This is to certify that the

dissertation entitled

Assessment of the Toxicologic, Pathologic and Carcinogenic Effects of 3,4,3',4'-Tetrabromobiphenyl in Rats

presented by

Darlene Dixon

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Pathology

Stuart D. Haught

Major professor

Date 9/20/85

O-12771

ASSESSMENT OF THE TOXICOLOGIC, PATHOLOGIC AND CARCINOGENIC EFFECTS OF 3,4,3',4'-TETRABROMOBIPHENYL IN RATS

By

Darlene Dixon

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Pathology

1985

ABSTRACT

ASSESSMENT OF THE TOXICOLOGIC, PATHOLOGIC AND CARCINOGENIC EFFECTS OF 3,4,3',4'-TETRABROMOBIPHENYL IN RATS

By

Darlene Dixon

Pitot's bioassay for hepatocarcinogenesis was used to determine if 3,4,3',4'-tetrabromobiphenyl (3,4-TBB) can promote or initiate gamma-glutamyl transpeptidase (GGT) positive enzyme-altered foci (EAF). Groups of 6 female 180-200 g rats were used for initiation and promotion assays. To test for initiation, rats were partially hepatectomized (PH) and given 1, 5, or 10 mg of 3,4-TBB/kg orally, or 10 mg of diethylnitrosamine (DEN)/kg ip as an initiator. Thirty days later, rats were promoted with 500 mg of phenobarbital (PB)/kg for 180 days. Results indicated that 3,4-TBB may have initiating potential as suggested by increased numbers of EAF in rats initiated with 3,4-TBB and promoted by PB compared to rats initiated with 3,4-TBB or DEN and fed basal diets. To test for promotion, PH rats were initiated with DEN and 30 days later diets containing 0.1, 1, or 5 mg of 3,4-TBB/kg were fed for 180 days. 3,4-TBB increased the number of EAF and thus appears to act as a hepatic tumor promoter.

3,4-TBB was not severely toxic in rats as evidenced by the histologic appearance of the liver, spleen, thymus and thyroid gland and ultrastructural changes in the liver. Rats fed 5 mg of 3,4-TBB/kg had significantly decreased hepatic retinyl esters concentrations compared to rats fed diets containing 0.1 or 1 mg of 3,4-TBB/kg or 500 mg of PB/kg. Serum thyroxine (T_4) and free T_4 concentrations were also decreased in rats fed diets containing 3,4-TBB.

To determine the effects of 3,4-TBB on hepatic glutathione (GSH) concentrations, groups of 3 male 160-180 g rats were given a single oral dose (17 mg/kg) of 3,4-TBB or a combination treatment consisting of a single oral dose (1 mg/kg) of 3,4,5,3',4',5'-hexabromobiphenyl (3,4,5-HBB) given 24 h before a single oral dose of 17 mg of 3,4-TBB/kg. 3,4-TBB had no effect on hepatic GSH concentrations compared to controls at 2, 4, 8, 24 or 48 h after dosing. Pretreatment of rats with 3,4,5-HBB did not alter the effects of 3,4-TBB on hepatic GSH concentrations.

DEDICATION

To my mother, Evelyn B. Dixon

"Guided by my heritage of a love of beauty and a respect for strength - in search of my mother's garden, I found my own."

Alice Walker

ACKNOWLEDGEMENTS

I would like to express my sincerest appreciation to Dr. Stuart D. Sleight, my major professor, for his guidance and support throughout my graduate studies. His friendship, tolerance, advice and criticisms are appreciated.

I wish to thank Drs. Steven D. Aust, Allan L. Trapp, Keiji Marushige and Tracie E. Bunton for serving as my guidance committee members and for excellent direction in completion of my research, academic program, and compilation of this dissertation.

In preparation of the research presented in this dissertation, I have received faculty and technical assistance from many persons at Michigan State University and would like to acknowledge the following individuals for their help: Dr. Ronald F. Slocombe, Dr. Gregory Fink, Dr. Raymond Nachreiner, Dr. Richard K. Jensen, Dr. Robert Leader, Dr. Margit Rezabek, Cynthia Millis, Fran Whipple, Mae Sunderlin and Donna Craft. I would especially like to thank Cheryl Assaff for typing this dissertation and Irene Brett and Dr. Esther Roege for their technical assistance.

