 48 mg/kg/day). Data in Table 2 demonstrate that increases in renal and hepatic AHH activities in mice were directly proportional to the concentrations of PBB and PCB in the diet. This suggests that enzyme

activities were increased in proportion to the amount of PBB or PCB absorbed or, possibly, in proportion to the amount of PBB and PCB which came into contact with renal and hepatic cells.

In general, renal AHH appeared less sensitive to induction by PBB and PCB than did hepatic AHH, a phenomenon that may reflect differences in the rates of delivery of PBB and PCB to kidney and liver. Being highly lipophilic, PBB and PCB would be expected to equilibrate rapidly across cell membranes (e.g., intestinal lumen to blood, blood to parenchymal cells) and achieve initial intracellular concentrations in proportion to the concentrations of PBB and PCB in blood perfusing the cells and the rate of organ perfusion. If hepatic extraction of PBB and PCB from hepatic, venous, portal blood significantly reduced the arterial blood concentrations of PBB and PCB (i.e., a "first-pass" effect as has been described for many drugs), then the rate of delivery of PBB and PCB to renal cells would be less than that to hepatic cells and the magnitude of enzyme induction would be correspondingly lower in kidney than in liver. In support of this theory, McCormack et al. (1979) have reported that the accumulation of PBB in rodent liver was greater than that in rodent kidney following chronic oral ingestion of dietary PBB. This effect, however, appears to be dose-related; the ratio of hepatic to renal PBB content declined as the daily intake of PBB increased (Robl et al., 1978). Intracellular concentrations after prolonged exposure to PBB and PCB may be proportional to the lipid content of the cell. Thus, the increase in hepatic lipid content produced by PBB and PCB (Table 11) may promote hepatic accumulation of these compounds.

Renal and hepatic AHH activities from mice ingesting PBB or PCB for 21 days were not significantly greater than AHH activities from mice ingesting PBB or PCB for 14 days (Table 3). This suggests that the magnitude of enhancement of AHH activities had plateaued within 14 days of dietary exposure to PBB and PCB. Although the actual tissue concentrations of PBB and PCB were not determined in this study, Fries et al. (1978) have reported that concentrations of hexa- and heptabromobiphenyl in bovine milk fat reached equilibrium levels within 15-20 days of daily treatment with 10 mg/animal of PBB. These data indicate that intracellular accumulation of PBB may plateau at a given daily dose of PBB and that enhancement of AHH activities may reflect the equilibrium concentrations of the inducing agents within the cell.

If the effects of PBB and PCB on renal AHH are indicative of the propensities of PBB and PCB to stimulate the activities of renal enzymes involved in toxification processes, then the effects of dietary PBB and PCB on the susceptibility of the kidney to the toxic effects of chemicals enzymatically activated within the kidney to proximate nephrotoxicants should be proportional to the concentration of PBB and PCB in the diet. The data in Figures 16 and 17, for example, suggest that the nephrotoxicity (and the hepatotoxicity) of $CHCl_3$ was directly proportional to the concentration of PBB in the diet. In contrast, there was an inverse correlation between induction of renal AHH activity and susceptibility to $CHCl_3$ nephrotoxicity by PCB (Figure 19). Thus, renal AHH activitiy alone is not a good indicator of susceptibility to $CHCl_3$ nephrotoxicity.

The data in Table 4 and Figures 2-4 clearly demonstrate that differences exist in the response of rodent kidney and liver to the inductive effects of PBB, PCB, NaPb and 3MC. These differences suggest that quantitative regulation of xenobiotic chemical metabolism is a local phenomenon, i.e., a process occurring directly within the individual tissues. The existence of multiple forms of P-450, with different affinities for various inducing substrates, may be the biochemical basis of local regulation (Ullrich and Kremers, 1977).

Of the 5 chemicals used as inducing agents (PBB, PCB, NaPb, 3MC, TCDD) only NaPb failed to induce renal P-450 and renal MFO activities. The insensitivity of rodent kidney (and some other extrahepatic tissues, as well) to the inductive effects of NaPb has been reported previously (Uehleke and Greim, 1968; Litterst et al., 1977) and may suggest that NaPb does not gain access to cells of the rodent kidney or that NaPb does not interact with the processes of enzyme synthesis in renal cells in the same manner as it does in hepatic cells. NaPb is freely filtered at the glomerulus and is avidly reabsorbed across renal tubular cells, though urinary excretion of unchanged NaPb is a major route of excretion in humans (Waddel and Butler, 1957; Whyte and Dekaban, 1977). In addition, Kuo and Hook (C.H. Kuo and J.B. Hook, personal communication) have determined that NaPb is actively accumulated by rat renal cortical slices. Thus, it would appear that NaPb gains access to the interior of renal as well as hepatic cells. The insensitivity of rodent kidney to NaPb, therefore, may indicate that the specific form(s) of P-450 or types of enzymes induced by NaPb in the liver are not inducible by NaPb, or are not present, in

the rodent kidney. NaPb does, however, induce MFO activities in rabbit kidney (Uehleke and Greim, 1968; Kluwe et al., 1978). Comparative studies of the interactions of NaPb and enzyme systems in lapine and rodent kidney may help to explain why rodent kidney is resistant to NaPb.

Single and multiple exposures to PBB, PCB and 3MC increased both renal and hepatic AHH activities (Figure 3). Renal AHH activity, however, was stimulated much more rapidly than was hepatic AHH activity following a single oral administration. The reason for the rapid change in renal AHH activity is not known. Since AHH activities, and presumably the concentration of the enzyme involved, however, are much lower in the kidney than in the liver one possible explanation for this difference in the time-sequence of AHH induction might be that a higher inducer-to-receptor ratio was initially achieved in the kidney than in the liver (assuming that the inducing agent must interact with a receptor to cause enzyme induction). Another might be that the rate of AHH turnover in the kidney is more rapid than that in the liver; renal AHH activities returned to non-induced levels more rapidly than did hepatic AHH activities (Figure 3). The unknown kinetics of distribution of the inducers within the body, organs and individual cells greatly complicate interpretation of these data. Whether rapid induction is a characteristic of renal MFOs in general or simply a characteristic of AHH in the rodent kidney remains to be determined.

PBB, PCB and 3MC-induced changes in the sensitivity of renal AHH to inhibition by ANF were also rapid (Table 7). Goujon et al. (1972) and Wiebel et al. (1971) have reported that the NaPb and 3MC-induced

alterations in sensitivity of hepatic AHH to inhibition by ANF are due to changes in the relative proportions of ANF-sensitive and ANF-resistant AHH and that such changes result from synthesis of new enzyme (AHH) and not from 3MC or NaPb-induced changes in cofactor requirements or the microenvironment of P-450. Thus, it would appear that the early increase in renal AHH activity and alterations in sensitivity to inhibition by ANF result from synthesis of new AHH. The lack of a stimulatory effect of NaPb on renal AHH activity, therefore, suggests that NaPb failed to increase the synthesis of renal AHH.

PBB and PCB have been referred to as "mixed inducers" because the spectrum of hepatic enzymes and the characteristics of P-450 induced by PBB and PCB bear resemblance both to those induced by NaPb and to those induced by 3MC. The effects of such mixed inducers on extra-hepatic metabolism, however, have not been clearly defined. Goujon *et al.* (1972) and Wiebel and Gelboin (1975) have reported that the form of AHH induced by 3MC in several strains of mice is more sensitive to inhibition by ANF *in vitro* (and less sensitive to inhibition by MET) than is AHH from naive animals. Hepatic AHH induced by NaPb exhibits the opposite characteristics; increased sensitivity to inhibition by MET *in vitro* and decreased sensitivity to inhibition by ANF. The data in Figure 4 confirm that NaPb and 3MC have the same effects on hepatic AHH from Fischer 344 rats as those reported on hepatic AHH from mice (Goujon *et al.*, 1972; Wiebel and Gelboin, 1975). PBB and PCB-induced hepatic AHH, however, exhibited no net alterations in sensitivities to inhibition by ANF or MET. Thus, the hepatic AHH induced by PBB and PCB appeared to be an equal mixture of both NaPb-inducible and

3MC-inducible forms of AHH. Dent et al. (1978) reported similar effects of PBB on hepatic MFOs in female Sprague-Dawley rats. Since the commercial brands of PBB and PCB used in these experiments were mixtures of several different congeners of polyhalogenated biphenyls it is possible that specific congeners induced only NaPb or 3MC-like effects in the liver.