A special thanks to Dr. Albert W. Dade, Patricia Lowrey, Dr. Charles Lowrey, my Tuskegee colleagues, Dr. David McConnell, Dr. Yasuko Marushige, Calvin and Deborah Moore,

Joyce Wright, and Karen Wettlin for their encouragement, advice and friendship.

My deepest appreciation to my mother for her unwithering love, understanding and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Polybrominated biphenyls (PBB) are lipophilic, water insoluble, slowly metabolized polyhalogenated aromatic hydrocarbons first used in the United States as flame retardants in plastic materials in 1970 (Brinkman and deKok, 1980). Two companies, the Michigan Chemical Company (St. Louis, MI), which later merged with the Velsicol Chemical Corporation (Chicago, IL) and White Chemical Corporation (Bayonne, NJ) produced commercial quantities of PBB. The Michigan Chemical Company produced approximately 11 million pounds of a PBB mixture sold under the tradename Firemaster (FM) BP-6 (Brinkman and deKok, 1980).

In 1973, approximately 1,000 pounds of FM BP-6 was inadvertently mixed into livestock feed at a mill located in Battle Creek, MI (Carter, 1976). Consumption of this feed by cattle, swine and poultry resulted in contamination of milk, meat, eggs and finished feed that was ultimately consumed by many of Michigan's residents (Dunckel, 1975). As a result of this accident, over 90% of Michigan's residents have detectable levels of PBB in their tissues (Selikoff and Anderson, 1979). To date, there is no conclusive evidence that PBB cause acute or short term illnesses in humans, but they pose possible long term or chronic health risks due to their persistence in the body

and environment. The FM mixture has been reported to cause hepatocellular carcinomas in rats (Kimbrough et al., 1981). FM and congeners of PBB have been reported to promote liver cancer in rats, and in the future may be associated with adverse long-term effects such as cancer in humans (Jensen et al., 1982, 1983b).

The FM BP-6 mixture is composed of a number of congeners differing from one another in the number and position of bromine substitutions on the biphenyl rings (Aust et al., 1981). The structural configuration and degree of bromination are important in predicting the toxicologic, metabolic and carcinogenic properties of these congeners. FM BP-6 and many congeners of PBB have been found to cause toxic responses in many different mammalian systems (Poland and Knutson, 1982). These include hepatocellular hypertrophy, vacuolation and necrosis, porphyria, chloracne, a wasting syndrome, menorrhea, immune suppression and thymic and splenic lymphoid depletion.

The first objective of the research described in this dissertation was to assess the carcinogenic effects of 3,4,3',4'-tetrabromobiphenyl (3,4-TBB), a minor component tentatively identified in the commercial FM BP-6 (Robertson et al., 1982). 3,4-TBB differs from the other congeners of PBB studied in our laboratory in that it is metabolized (in vitro and in vivo), and therefore it is not very persistent in the tissues of the body (Millis et al., 1985a; Mills et al., 1985). This characteristic of 3,4-TBB allowed us to

evaluate the congener as both an initiator and promoter in a two-stage initiation/promotion assay for hepatocarcinogenesis in rats (Pitot et al., 1978). To date, all other congeners of PBB studied in our laboratory have acted as promoters in Pitot's assay (Jensen et al., 1982). It is impossible to assess these congeners as initiators due to their lipid solubility and persistence within fat and liver parenchymal cells of mammals. It is hypothesized that if persistent congeners capable of tumor promotion and a nonpersistent congener or other congeners capable of tumor initiation are within FM BP-6, this mixture can be classified as a complete carcinogen. In the future this may have significance in the development of cancer in Michigan residents exposed to PBB.

The second objective of the research presented in this dissertation was to evaluate the toxicologic effects of 3,4-TBB in the rat as evidenced by light and electron microscopic tissue changes and propose a mechanism for toxicity. It is hypothesized that 3,4-TBB is metabolized by the hepatic microsomal monooxygenase system to a toxic intermediate, an arene oxide or epoxide. This metabolite is very reactive and can be detoxified by enzymatic or nonenzymatic conjugation with reduced glutathione (GSH). It is suggested that in the event of hepatocellular GSH depletion, acute toxicity may occur as a result of this electrophilic intermediate binding to macromolecules within the cellular cytosol, membrane, or nucleus.

The third objective of the research presented in this dissertation was to characterize the effects of 3,4-TBB on serum and hepatic vitamin A homeostasis and serum triiodothyronine (T_3) and thyroxine (T_4) concentrations in rats.