Renal AHH induced by PBB and PCB, in contrast to that induced in the liver, exhibited marked increases in susceptibility to the inhibitory effects of ANF. Increased susceptibility to ANF inhibition was exhibited also by renal AHH induced by 3MC, but not by renal AHH from NaPb-treated animals (Figure 4). PBB and PCB, therefore, do not appear to be mixed inducers in the kidney, a phenomenon which may indicate the nonresponsiveness (or absence) in the rodent kidney of drug-metabolizing enzyme components affected in the liver by NaPb. These results, however, should not be interpreted as indicating that the net inductive effects of PBB, PCB and 3MC on the rodent kidney are the same. Although the effects of these agents on renal AHH were indistinguishable the data in Figures 17 and 19 demonstrate that PBB and PCB affected the nephrotoxicity of $CHCl_3$ in dissimilar manners and suggest that subtle differences may exist in modification of renal enzymes by PBB and PCB.

Quantitative differences in the response of renal and hepatic microsomal AHH activities to inhibition by SKF 525-A, MET and ANF in vitro were apparent but differences to inhibition by PB and AIA were not (Figure 5). The detergent used for dissolution of AIA and PB, Tween 80, present at a concentration of only 0.01%, probably inhibited

both renal and hepatic AHH activities by disrupting the integrity of the microsomal membrane. The relatively greater resistance of renal than hepatic AHH to inhibition by Tween 80 may be related to the higher concentration of microsomes in the renal incubation mixture than in the hepatic incubation mixture. That is, the ratio of detergent molecules to membrane molecules was greater in the hepatic incubation mixtures than it was in the renal incubation mixtures.

AIA was employed as a negative control. DeMatteis (1971) reported that AIA appeared to inhibit microsomal enzyme activity by destroying P-450 and that a finite period of time accrued between exposure of the animal to AIA and a decrease in hepatic P-450 concentration. Consistent with this theory, AIA alone did not inhibit AHH activities in vitro (Figure 5).

PB, SKF 525-A and MET are believed to inhibit microsomal AHH activities in a biphasic manner, initially by competitive inhibition and later by non-competitive inhibition (Franklin, 1977). These compounds appear to be substrates for P-450-dependent MFO oxidations (source of competitive inhibition) and the product or an intermediate of oxidative metabolism appears to bind tightly to the cytochrome and prevent subsequent involvement of the cytochrome in oxidative metabolism (source of non-competitive inhibition). Inhibition in vitro probably occurred initially in a competitive manner and later in both competitive and non-competitive manners (Figure 5). Although renal and hepatic microsomal AHH activities appeared to be equally sensitive to inhibition by PB in vitro, renal AHH appeared to be more resistant than hepatic AHH to inhibition by SKF 525-A and MET in vitro (Figure 5).

One explanation for the organ-related differences in sensitivity to inhibition by SKF 525-A and MET *in vitro* might be that the ratio of cytochrome to non-cytochrome protein, which was lower in renal homogenates than in hepatic homogenates, resulted in greater non-specific binding of SKF 525-A and MET to noncatalytic proteins and, therefore, less inhibition of renal AHH. The high concentration of inhibitor ($1x10^{-3}$M) in the incubation mixture, however, makes this unlikely. An alternate explanation might be that the population of the various forms of P-450 (Ryan *et al.*, 1975; Guengerich, 1977; Ullrich and Kremers, 1977) present in the kidney had a lower composite affinity for SKF 525-A and MET than did the population of the various forms of P-450 in the liver. Hepatic cytochromes induced by NaPb, for example, appear to have greater affinities for SKF 525-A and MET than do the native forms of P-450 (Sladek and Mannering, 1969; Goujon *et al.*, 1972; Grasdalen *et al.*, 1975; Franklin, 1977). This suggests that the sensitivity of AHH to inhibition by SKF 525-A and MET *in vitro* may be proportional to the relative percentages of total P-450 that are cytochromes with high affinity for these inhibitors. That is, renal AHH activities may be more resistant than hepatic AHH activities to SKF 525-A and MET because cytochromes with high affinities for these inhibitors are present in only small proportions, if at all, in the kidney.

Low concentrations of ANF stimulated hepatic AHH activity *in vitro* (Figure 5), an effect reported previously by Goujon *et al.* (1972) and Wiebel and Gelboin (1975). Renal AHH activity, in contrast, was inhibited by ANF *in vitro* in a concentration-dependent manner (Figure

5). Wiebel et al. (1971) reported that ANF was a potent inhibitor of hepatic microsomal AHH from 3MC-treated rats but not of hepatic microsomal AHH from NaPb-treated rats and hypothesized that two forms of AHH existed in the liver; the activity of the form sensitive to induction by 3MC was inhibited by ANF in vitro, and the activity of the form sensitive to induction by NaPb was increased by low concentrations of ANF in vitro (Wiebel et al., 1971; Wiebel and Gelboin, 1975). In light of this information the data in Figure 5 may indicate that the predominant form of AHH present in rat kidney was the 3MC-inducible type. Furthermore, the data in Figure 4 indicate that "mixed inducers" such as PBB and PCB increase the activities of both forms of AHH in rat liver but increase only the activity of the 3MC-inducible form of AHH in rat kidney, perhaps because the NaPb-inducible form of the enzyme does not exist in rodent kidney.

Intraperitoneal administration of SKF 525-A and PB reduced renal and hepatic AHH activity and, to a lesser extent, hepatic PCNMA activity as measured in vitro (Figure 6). Inhibition of MFO activities by SKF 525-A and PB, as discussed previously, is biphasic. Based on the studies of Philpot and Hodgson (1971a), Buening and Franklin (1976) and Schenkman et al. (1972) it would appear that the reduction of AHH activity observed in vitro after i.p. injection of SKF 525-A and PB was due primarily to non-competitive inhibition. Spectral evidence indicates that non-competitive inhibition by SKF 525-A and PB is the result of a ligand interaction between the metabolic intermediate of the oxidation of SKF 525-A or PB and the heme moiety of P-450, perhaps at the CO/O_2 binding site (Franklin, 1971;

Philpot and Hodgson, 1971b; Beuning and Franklin, 1976). Non-competitive inhibitor P-450 complexes formed *in vivo*, furthermore, are stable to cell membrane disruption and microsomal isolation (Franklin, 1977). Thus, the activities of AHH and PCNMA, as determined *in vitro* after injection of SKF 525-A and PB into intact animals, should be indicative of relative AHH and PCNMA activities in kidney and liver at the time of sacrifice.

In general, hepatic MFO activities appeared more sensitive than renal MFO activities to reduction by i.p. injections of SKF 525-A and PB. The differences may be related to pharmacokinetics; compounds administered i.p. are largely absorbed into veins draining the peritoneal cavity that flow into the hepatic portal vein and the liver. If significant fractions of SKF 525-A and PB are extracted by the liver in a "first-pass" effect, then the concentration of inhibitor in blood perfusing the kidney would be much lower than that in blood perfusing the liver and the magnitude of effect of SKF 525-A and PB on the kidney might be less than that on the liver. Alternatively, active accumulation of SKF 525-A and PB by hepatocytes, but not by renal cells, could result in higher intracellular concentrations of SKF 525-A and PB in liver than in kidney and, therefore, a greater inhibitory effect. In addition, the possible presence in kidney of endogenous or dietary substances that compete with SKF 525-A and PB for interaction with P-450, and the possibility of non-specific (e.g., hydrophobic) binding of the inhibitors to noncatalytic proteins (the ratio of noncatalytic proteins to P-450 was greater in kidney than in liver) are examples of phenomena which may be responsible for the greater

sensitivity of liver than kidney to SKF 525-A and PB. Finally, the composite affinity of renal P-450 for SKF 525-A may be less than that of hepatic P-450 for SKF 525-A, as suggested earlier.

It is apparent that differences exist in renal and hepatic response to chemicals that stimulate and inhibit P-450-dependent microsomal MFO activities. The biochemical basis of such differences may be organ-specific differences in the subpopulations of the various forms of P-450. It may be possible to exploit the qualitative and quantitative differences in organ response to inducers and inhibitors as tools with which to study the interrelationships between renal and hepatic metabolism of nephrotoxicants and to study how these events may modulate chemical injury to the kidney. The in vitro methodology currently used to study renal biotransformation processes frequently focuses upon singular aspects of the interactions of chemicals with the kidney and tends to underestimate the importance of the rate and chemical form in which the potential toxicant is delivered to the kidney. The perfused, isolated kidney preparation is useful for the study of renal biotransformation but suffers from a lack of integration with other organ systems. Evaluation of renal biotransformation in the intact animal, on the other hand, is complicated by the inaccessibility of the kidney in non-anesthetized animals and by the apparent dominance of the liver in the formation of stable metabolites appearing in easily-collected excreta (urine, feces, bile, expired air). With judicious use of organ-specific modulators of microsomal MFO activities, however, it may be possible to discern the roles of hepatic and

renal biotransformation processes in renal chemical injury in intact animals.

In summary, there is currently much interest in organ-specific P-450-dependent MFO systems and their relationships to metabolic activation of toxicants. So-called "toxification" enzymes, primarily P-450-dependent MFOs, appear to be present in the kidney in only small amounts though so-called "detoxification" enzymes, catalyzing primarily synthetic reactions, appear to be more equally distributed in kidney and liver (Litterst et al., 1975, 1977; Fry et al., 1978). The low renal MFO activities measured in vitro, however, may be artifacts of current methodology; the distribution of cell types in the liver is relatively homogenous but the anatomical separations of cell types in the kidney are distinct (though complex). In general, the distribution of MFO activities (renal MFO activities determined in vitro with known hepatic MFO substrates) in the kidney appears to be similar to that of proximal tubular cells (Fowler et al., 1977; Zenser et al., 1978; K. Hilliker, W.M. Kluwe and J.B. Hook, unpublished observations). Proximal tubular cells, accordingly, are the renal cells most sensitive to the toxicities of chemicals believed to be enzymatically activated to toxicants (Schreiner and Maher, 1965). In addition, there is evidence to indicate that some inducers of MFO activities may selectively stimulate MFO activities in one particular renal cell type, the S_3 cell, that is located primarily in the straight portion of the proximal tubules (Fowler et al., 1977) and that the specific activities of MFOs in S_3 cells may approach those

in hepatocytes. Thus, measurements of MFO activities in whole kidney preparations may underestimate the biotransformation capacities of specific renal cell types. The methodology to mechanically separate S_3 cells from other renal cell types, unfortunately, is not currently available.

The effects of $CHCl_3$ and similar compounds on renal morphology and function closely resemble the effects of temporary renal anoxia on these same parameters. Berndt (1976) and Venkatachalam et al. (1978) have demonstrated that S_3 cells are more sensitive than other renal cell types to anoxic ischemia. These results suggest that the syndrome of nephrotoxic renal failure may originate from selective damage to S_3 cells. Activation of chemicals to toxicants within the S_3 cell, therefore, may be an important factor in chemical nephrotoxicity.

The effects of known inducers and inhibitors of hepatic MFO activities on renal MFO activities and the effects of PBB and PCB on the soret maxima of renal and hepatic P-450 suggest that the types of enzyme activities and cytochromes affected by these agents in the kidney may not be the same as those affected in the liver. Furthermore, the relative concentrations of the various cytochromes in the kidney may be different than in the liver and the metabolism of xenobiotic compounds in the kidney may therefore differ, qualitatively as well as quantitatively, from that in the liver. These differences, however, may be useful in evaluating renal metabolism of xenobiotic chemicals and the relationship of this function to chemical nephropathy.

B. Renal Drug Metabolism and CCl_4 Nephrotoxicity

Several investigators have demonstrated that the hepatotoxicity of CCl_4 can be greatly modified by induction and inhibition of hepatic microsomal drug metabolism. Their results suggest that CCl_4 is metabolically activated to a proximate hepatotoxicant (Cignola and Castro, 1971; Pitchumoni et al., 1972; Suarez et al., 1972; Reynolds and Moslen, 1974; Carlson, 1975; Suriyachan and Thithipandha, 1977). Recknagel and co-workers have proposed that CCl_4 is homolytically cleaved to free radical products, $CCl_3\cdot$ and $Cl\cdot$, that can abstract $H\cdot$ from methylene carbons on unsaturated membrane lipids to form $CHCl_3$, HCL and lipid free radicals. The resulting lipid free radicals can attack molecular oxygen and form lipid peroxide free radicals which abstract $H\cdot$ from adjacent methylene carbons on unsaturated membrane lipids, thus perpetuating the process (Recknagel, 1967; Recknagel and Glende, 1973). The result is trichloromethyl free radical-initiated autocatalytic, peroxidative destruction of membranes and, presumably, hepatocellular necrosis. Many compounds that alter CCl_4 hepatotoxicity affect CCl_4-induced lipid peroxidation in a similar manner. The ability of liver to homolytically cleave CCl_4 to free radicals is indicated indirectly by the presence of $CHCl_3$ (the product of condensation of $H\cdot$ and $CCl_3\cdot$) and C_2Cl_6 (hexachloroethane, the product of condensation of $CCl_3\cdot$ and $CCl_3\cdot$) in tissues of mammals exposed to CCl_4 (Butler, 1961; Fowler, 1969). In addition, Poyer et al. (1978) have recently isolated $CCl_3\cdot$ (by spin trapping with phenyl-t-butyl nitrone) from livers of rats treated with CCl_4. Generation of $CCl_3\cdot$ by rat liver homogenates was found to be an enzyme-catalyzed process that was

dependent on the presence of NADPH, suggesting that homolytic cleavage of CCl_4 may be a P-450-dependent process (Poyer et al., 1978).

Although the correlation between homolytic cleavage of CCl_4 and hepatic lipid peroxidation appears strong, considerable doubt that lipid peroxidation is the ultimate cause of CCl_4 toxicity exists. Cignalo and Castro (1971), for example, were able to block CCl_4-induced hepatic necrosis by prior treatment of rats with chemicals that failed to block CCl_4-induced lipid peroxidation, and Diaz Gomez et al. (1975) and Villarruel et al. (1977) reported a poor correlation between the susceptibilities of several species of mammals and birds to CCl_4-induced hepatic necrosis and CCl_4-initiated lipid peroxidation. Furthermore, CCl_4 does not appear to cause lipid peroxidation in the rodent kidney, though this organ is a primary target of CCl_4 toxicity (Villarruel et al., 1977).

Reynolds (1967) reported that administration of (^{14}C)-CCl_4 and (^{36}Cl)-CCl_4 to rats resulted in covalent binding of radioactivity to hepatic proteins and lipids and, furthermore, that the relative distributions of binding within liver cells was similar for ^{14}C and ^{36}Cl radioactivity. These findings suggest that homolytic cleavage of CCl_4 to free radical metabolites may result in covalent binding to lipids and proteins. A good correlation appears to exist between the effects of MFO inducers and inhibitors and alcohols on covalent binding of CCl_4 metabolites and the hepatotoxicity of CCl_4 (Diaz Gomez et al., 1973; Sipes et al., 1973; Maling et al., 1975). Sipes et al., (1977) have reported that covalent binding of CCl_4 metabolites to microsomal protein in vitro was dependent on P-450 and was inhibited by CO,

but was enhanced by the absence of O_2 (100% N_2 atmosphere), suggesting that the reactive CCl_4 metabolite was generated by reductive, microsomal metabolism (presence of O_2 would tend to increase competition for reducing equivalents via oxidative metabolism and thereby reduce NADPH-dependent reductive metabolism *in vitro*). Castro and Diaz Gomez (1972) have also reported that metabolites of CCl_4 were covalently bound to renal proteins and lipids following i.p. injection, though to a lesser extent than to hepatic components. Since free radicals are not readily transported from tissue to tissue (Jollow and Smith, 1977), the data of Castro and Diaz Gomez (1972) may indicate that CCl_4 is metabolized to reactive intermediates directly within the kidney.