LITERATURE REVIEW

Chemical and Physical Properties of Polybrominated Biphenyls (PBB)

Congeners of PBB were introduced into the United States in 1970 and were used as flame retardants in various plastic and electrical materials found in business machines, industrial equipment, thermostats and radio and television parts (Brinkman and deKok, 1980). In 1974, the production of FM BP-6, the only PBB product to reach large-scale commercial production in the United States, was halted.

PBB are polyhalogenated aromatic hydrocarbons (PHAH). Also belonging to this group of PHAH are the polychlorinated dibenzo-p-dioxins, classically 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the polychlorinated biphenyls (PCB) and the polychlorinated dibenzofurans (PCDF). These toxicants, along with PBB, are all environmental contaminants and have been shown to possess a similar structure-activity relationship which dictates the carcinogenic, metabolic, toxicologic and biochemical effects of these compounds in mammalian systems (Poland and Knutson, 1982).

Congeners of PBB consist of 2 polycyclic ring structures joined by a carbon-carbon bridge (Brinkman and deKok, 1980). The empirical formula for PBB is $C_{12}H_{10-n}Br_n$, with n varying from 1 to 10 bromines. The bromines may be

ortho (2,2';6,6'), meta (3,3';5,5') or para (4,4') to the carbon-carbon bridge as shown in Figure 0-1.

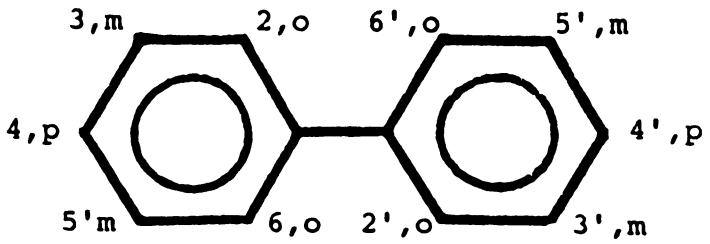

Figure 0-1. Chemical structure of polybrominated biphenyls (PBB).

The average composition (wt. %) of the different congeners within the FM BP-6 mixture is as follows: tetra 2-5; penta 5-10; hexa 70-80; hepta 12-18; remainder 0-1 (Brinkman and deKok, 1980). Gas chromatographic analysis of FM BP-6 has shown the mixture to consist of 12 to 14 major congeners, 10 of these congeners have been structurally characterized (Dannan et al., 1982b; Moore and Aust, 1978; Moore et al., 1980). 2,4,5,2',4',5'-Hexabromobiphenyl (2,4,5-HBB) is the major congener within FM BP-6 and comprises approximately 50 to 70% of the mixture (Brinkman and deKok, 1980; Jacobs et al., 1976; Moore and Aust, 1978; Sundström et al., 1976).

Commercial mixtures of PBB are typically white, off-white or beige powdered solids. These chemicals are soluble in organic solvents, insoluble in water, slowly metabolized and highly lipophilic (Brinkman and deKok, 1980; Matthews, 1981; Tuey and Matthews, 1980). Congeners of PBB are very

persistent in the environment and upon U.V. irradiation highly brominated, nontoxic congeners may be rapidly photolyzed to lower brominated, toxic congeners (Millis et al., 1985b).

PBB as Environmental Contaminants

During the summer of 1973, FM BP-6 was accidently mixed into livestock feed in place of "Nutrimaster" or magnesium oxide, a compound with a similar physical appearance used as a feed additive (Carter, 1976). At the Michigan Chemical Company plant, where "Nutrimaster" also was manufactured, there was a shortage of preprinted, red lettered bags normally used to package FM BP-6. Both FM BP-6 and "Nutrimaster" were packaged in brown bags on which tradenames were stenciled in black. Ten to twenty 50 pound bags of FM BP-6 were included into a truck load of "Nutrimaster" and distributed to a large feed mill operated by Farm Bureau Services, Inc. in Battle Creek, MI. This incident went undetected until the spring of 1974 and resulted in widespread contamination of meat, poultry and milk products with ultimate human exposure (Bekesi et al., 1978; Dunckel, 1975).

Some 30,000 livestock and 1,600,000 poultry on farms throughout Michigan became contaminated at levels requiring their destruction. Damages for livestock and poultry losses were estimated at approximately 100 million dollars or more (Carter, 1976; Dunckel, 1975).