Treatment of rats with PBB, PCB and HCB, potent inducers of renal and hepatic MFOs, greatly increased CCl_4 toxicity (Tables 12 and 13, Figures 8-13). Indeed, all aspects of CCl_4 toxicity in male rats were potentiated by organohalide treatment. Thus, it does not appear that the mechanism of CCl_4 toxicity was altered by treatment with the aromatic organohalides. Rather, the aromatic organohalides appeared to magnify the biochemical mechanism of CCl_4 toxicity. That is, PBB, PCB and HCB treatments may have increased the rate or extent of metabolism of CCl_4 to toxic metabolites in both kidney and liver. The effects of PBB, PCB and HCB on CCl_4 metabolism, however, were not determined. Suarez *et al.* (1972) have reported that NaPb and 3MC, both inducers of hepatic MFOs, enhanced and inhibited, respectively, CCl_4 hepatotoxicity in rats. Thus, CCl_4 may be a substrate for competing pathways of hepatic biotransformation, one pathway generating a toxic metabolite and another generating a non-toxic metabolite, as

illustrated in Figure 1. In addition, Carlson (1975) has shown that PCB, a "mixed" hepatic inducer, potentiated CCl_4 hepatotoxicity, presumably by stimulating the activity of an enzyme system(s) responsible for CCl_4 toxification. The data in Figures 9-13 indicate that PBB and HCB, as well as PCB, potentiated CCl_4 hepatotoxicity. Similar potentiating effects were produced when developing rats were exposed to PBB in utero and via maternal milk (Figure 15). Thus, these mixed inducers appear to affect hepatic response to CCl_4 in a manner more similar to that of NaPb than to that of 3MC.

The data in Table 14 indicate that PBB and PCB did not stimulate the activities of hepatic GOT and GPT. Since liver size was increased relative to body size, however, hepatic GOT and GPT activities per 100 g of body weight were slightly elevated. Assuming that total blood volume per 100 g of body weight was not altered by PBB and PCB the background activities of GPT and GOT in serum (from leakage across hepatocyte membranes) might be expected to be slightly elevated by PBB and PCB. Although increases in background activities of SGPT and SGOT in PBB, PCB and HCB-treated rats were not apparent (Figures 9 and 15), ingestion of 100 ppm of PBB by mice for 14 days increased GOT activities in the serum (Figure 16). The PBB-related increase in SGOT activity in mice, therefore, may be a result of increased liver size rather than of liver damage. The slight increase in hepatic GOT and GPT activities per 100 g body weight appeared too small to contribute greatly to aromatic organohalide potentiation of CCl_4 hepatotoxicity.

Histological examination of liver sections revealed that CCl_4 produced centrilobular necrosis (necrosis extending radially from central veins) while the aromatic organohalides produced degenerative changes (vacuolation, cell swelling) that were most intense in the periphery of the hepatic lobules (Figures 10-13). Aromatic organohalide-treatment appeared to increase the extent of centrilobular necrosis at each dose of CCl_4. Thus, the hepatic lesion produced by CCl_4 in aromatic organohalide-treated rats resembled that produced by CCl_4 in naive rats but occurred at lower doses of CCl_4. These data support the hypothesis that the mechanism of aromatic organohalide potentiation of CCl_4 hepatotoxicity was increased enzymatic conversion of CCl_4 to a proximate hepatotoxicant. That is, PBB, PCB and HCB magnified, rather than altered, the mechanism of CCl_4 hepatotoxicity.

The renal lesion produced by CCl_4 is more subtle than that produced by CCl_4 in the liver. Histological examination by light microscopy of renal sections removed 48 hr after injection of CCl_4 (the time when CCl_4-induced renal dysfunction was most severe) failed to reveal signs of morphological injury. Striker et al. (1968) reported similar finding when kidneys were removed and examined 3, 6, 12, 24, 48 and 96 hr after CCl_4 administration, though transient alterations in the structure of renal mitochondria were evident by electron microscopy shortly after CCl_4 administration. In addition, CCl_4 failed to alter consistently urinary pH, protein and glucose concentrations or BUN concentration. Thus, CCl_4-induced reduction in the abilities of renal cortical slices to accumulate organic ions (PAH and TEA) suggests that CCl_4 selectively injures proximal tubular cells. Frank renal

necrosis is similarly absent in biopsies of kidneys from human intoxications with CCl_4, but Sirota (1949) has reported that CCl_4 intoxication produced loss of selected proximal tubular functions in humans.

The increase in renal cellular respiration (QO_2) in tissue from CCl_4-intoxicated rats may indicate that CCl_4 uncoupled renal mitochondrial oxidative phosphorylation. Uncoupled mitochondrial respiration was reported previously in liver preparations from CCl_4-intoxicated rats (Reynolds et al., 1962; Reynolds, 1965) but was attributed by at least one investigator to the presence in the liver of large amounts of calcium from calcified, necrotic cells (Smuckler, 1976). The preparations used to generate the data in Figure 8, however, contained intact, non-necrotic cells. The increase in renal QO_2, therefore, may have been the result of direct CCl_4-induced mitochondrial damage. It is unlikely that the increase in QO_2 was due to lipid peroxidation since Villarruel et al. (1977) have reported that CCl_4 intoxication does not initiate lipid peroxidation in rat kidney.

Whether the proposed nephrotoxic metabolite of CCl_4 was formed in the kidney or the liver is unknown. If, as has been suggested for the liver, the nephrotoxic product of CCl_4 metabolism is a highly-reactive chemical species, then it is likely generated in close proximity to the site of injury. That is, a reactive metabolite that produces damage to the kidney is likely to be generated directly within the kidney. There is little direct evidence that CCl_4 nephrotoxicity is mediated by a reactive metabolite. Castro and Diaz Gomez (1972), however, have reported covalent binding in vivo of CCl_4 metabolites to renal macromolecules in the rat, suggesting that activation

to a reactive species may occur directly in the kidney. In addition, Cawthorne et al. (1971) reported that CCl_4 intoxication reduced glucose-6-phosphatase activity in rat kidney. CCl_4-induced loss of glucose-6-phosphatase activity is thought to result from the generation of a reactive CCl_4 metabolite that destroys ER membranes (Recknagel and Glende, 1973). Since CCl_4 reduced glucose-6-phosphatase activities in livers of male and female rats but in kidneys only of male rats (Cawthorne et al., 1971) and CCl_4 intoxication produced toxic effects in livers of male and female rats but in kidneys only of male rats (Ross et al., 1978; W.M. Kluwe and J.B. Hook, unpublished observations) a correlation between CCl_4 nephrotoxicity and renal generation of reactive CCl_4 metabolites may exist.

In summary, treatment with several inducers of renal and hepatic MFOs potentiated the nephrotoxicity and hepatotoxicity of CCl_4 in male rats. The mechanism of potentiation appears to be stimulation of the metabolism of CCl_4 to a proximate toxicant. The effects of PBB, PCB and HCB on CCl_4 metabolism, however, were not directly determined.

The hypothesis that a toxic CCl_4 metabolite that produces renal injury is formed directly in the kidney rather than in the liver is consistent with the reports that sex differences exist in the susceptibility of rats to CCl_4 nephrotoxicity but not to CCl_4 hepatotoxicity. Since the renal lesion produced by CCl_4 does not resemble the hepatic lesion induced by CCl_4, however, the possibility exists that the nephrotoxic and hepatotoxic metabolites of CCl_4 are different compounds and that both may be formed primarily in the liver. Further studies will be needed to determine the roles of renal and hepatic CCl_4 metabolism in CCl_4 nephrotoxicity.

C. Renal Drug Metabolism and $CHCl_3$ Nephrotoxicity

$CHCl_3$ produces centralobular necrosis in the liver and proximal tubular necrosis in the kidney. The hepatic lesion appears to be dependent on metabolism of $CHCl_3$ to a toxic, reactive intermediate (Brown et al., 1974b; Docks and Krishna, 1976). Renal necrosis may also be dependent on the generation of a toxic, reactive intermediate; renal injury occurs specifically in that portion of the nephron (proximal tubule) where cells containing the highest activities of P-450-dependent MFOs are found (Deringer, 1953; Fowler et al., 1977; Venkatachalam et al., 1978) and $CHCl_3$ metabolites are covalently bound to proximal tubules after intoxication with $CHCl_3$ (Ilett et al., 1973).