The "PBB mix-up", as stated earlier, went undetected for approximately 1 year. Within this time, not only was there direct contamination of livestock through consumption of tainted feed, but also cross-contamination occurred to other livestock and poultry as a result of feed handled in facilities that had been exposed to PBB. Many of the barns and pastures on which the animals were kept were also contaminated with PBB.

PBB may not be a problem to Michigan alone. To date, PBB have been found in catfish in the Ohio River and in plants, fish, soil, water and human hair in New York and New Jersey (Culliton, 1977). PBB have not been conclusively linked to short term illnesses in humans (Stross et al., 1981; Kay, 1977), but because of their persistence in the body and environment and their ubiquitousness in various ecosystems, they pose a potential threat to human health.

Xenobiotics and Xenobiotic Metabolism by the Hepatic Microsomal Monooxygenase System

The prefix "xeno" is derived from the Greek term "xenos" meaning stranger or foreigner. Therefore, xenobiotics are compounds that are foreign to life or to an organism. Examples of xenobiotics are environmental contaminants such as PBB, PCB and TCDD. Animals are exposed to many xenobiotics through ingestion of food and water, or by inhalation of aerosols containing these foreign compounds. Many xenobiotics may also enter an organism by mere contact and absorption through the skin. Animals are

unable to separate foreign compounds from substances utilized for production of energy or for building of tissue components during the processes of ingestion, inhalation or absorption, but through metabolism the animal is capable of eliminating them from the body. In some instances during the process of elimination, some compounds are metabolized to toxic intermediates that may do more damage to the host's cells than the parent compound (Conney and Burns, 1972).

The liver is the primary site of metabolism of foreign and endogenous compounds (Conney, 1967). Secondary sites of metabolism are the lung, kidney, gastrointestinal tract and the skin (Parke, 1968). The hepatic microsomal monooxygenase system (MMS) is a nonspecific metabolizing system which functions to convert lipid-soluble, nonpolar substrates to more polar, water soluble, readily excretable compounds. The MMS are contained in microsomes which contain complex enzymes associated with the lipid bilayer of endoplasmic reticulum of liver cells and cells of other tissues. The MMS enzyme complex consists of a flavoprotein (F_p), known as cytochrome P450 reductase (Yasukochi and Masters, 1976; Guengerich, 1977), hemoproteins, collectively termed cytochromes P450 (Cooper et al., 1965; Garfinkel, 1957; Klingenberg, 1958; Lu and West, 1980) and a phospholipid, phosphatidylcholine (Strobel et al., 1970).

Cytochrome P450 reductase is thought to be exposed to the exterior of the lipid bilayer of endoplasmic reticulum, whereas the forms of cytochrome P450 are embedded in the

cellular membranes as shown in Figure 0-2 (Vermilion and Coon, 1978). The reductase has one flavin adenine dinucleotide (FAD) molecule and one flavin mononucleotide (FMN) molecule and a molecular weight of approximately 78 K. The exterior portion of the reductase molecule is considered to be the active site. There is also a membrane binding fraction (also called foot or tail) having a molecular weight of 8-9 K, which is essential for reconstitution of microsomal monooxygenase activity (Yasukochi and Masters, 1976).

The hemoproteins, cytochrome P450, is a carbon monoxide (CO) binding pigments, that in their reduced form binds CO to form a difference spectrum with a maximum around 450 nM, hence P450 (Garfinkel, 1957; Klingenberg, 1958). Garfinkel and Klingenberg each identified this pigment as a b-type cytochrome with one protoporphyrin IX prosthetic group per molecule (hemoprotein). A multiplicity of hepatic microsomal cytochrome P450 isozymes have been purified and characterized in various mammalian species (Dannan et al., 1983; Lu and West, 1980; Nebert et al., 1981; Waxman and Walsh, 1982; Welton and Aust, 1974). Some of the isozymic forms of cytochrome P450 have been shown to have identical residue sequences at the NH_2 terminal which are thought to be involved in anchoring these molecules to the lipid bilayer of the endoplasmic reticulum as shown in Figure 0-2 (Waxman and Walsh, 1982).

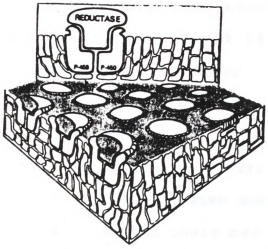

Figure 0-2. Modified hypothetical diagram of the relationship of cytochrome P450 reductase and forms of cytochrome P450 in membrane of endoplasmic reticulum. Taken from Nebert et al., 1981.