Experimental $CHCl_3$ nephrotoxicity in male mice was characterized by an increase in kidney weight, an increase in BUN concentration and a decrease in the ability of renal cortical slices to accumulate organic ions. These three parameters appeared to be reliable and sensitive indices of $CHCl_3$ nephrotoxicity. The increase in kidney weight was probably due to tissue edema since the dry weight of the kidney was not affected by $CHCl_3$ intoxication (W.M. Kluwe and J.B. Hook, unpublished observations). The increase in BUN, which occurred in the absence of detectable hepatic damage in some cases, was likely due to a decrease in glomerular filtration. While BUN and kidney weight are relatively non-specific tests, in vitro accumulation of organic ions (S/M) appears to be a sensitive test for specific effects of toxicants on proximal tubular cells (Plaa and Larson, 1965; W.M. Kluwe and J.B. Hook, unpublished observations). The $CHCl_3$-induced decreases in PAH and TEA S/Ms, therefore, are probably indicative of proximal tubular injury.

The data in Figure 17 indicate that PBB greatly potentiated $CHCl_3$ nephrotoxicity. The degree of potentiation appeared to be directly proportional to the concentration of PBB in the diet, as was the magnitude of induction of renal and hepatic AHH activities (Table 2). These results suggest that PBB may have increased the rate or extent of renal (and hepatic) metabolism of $CHCl_3$ to a toxic product and, thereby, increased the relative nephrotoxicity of $CHCl_3$ in mice. The effects of NaPb, 3MC, TCDD and PCB on renal and hepatic enzyme induction and the nephrotoxicity and hepatotoxicity of $CHCl_3$ are shown in Table 4 and Figures 18 and 19. NaPb had no effect on renal drug metabolism but greatly increased the metabolic capacity of the liver. Accordingly, NaPb enhanced $CHCl_3$ hepatotoxicity but not $CHCl_3$ nephrotoxicity. These results suggest that the nephrotoxic metabolite was generated in the kidney and not in the liver. That is, if the nephrotoxic metabolite was generated in the liver then NaPb would likely have enhanced the nephrotoxicity as well as the hepatotoxicity of $CHCl_3$. 3MC, TCDD and PCB increased renal and hepatic MFO activities but sharply reduced the nephrotoxicity of $CHCl_3$ in mice, suggesting that renal biotransformation of $CHCl_3$ was altered by treatment with these agents in such a manner that relatively less $CHCl_3$ was converted to nephrotoxic metabolites. The hepatotoxicity of $CHCl_3$, however, was not markedly affected by 3MC, TCDD or PCB (though PCB appeared to enhance hepatotoxicity slightly and TCDD appeared to decrease hepatotoxicity slightly) suggesting that the net conversion of $CHCl_3$ to a hepatotoxicant was not greatly altered by treatment with these agents. If nephrotoxic metabolites were generated in the liver,

then modulators of MFO activities that reduced $CHCl_3$ nephrotoxicity would have been expected to have a similar effect on $CHCl_3$ hepatotoxicity. Since this was not the case it appears that the liver is not responsible for the generation of the nephrotoxic metabolite. An alternate explanation of these data, however, might be that nephrotoxic and hepatotoxic metabolites of $CHCl_3$ are generated in the liver but by different metabolic pathways. That is, the nephrotoxic and hepatotoxic metabolites of $CHCl_3$ may be different molecular species and their enzymatic formation might be affected by PCB, 3MC, TCDD and NaPb in different manners.

It is apparent from the effects of PBB, PCB, 3MC and TCDD on $CHCl_3$ nephrotoxicity that induction of MFO activities *per se* does not have a predictable effect on the metabolism of $CHCl_3$ to nephrotoxic products. Rather, the net effects of such compounds on $CHCl_3$ nephrotoxicity may be the result of quantitative changes in the balance between toxification and detoxification pathways of $CHCl_3$ metabolism. Nearly 80% of $CHCl_3$ is converted to CO_2 and exhaled as such in the mouse (Brown *et al.*, 1974a), indicating substantial degradation via this pathway. CO and unidentified non-volatile urinary metabolites have also been reported, however (Ahmed *et al.*, 1977; Brown *et al.*, 1974a), suggesting that alternate pathways of $CHCl_3$ biotransformation exist in mammals and that selective stimulation or inhibition of one or more pathways of metabolism may alter the amount or rate of $CHCl_3$ metabolized to a proximate toxicant, as indicated in Figure 1.

The existence of competing pathways of $CHCl_3$ metabolism is supported by differential effects of SKF 525-A and PB on $CHCl_3$ toxicity

(Figure 20). The failure of pretreatment with SKF 525-A to prevent or reduce $CHCl_3$ injury to the kidney and liver was not unexpected since Lavigne and Marchand (1974) had reported that SKF 525-A did not reduce the hepatotoxicity of $CHCl_3$ in rats. Inhibition of P-450-dependent MFOs by SKF 525-A is selective (Mannering, 1971), perhaps because the various forms of P-450 identified in rodent tissues exhibit different affinities for various substrates, including SKF 525-A. Since SKF 525-A does not block $CHCl_3$ toxicity, metabolism of $CHCl_3$ to a proximate toxicant may occur via a cytochrome with low affinity for SKF 525-A. Lavigne and Marchand (1974) have reported that SKF 525-A inhibited the metabolism of $CHCl_3$ to CO_2 in intact rats. Thus, there may exist one pathway of $CHCl_3$ metabolism that converts $CHCl_3$ to CO_2 and another pathway that converts $CHCl_3$ to a proximate toxicant. SKF 525-A may have enhanced the nephrotoxicity and hepatotoxicity of $CHCl_3$ by directly inhibiting a pathway forming non-toxic metabolites (perhaps CO_2), thereby increasing the availability of substrate ($CHCl_3$) for the toxification reaction and indirectly increasing the amount of $CHCl_3$ metabolized to a proximate toxicant (see Figure 1).

Preadministration of PB greatly reduced the nephrotoxicity and hepatotoxicity of $CHCl_3$, suggesting that PB may have directly inhibited an enzymatic pathway forming toxic $CHCl_3$ metabolites in both kidney and liver. The inability of PB to reduce $CHCl_3$ toxicity when administered 1 hr after $CHCl_3$ may be an indication that conversion to a toxic product occurs very rapidly after $CHCl_3$ administration. An explanation for the enhancement of $CHCl_3$ toxicity when PB was administered 1 hr after $CHCl_3$ is not immediately apparent. After receiving

the same dose of $CHCl_3$, however, mice treated with SKF 525-A before $CHCl_3$ and mice treated with SKF 525-A or PB after $CHCl_3$ remained unconscious for longer periods of time than did control mice or those pretreated with PB. These results indicate that post-administration of SKF 525-A and PB, as well as preadministration of SKF 525-A, may have greatly altered the pharmacokinetics of $CHCl_3$ in the mice.