The microsomal monooxygenase system is an NADPH-dependent transport chain that inserts one atom of atmospheric oxygen (O_2) into their substrates (Conney, 1967). This electron transport pathway transfers electrons or reducing equivalents through the reductase from NADPH to the terminal oxidases, cytochromes P450 (Cooper, 1965; Lu et al., 1969). Another name for this system is mixed function oxidase (MFO) system, in that it requires both oxidizing and reducing equivalents, not derived from the substrate, but from NADPH (Mason, 1957).

During the initial processes of xenobiotic metabolism, the foreign compound forms a complex with the oxidized form of cytochrome P450, which is then reduced by a flow of electrons from NADPH to cytochrome P450 reductase (Parke, 1968). The reduced cytochrome P450-substrate complex then interacts with molecular oxygen to form a hydroxylated

substrate and water. During this reaction there is simultaneous regeneration of oxidized P450 for further substrate binding (Figure 0-3). There is also another electron transport system in microsomes. This system is NADH-dependent and is thought to play a role in the fatty acid desaturase system (Sato et al., 1969). There appears to be a relationship between this system and the microsomal drug metabolizing system in that there may be a process of electron crossover from one pathway to the other and the NADH-dependent system may supply some reducing equivalents for the terminal oxidases (cytochromes P450) as shown in Figure 0-3.

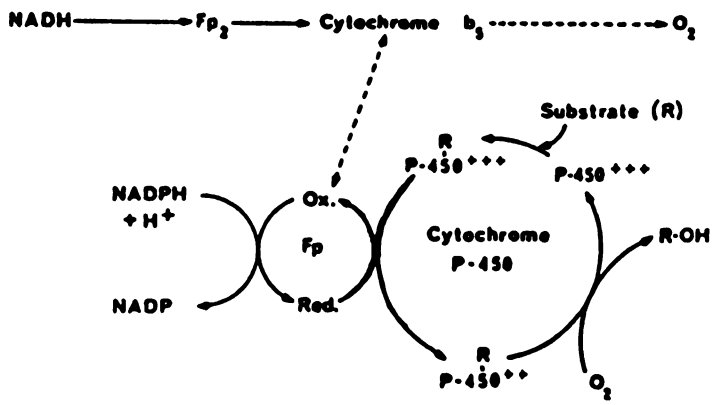

Figure 0-3. Modified microsomal electron transport chain. Taken from Wilkinson and Brattsten, 1972-73.

Hepatic xenobiotic metabolism is divided into microsomal and nonmicrosomal reactions (Ingelman-Sundberg, 1980; Kappas and Alvares, 1975; Parke, 1968). Phase I or primary reactions occur within the microsomes and may be accomplished by one or a combination of the following

reactions: 1) side-chain oxidations, 2) hydroxylations, 3) nitrogen oxidations, 4) sulfoxidations, 5) dealkylations, 6) nitroreductions, 7) azoreductions, 8) dehalogenations, 9) epoxidation and alcohol oxidations. Phase II or secondary reactions occur subsequent to primary reactions. They include reactions with enzymes such as epoxide hydratase, conjugation reactions with UDP glucuronic acid, sulfate, endogenous amines and glutathione (GSH). The phase I reactions are responsible for converting nonpolar foreign substrates into less complexed polar substances that can be further processed during phase II of xenobiotic metabolism and excreted from the organism. There are many factors which dictate the fate of a compound once introduced into an organism. These factors will be discussed in more detail in the following section.

Pharmacokinetics of Polyhalogenated Aromatic Hydrocarbons (PHAH)

Many factors must be considered when one looks at the fate of xenobiotics upon entry into an organism. Of primary importance are factors such as absorption and distribution of a foreign compound prior to its metabolism and excretion from the body. This section will briefly review the pharmacokinetics of PHAH.

Congeners of PBB are lipid soluble PHAH. The lipophilic properties of these compounds and other PHAH allow their absorption across cellular membranes by passive diffusion. In the gastrointestinal tract this absorption is

favored by a concentration gradient and accounts for the passage of these compounds from the gastrointestinal mucosa into the blood (Surak and Bradley, 1976). Upon entry into the blood, PHAH are almost completely adsorbed onto various blood proteins (Matthews et al., 1977). Adsorbtion of PHAH onto blood proteins does not interfere with the partitioning of these compounds to other tissue proteins or lipid, but does inhibit their excretion from the body.