Pohl et al. (1977) and Mansuy et al. (1977) have demonstrated that $CHCl_3$ can be metabolized by rat microsomal protein in vitro to an electrophilic species that readily combines with reduced, non-protein thiols. In addition, Brown et al. (1974b) and Docks and Krishna (1976) have reported that administration of $CHCl_3$ to intact rodents resulted in a rapid depletion of hepatic glutathione (GSH), a cytosolic tripeptide thiol believed to protect hepatocytes from electrophile injury by combining covalently, enzymatically or non-enzymatically, with potentially-toxic electrophiles (Mitchell et al., 1973; Brown et al., 1974b). Intraperitoneal administration of $CHCl_3$ produced dose-dependent decreases in the concentrations of reduced GSH in kidney as well as in liver (Figure 21). Maximum $CHCl_3$-induced depletion of GSH occurred 2 hr after $CHCl_3$ administration in control and in PBB and PCB-treated mice. If, in fact, the product of $CHCl_3$ metabolism via the toxic pathway is a strong electrophile that is detoxified by conjugation with GSH, then the amount of electrophile formed, as estimated by the degree of depletion of tissue GSH, should reflect the relative activity of the toxification reaction. Similarly, treatments which enhance or reduce organ-specific $CHCl_3$ toxicity should accordingly enhance or reduce $CHCl_3$-induced depletion of tissue GSH. In

agreement with this hypothesis treatment of mice with PBB enhanced the hepatotoxicity of $CHCl_3$ (Figure 16) and greatly increased $CHCl_3$-dependent depletion of hepatic GSH (Figure 21) while treatment with PCB did not alter the hepatotoxicity of $CHCl_3$ (Figure 18) and failed to consistently alter $CHCl_3$-dependent depletion of hepatic GSH (Figure 21). Similarly, treatment of mice with PBB enhanced $CHCl_3$ nephrotoxicity (Figure 17) and increased $CHCl_3$-dependent depletion of renal GSH (Figure 21) while treatment with PCB greatly reduced $CHCl_3$ nephrotoxicity (Figure 18) and blocked $CHCl_3$-dependent depletion of renal GSH (Figure 21).

These strong correlations between the susceptibilities of kidney and liver to $CHCl_3$ toxicity and the susceptibilities of kidney and liver to $CHCl_3$-dependent depletion of GSH support the hypothesis that a GSH-depleting metabolite of $CHCl_3$ may be a proximate toxicant and that conjugation with GSH may be a detoxification reaction. Stoichiometric evaluations, however, revealed that several moles of GSH (up to 15) were consumed for each mole of $CHCl_3$ injected into the mice. Therefore, interactions other than, or in addition to, conjugation of GSH with an electrophilic $CHCl_3$ metabolite appear to be responsible for $CHCl_3$-dependent depletion of GSH. Further support for the involvement of GSH in protection against $CHCl_3$ nephrotoxicity (and hepatotoxicity) is provided by experiments demonstrating that diethyl maleate, a depletor of renal GSH, increased the susceptibility of the kidney and liver to $CHCl_3$ injury (Figures 22 and 23). The degree of increase in susceptibility to $CHCl_3$ toxicity did not appear to be as great as might be expected in view of the dramatic recuction of

tissue GSH produced by diethyl maleate. Anders (1978) and Chuang et al. (1978), however, have reported that diethyl maleate inhibited the activities of some microsomal MFOs. The effects of diethyl maleate on $CHCl_3$ toxicity, therefore, may have been the net result of concomitant depletion of GSH and inhibition of enzymatic transformation of $CHCl_3$ to a toxic metabolite.

Several investigators have proposed that covalent binding of halogenated aliphatic hydrocarbon metabolites to critical cellular macromolecules may be the mechanism by which these agents initiate tissue injury (Reynolds, 1967; Reid, 1973; Gillette et al., 1974; Uehleke, 1977). Non-specific alkylation of macromolecules (both critical and non-critical macromolecules) by reactive metabolites could conceivably lead to cell dysfunction, cell death and tissue necrosis. Brown et al. (1974b) and Docks and Krishna (1976) have proposed that $CHCl_3$ is metabolized in the liver to a strong electrophile that is detoxified by preferential conjugation to hepatic GSH. When hepatic GSH content is depleted, however, the reactive metabolite more readily combines with nucleophilic sites on other hepatic molecules. Accordingly, the threshold of $CHCl_3$ hepatotoxicity appears to coincide with depletion of hepatic GSH (Brown et al., 1974b; Docks and Krishna, 1976). Ilett et al. (1973) reported that $CHCl_3$ metabolites are covalently bound in the renal proximal tubules as well as in the liver and that covalent binding of $CHCl_3$ metabolites in the kidney appeared to correlate with susceptibility to $CHCl_3$ nephrotoxicity; male and female mice were equally susceptible to the hepatotoxic effects of $CHCl_3$ and bound equal amounts of $CHCl_3$ metabolites to hepatic

proteins, but female mice were markedly less susceptible to $CHCl_3$ nephrotoxicity than were male mice and bound much less $CHCl_3$ metabolites to renal proteins. Thus, it appeared that $CHCl_3$ nephrotoxicity, as well as $CHCl_3$ hepatotoxicity, may have resulted from alkylation of cellular macromolecules by reactive $CHCl_3$ metabolites.

Figure 25 indicates that maximum binding in male mice of radioactivity from (^{14}C)-$CHCl_3$ occurred within 3 hr after $CHCl_3$ administration in both the kidney and the liver. The time sequence of covalent binding was similar to that reported previously (Ilett et al., 1973). As discussed earlier, highly-reactive metabolites that non-specifically alkylate cellular macromolecules probably possess little mobility; their movements are likely to be severely restricted by biological membranes. For this reason the relatively large amount of covalent binding in the kidney is consistent with activation of $CHCl_3$ to a reactive metabolite in kidney as well as in liver. A comparison of Figure 25 with Figures 16-19, however, indicates that discrepancies exist between the effects of PBB and PCB on organ-specific $CHCl_3$ toxicity and the effects of these agents on organ-specific binding of reactive $CHCl_3$ metabolites. Ingestion of PCB appeared to enhance the hepatic biotransformation of $CHCl_3$ to alkylating metabolites but did not greatly alter $CHCl_3$ hepatotoxicity. In addition, PBB markedly potentiated $CHCl_3$ nephrotoxicity despite a decrease in alkylation of renal proteins by reactive $CHCl_3$ metabolites. These results suggest that reactive $CHCl_3$ metabolites that bind to renal and hepatic proteins may not be the $CHCl_3$ metabolites responsible for acute tissue injury. That is, reactive $CHCl_3$

metabolites may be formed by a different pathway of metabolism than toxic $CHCl_3$ metabolites.

An alternate explanation for the lack of correlation between binding and toxicity might be that PBB and PCB altered the mechanism of $CHCl_3$ toxicity (Gillette, 1974) and, therefore, expression of $CHCl_3$ injury. This, however, appears unlikely since the symptomatologies of $CHCl_3$ intoxication were the same in PBB and PCB-treated mice as in control mice, and the effects of PBB and PCB on $CHCl_3$-dependent depletion of GSH were the same as their effects on organ-specific $CHCl_3$ toxicity (Figure 21). PBB and PCB probably increased hepatic binding of $CHCl_3$ metabolites by increasing the rate of hepatic $CHCl_3$ biotransformation to a reactive product and also be increasing hepatic extraction of $CHCl_3$ from the blood (since total radioactivity in the liver appeared to be increased by nearly as great a factor as that covalently bound to protein, Table 16). Covalent binding in the kidney may have been reduced by treatment with PBB and PCB, therefore, because relatively less $CHCl_3$ was being delivered to the kidney rather than because PBB and PCB reduced the conversion of $CHCl_3$ to a reactive metabolite in the kidney. Total radioactivity in the kidney, for example, was reduced by the same factor as that covalently bound to protein (Table 16). $CHCl_3$ injected into the peritoneal cavity would be largely absorbed by local venous systems and initially enter the hepatic portal vein and the liver. Thus, most of the $CHCl_3$ supplied to the kidney via the blood would be that which escaped hepatic biotransformation despite passage through the liver. Figure 26 indicates that the clearance of radioactivity ($CHCl_3$ and metabolites)

from the blood occurred rapidly and that the rate of clearance was increased by PBB and PCB-treatment by nearly a factor of 2. Assuming that most radioactivity disappeared from the blood as a result of exhalation of CO_2 (nearly 80% of $CHCl_3$ is exhaled as CO_2 in the mouse, only 10% as $CHCl_3$, Brown et al., 1974a) and that clearance from the blood is proportional to the rate of hepatic metabolism of $CHCl_3$ to CO_2, then it would appear that PBB and PCB enhanced hepatic metabolism of $CHCl_3$ and that the rate of delivery of $CHCl_3$ to the kidney may subsequently have been reduced. Studies were not specifically conducted to determine whether or not a decrease in rate of delivery of $CHCl_3$ to the kidney could affect the rate of renal metabolism of $CHCl_3$. That is, that $CHCl_3$ metabolism in the kidney was not saturated at the dose of $CHCl_3$ used in these experiments (1.75 mmole/kg). Treatment with PBB and PCB also reduced total radioactivity present in the kidney, suggesting that PBB and PCB did not reduce renal binding solely by shifting the major pathway of renal $CHCl_3$ metabolism from one that produced a reactive metabolite to one that produced non-reactive metabolites nor by inhibiting renal $CHCl_3$ metabolism in general. The covalent binding of $CHCl_3$ metabolites in these experiments, therefore, may have been a function of the rate of delivery of $CHCl_3$ to the target organs as well as the relative activity of the enzyme systems generating the reactive metabolites. That is, the enzyme system responsible for metabolism of $CHCl_3$ to a reactive, alkylating intermediate in both liver and kidney was not saturated under the conditions of the experiment and formation of product (reactive metabolite) was proportional to the rate of delivery of substrate ($CHCl_3$).