Initial distribution of PHAH to various tissues is determined by tissue volume, rate of tissue perfusion and affinity of a given tissue for a specific compound (Matthews, 1981). The liver has a high rate of blood perfusion and moderate to high affinity for specific PHAH and is commonly a site of initial distribution for PHAH compounds. On the other hand, muscle has a low affinity for lipid soluble compounds and a moderate rate of blood perfusion, but due to its large tissue volume (40-50% total tissue volume), it is also a primary site of initial distribution of PHAH (Matthews and Anderson, 1975). Initial distribution of PHAH to adipose tissue is surprisingly low. This is due to the small tissue volume of fat in the body (usually less than 10% of total tissue volume) and the slow rate of blood perfusion (Matthews, 1981). However, it has been shown that PHAH may accumulate in adipose tissue under conditions of chronic exposure to high concentrations of a compound, or if an acute high dose of a compound cannot be

effectively excreted from the body (Tuey and Matthews, 1980).

Once PHAH enter the body, there is establishment of a dynamic equilibrium between the PHAH, the blood and all tissues of the body (Matthews, 1981). The rate at which this equilibrium is reached is determined by the affinity of the compound for a particular organ and the rate of perfusion of that organ. Other factors that may influence the rate of equilibrium are receptor binding, lipid solubility of the PHAH and lipid content of the tissue (Lutz et al., 1977). Once equilibrium is reached, PHAH are free to redistribute from one tissue compartment to another with the blood. If there is an increase or decrease in the concentration of PHAH in one tissue compartment, this change will be reflected in the concentration of all tissues (Matthews, 1981). The rate at which this change is seen is proportional to the rate of tissue perfusion with blood, hence once equilibrium is established, removal of PHAH from slowly perfused sites like adipose tissue is extremely slow. This factor attributes to the persistence of many PHAH in the fat deposits of the body.

Excretion of PHAH occur via the feces and urine. As mentioned earlier, the parent PHAH is usually a nonpolar and lipid-soluble molecule which readily adsorbs to various blood proteins. The protein-xenobiotic complex is too large to enter the renal glomerular filtrate, so renal excretion of the adsorbed PHAH does not occur (Matthews, 1981). If

the parent PHAH is metabolized to an intermediate metabolite, the metabolite formed is usually more water soluble and may enter the renal glomerular filtrate and be excreted in the urine. Excretion of PHAH into the feces is dependent upon the formation of amphipathic molecules during metabolism (Smith, 1973). These molecules may then be actively excreted from the liver into the bile. Fries (1978) showed that PBB are also excreted from the body of animals into fat containing products such as milk fat and eggs. This author also found that molecular size and weight of congeners of PBB made a major quantitative difference as to the accumulation and/or excretion of these compounds in the body.

PBB and Induction of Hepatic Microsomal Drug-Metabolizing Enzymes

The hepatic MMS is a nonspecific drug-metabolizing system that functions through the elaboration of principally oxidative enzymes to convert nonpolar, lipid soluble substrates to more polar and readily excretable compounds. There are numerous xenobiotic and endogenous substrates known to stimulate the activity of drug-metabolizing enzymes in liver microsomes, as well as microsomes located in other tissues (Conney, 1967; Kappas and Alvares, 1975). Examples of compounds that induce hepatic microsomal enzymes include steroid hormones, carcinogens, environmental toxicants such as PBB, PCB, TCDD and a variety of drugs including

hypnotics, sedatives, anesthetic gases, anticonvulsants, and muscle relaxants (Conney, 1967).

There are basically two types of hepatic microsomal enzyme induction exemplified by phenobarbital (PB) and 3-methylcholanthrene (3-MC) which are designated "PB type" and "3-MC type", respectively (Conney, 1967). These two types of microsomal enzyme induction differ with respect to substrate specificity and the absorbtion maxima of their reduced cytochrome-CO complex and ethylisocyanide difference spectra (Lu and West, 1978; Omura and Sato, 1964a,b).