The homogeneity of binding to renal and hepatic endoplasmic reticulum (ER), mitcochondria (M), and cytosolic protein (CP) suggests that the direct alkylating species, if in fact generated at the ER membrane, possessed sufficient stability to be distributed throughout much of the cell (Table 17). Generation of the reactive, alkylating metabolite at the ER is supported by data in Table 17 showing that covalent binding to hepatic M and CP was 2-3 times greater in PBB and PCB-treated mice than in control mice, an increase equivalent to the increase in liver microsomes (largely ER membrane fragments) produced by treatment with PBB and PCB (data not shown). The magnitude of covalent binding to microsomal proteins and lipids in vivo was not increased by PBB or PCB when expressed as pmoles bound/mg protein or lipid, suggesting that PBB and PCB enhanced hepatic activation of $CHCl_3$ primarily by increasing the amount of ER present in the liver cells. PBB and PCB did not alter the amount of microsomes (ER) isolated per g of kidney (data not shown). Due to the non-homogenous anatomical distribution of renal cell types, however, it is possible that PBB and PCB increased ER concentrations in a particular renal cell type. Covalent binding of $CHCl_3$ metabolites to renal ER, as well as binding to M and CP were reduced by a factor of 2 in mice treated with PBB and PCB. This suggests that less $CHCl_3$ was metabolized to reactive, alkylating intermediates at the ER in PBB and PCB-treated mice than in control mice. Such an effect could result from a decreased delivery of $CHCl_3$ to the kidney, as discussed previously.

PBB and PCB-treatment had no effect on the binding of $CHCl_3$ metabolites to hepatic nucleotides (Table 18). Since it is unlikely that a reactive metabolite would be able to cross the nuclear membrane, and since nuclear MFO activity has been described (Kashnig and Kasper, 1969; Khandivala and Kasper, 1973), $CHCl_3$ metabolites binding to nuclear RNA and DNA may have been formed directly on or in the nuclear envelope. Failure of PBB and PCB to alter hepatic nuclear binding of $CHCl_3$ metabolites may indicate that the proposed nuclear metabolism of $CHCl_3$ was not affected by PBB and PCB. Covalent binding of reactive $CHCl_3$ metabolites to renal nucleotides, in contrast, was reduced by treatment with PBB and PCB, providing further support for the theory that PBB and PCB indirectly reduced delivery of $CHCl_3$ to the kidney.

Ilett et al. (1973) reported that $CHCl_3$ metabolites are covalently bound to hepatic and renal microsomal protein following incubation of microsomes with (^{14}C)-$CHCl_3$ in vitro. Binding to hepatic protein in vitro was dependent on P-450, O_2 and NADPH and was inhibited by CO and 100% N_2. These results suggest that the reactive, alkylating metabolite was generated by microsomal oxidation of $CHCl_3$. The specific activity of covalent binding to renal microsomal protein, however, was less than 10% of that to hepatic microsomal protein, despite nearly equal amounts of binding in vivo, and the effects of P-450, O_2, N_2, NADPH and CO on renal binding in vitro were equivocal (Ilett et al., 1973; Sipes et al., 1977). Thus, renal microsomal binding of $CHCl_3$ metabolites in vitro has not been demonstrated to be a consequence of renal P-450-dependent microsomal oxidation of $CHCl_3$.

The data in Figure 24 suggest that PBB and PCB increased the rate of metabolism of $CHCl_3$ by hepatic MFOs to reactive products. These results are consistent with the effects of PBB and PCB on covalent binding of $CHCl_3$ metabolites to hepatic components *in vivo*. PCB-treatment did not increase $CHCl_3$ hepatotoxicity, however, suggesting that non-specific covalent binding of reactive $CHCl_3$ metabolites to hepatic components may not be the mechanism of $CHCl_3$ hepatotoxicity. Similarly, the effects of PBB and PCB on covalent binding to renal microsomal protein *in vitro* (Figure 24) are inconsistent with the effects of PBB and PCB on $CHCl_3$ nephrotoxicity. Since the dependency of covalent binding to renal microsomal protein *in vitro* on oxidative metabolism has not been shown, however, these results are difficult to interpret.

A further complication of the *in vitro* $CHCl_3$ binding studies, both those illustrated in Figure 24 and those reported by Ilett *et al.* (1973) is that the percentage of total radioactivity [(^{14}C)-$CHCl_3$] added to the incubation mixture that is converted to a quantitatable alkylating metabolite (≃0.1% with hepatic microsomes, ≃0.005% with renal microsomes) is less than the estimated concentration of impurities (≃1%) in the radiolabelled $CHCl_3$ used in these studies. Thus, much of the material bound may actually have been derived from non-$CHCl_3$ contaminants.

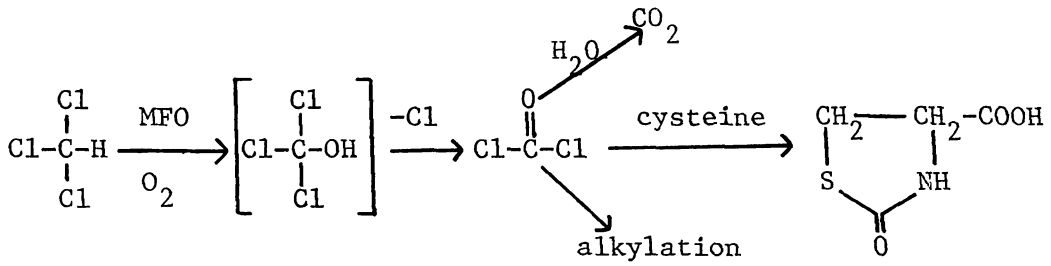
In summary, several inducers and inhibitors of renal and hepatic drug metabolism alter organ-specific $CHCl_3$ toxicity, perhaps by altering the relative proportions of $CHCl_3$ metabolized to proximate toxicants in the kidney and liver. The effects of specific inducers

on organ-specific $CHCl_3$ toxicity are consistent with the theory that the nephrotoxic $CHCl_3$ metabolite is formed directly in the kidney. $CHCl_3$ toxicity in both kidney and liver is associated with a loss of GSH and toxicity is enhanced when GSH is depleted by diethyl maleate treatment. GSH depletion following $CHCl_3$ exposure, however, does not appear to result solely from conjugation with electrophilic $CHCl_3$ metabolites. Finally, a correlation was not shown between alkylation of renal and hepatic proteins and lipids *in vivo* or *in vitro* and $CHCl_3$ toxicity.

SPECULATION

A. Phosgene and $CHCl_3$ Toxicity

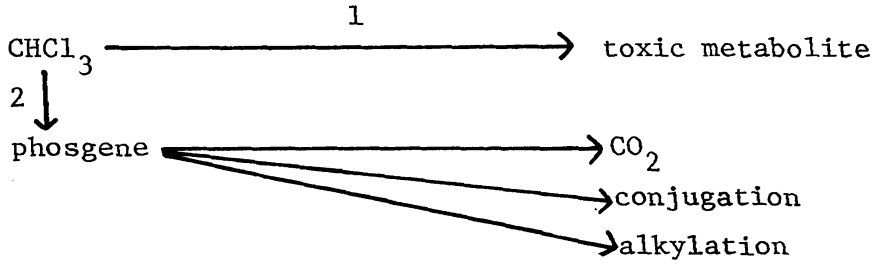
Pohl et al. (1977) and Mansuy et al. (1977) were able to trap phosgene ($COCl_2$) as a metabolite of $CHCl_3$ by the addition of high concentrations of cysteine to in vitro incubations of $CHCl_3$ and rat liver microsomes. The product of non-enzymatic condensation of phosgene with cysteine, 2-oxathiazolidine-4-carboxylic acid, was isolated from the in vitro incubation mixture and identified by mass spectroscopy. Pohl et al. (1977), furthermore, determined that molecular oxygen was the source of the oxygen atom in the phosgene molecule and proposed that $CHCl_3$ is metabolized to phosgene by P-450-dependent MFOs as shown below:



2-oxathiazolidine-4-carboxylic acid

2-Oxathiazolidine-4-carboxylic acid has also been isolated from the livers of rats injected with cysteine and $CHCl_3$ (separate injection sites), leading to speculation that phosgene may be the toxic $CHCl_3$ metabolite (Pohl and Krishna, 1978). The effects of PBB and PCB on $CHCl_3$ toxicity and the generation of alkylating metabolites, however,

are dissimilar in some cases, suggesting that the alkylating metabolite, very likely phosgene, may not be responsible for acute $CHCl_3$ toxicity. Pohl and Krishna (1978) have reported that the formation of phosgene *in vitro* is inhibited by SKF 525-A, as is the covalent binding of a $CHCl_3$ metabolite to microsomal protein *in vitro* (Sipes *et al.*, 1977). In contrast, SKF 525-A does not reduce $CHCl_3$ toxicity in rats (Lavigne and Marchand, 1974) and potentiates $CHCl_3$ toxicity in mice (Figure 20). Thus, phosgene is very likely the source of tissue alkylation *in vivo* and *in vitro* but does not appear to be the acutely toxic metabolite of $CHCl_3$. Rodents and humans metabolize a significant portion (40-80%) of absorbed $CHCl_3$ to CO_2, a process also inhibited by SKF 525-A (Lavigne and Marchand, 1974). Thus, microsomal oxidation of $CHCl_3$ appears to be a major pathway of $CHCl_3$ metabolism but not necessarily a toxification reaction. The effects of inhibitors and inducers of renal drug metabolism on $CHCl_3$ nephrotoxicity are consistent with the existence of at least two competing pathways of $CHCl_3$ metabolism, as illustrated below; one pathway generating an unidentified, toxic metabolite (reaction 1, induced by PBB and inhibited by PB) and another generating a non-toxic metabolite (reaction 2, induced in the kidney by 3MC and PCB, inhibited by SKF 525-A), possibly phosgene, that is ultimately responsible for the generation of CO_2 and the alkylating metabolite.



Additional studies would be needed to test the validity of the model proposed. It is, however, consistent with the data of Pohl and co-workers as well as with the results of this dissertation.

SUMMARY

The experiments in this dissertation were designed to elucidate potential roles of renal biotransformation in the development of halogenated aliphatic hydrocarbon nephropathy. Summarized below are the major findings of this study and their relevance to chloroform and carbon tetrachloride nephrotoxicity.

Induction of renal and hepatic AHH activities in male, ICR mice by continued dietary ingestion of PBB and PCB appeared to be maximal within 14 days. The magnitude of increase in renal and hepatic AHH activities was proportional to the concentration of the inducing agent in the diet over a concentration range of 25-200 ppm for PBB and 25-400 ppm for PCB. Additional studies demonstrated that dietary PBB potentiated $CHCl_3$ and CCl_4 toxicities in mice and that the degrees of potentiation of $CHCl_3$ and CCl_4 toxicities were proportional to the concentrations of PBB in the diets.

3MC and TCDD, as well as PBB and PCB, induced P-450-dependent MFO activities and cytochrome P-450 concentration in rodent kidney and liver. NaPb, however, increased hepatic but not renal enzyme activities and cytochrome P-450 content. The non-inducibility of renal drug metabolism by NaPb was consistent with the lack of effects of NaPb on $CHCl_3$ nephrotoxicity.

As has been demonstrated previously in the liver, 3MC induced forms of cytochrome P-450 and AHH in kidney and liver that were distinct from

those induced in liver by NaPb. NaPb did not alter the spectral characteristics of renal cytochrome P-450 nor the forms of AHH present in the kidney. The forms of cytochrome P-450 and AHH induced in the liver by PBB and PCB appeared to be mixtures of the forms normally induced by NaPb and by 3MC. In the kidney, however, cytochrome P-450 and AHH induced by PBB and PCB closely resembled that induced by 3MC. Thus, agents that have mixed inductive effects on liver cytochrome P-450 and MFO activities may not have such effects on kidney cytochrome P-450 and MFO activities.

Reduction of renal and hepatic P-450-dependent MFO activities by SKF 525-A and piperonyl butoxide were demonstrated in vivo and in vitro. Liver P-450-dependent MFOs appeared more sensitive than kidney P-450-dependent MFOs to reductions in activities by intraperitoneal injection of SKF 525-A and piperonyl butoxide. The differential susceptibilities of renal and hepatic AHH activities to inhibition by several compounds in vitro indicate that renal cytochromes may respond differently than hepatic cytochromes to the presence of inhibitors of P-450-dependent MFO activities.

Several inducers (PBB, PCB, HCB) of renal and hepatic P-450-dependent MFOs increased the susceptibilities of male, Sprague-Dawley rats to the nephrotoxic and hepatotoxic effects of CCl_4. These agents did not appear to alter the mechanism of CCl_4 toxicity and may have potentiated CCl_4 toxicity by increasing the rate of extent of metabolism of CCl_4 to a proximate toxicant.

Treatment with PBB potentiated $CHCl_3$ toxicity in ICR, male mice while treatment with PCB, 3MC and TCDD inhibited $CHCl_3$ toxicity. In

some instances the effects of inducing agents (PCB, 3MC, NaPb) on the renal and hepatic toxicities of $CHCl_3$ were dissimilar. If it is assumed that a single molecular species produces both renal and hepatic injury, then these results suggest that organ-specific $CHCl_3$ toxicity may be a consequence of organ-specific $CHCl_3$ metabolism. Alternatively, the nephrotoxic and hepatotoxic metabolites of $CHCl_3$ may be different molecular species and both may be formed primarily in liver. Enzymatic formation of the nephrotoxic and hepatotoxic $CHCl_3$ metabolites, however, may be affected differently by treatment with inducers such as PCB, 3MC and NaPb. Pretreatment with SKF 525-A potentiated while pretreatment with piperonyl butoxide inhibited $CHCl_3$ toxicity in mice. The different effects of these inducers and inhibitors of drug metabolism on $CHCl_3$ toxicity are consistent with the existence of competing enzymatic pathways of $CHCl_3$ metabolism; one pathway leading to a toxic metabolite and another pathway leading to a nontoxic metabolite.

Renal and hepatic GSH concentrations in intact mice were depleted by $CHCl_3$ in a dose-dependent manner and correlations were demonstrated between $CHCl_3$-induced tissue injury and $CHCl_3$-induced depletion of GSH. Loss of GSH, however, did not appear to occur solely through conjugation with electrophilic $CHCl_3$ metabolites.

Alkylations of renal and hepatic components by $CHCl_3$ metabolites *in vivo* appeared to be proportional to the rate of delivery of $CHCl_3$ to the liver and kidney and to the rate of metabolism within the liver or kidney to reactive intermediates. A consistent correlation between covalent binding of $CHCl_3$ metabolites to liver and kidney and susceptibility to $CHCl_3$ injury was not demonstrated. These results do not

support the hypothesis that nonspecific alkylation of renal and hepatic macromolecules by reactive $CHCl_3$ metabolites is the mechanism by which $CHCl_3$ produces acute renal and hepatic injury. The reactive $CHCl_3$ metabolite (possibly phosgene) that binds to cell constituents may be an intermediate in the metabolism of $CHCl_3$ to CO_2. Another $CHCl_3$ metabolite, possibly formed in the kidney, may be responsible for the acute nephrotoxic effects of $CHCl_3$.

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