Treatment of rat hepatic microsomes with PB results in elevated synthesis of a hemoprotein that in its reduced form binds CO at a maximum difference spectrum of 450 nM. This hemoprotein is called cytochrome P450 and has the same spectral characteristics as cytochrome P450 from untreated rats (Lu and West, 1980). Conversely, treatment of rat hepatic microsomes with 3-MC results in the synthesis of a new hemoprotein, which in its reduced form binds CO at a maximum difference spectrum of 448 nM. This new hemoprotein is designated as cytochrome P448 (Alvares et al., 1967) or cytochrome P_1450 (Sladek and Mannering, 1966). Endogenous or exogenous compounds that induce isozymes similar to those induced by PB or 3-MC are classified as PB type or 3-MC type of microsomal enzyme inducers.

There are multiple inducible isozymes of cytochrome P450 and P448. These isozymes have been shown to have different spectral properties, catalytic activity,

immunological properties and amino acid sequences (Dannan et al., 1983; Lu and West, 1980; Nebert et al., 1981). The major inducible isozymes of cytochromes P450 and P448 appear to vary among mammalian species and may differ between sexes, strains and individuals within a given species (Dent et al., 1980; Guengerich et al., 1981; Kamataki et al., 1983; Lu and West, 1980; Nebert et al., 1981). The induction of polysubstrate monooxygenase activities is thought to be genetically controlled by a combination of regulatory, structural and possibly temporal genes located at the Ah locus as shown in certain strains of mice (Greenlee and Poland, 1979; Nebert et al., 1981; Poland and Glover, 1980). This concept will be discussed later in this dissertation under "Toxicity and Microsomal Enzyme Induction - Effects of PBB".

The commercial PBB mixture, FM BP-6, can induce PB and 3-MC types of hepatic microsomal enzymes (Dent et al., 1976a,b) and this type of microsomal enzyme induction is termed mixed-type. The mixed-type of hepatic microsomal enzyme induction seen with FM is due to its content of individual congeners of PBB capable of inducing several isozymes of cytochrome P450 or P448, or a combination of both isozymes (Dannan et al., 1982d, 1983). The individual congeners are classified as either strictly PB type (P450), strictly 3-MC type (P448) or mixed-type (P450 and P448) of microsomal enzyme inducers. There is a structure-activity

correlation suggested for congeners of PBB and microsomal enzyme induction.

Congeners of PBB such as 2,4,5-HBB, 2,3,4,5,2',4',5'-heptabromobiphenyl, 2,3,4,5,2',3',4'-heptabromobiphenyl and 2,3,4,5,2',3',4',5'-octabromobiphenyl are all strictly PB type of hepatic microsomal enzyme inducers (Aust et al., 1981; Besaw et al., 1978; Moore et al., 1978a,b, 1979; Render et al., 1982). These congeners of PBB have 2 bromines at the ortho (2,2') positions on the biphenyl rings and this configuration is thought to be necessary for strictly PB type of hepatic microsomal enzyme induction (Besaw et al., 1978; Moore et al., 1978b, 1979; Render et al., 1982). Dannan et al. (1983) found that the presence of bromine at carbons ortho to the biphenyl bridge favored the induction of several isozymes of P450 in rat hepatic microsomes, but this structural arrangement did not eliminate the ability of congeners to induce isozymes of cytochrome P448 ($P450_{BNF-B}$ or $P450_{BNF/ISF-G}$). PB type of microsomal enzyme induction results in increased activity of enzymes such as NADPH-cytochrome P450 reductase, aminopyrine-n-demethylase and epoxide hydrase (Dannan et al., 1982d, 1983; Moore et al., 1979). Other microsomal enzymes induced are UDP-glucuronyltransferase and glutathione-S- transferases (Bock et al., 1973; Kaplowitz et al., 1975). Congeners of PBB that are strictly PB type of microsomal enzyme inducers are not toxic, but have been found to cause extensive proliferation of hepatic

endoplasmic reticulum as evidenced by electron microscopic evaluation (Besaw et al., 1978; Moore et al., 1978b, 1979; Render et al., 1982).

A strictly 3-MC type of microsomal enzyme inducer, 3,4-TBB has been tentatively identified within FM (Robertson et al., 1982). These authors reported that 3,4,5-HBB, 3,4,4'-tribromobiphenyl, 3,4,5,4'-TBB and 3,4,3',4',5'-pentabromobiphenyl were also capable of 3-MC type of induction. They suggested for 3-MC type of hepatic microsomal enzyme induction, congeners must possess halogen substitutions at both para (4,4') positions and at one, two, three or four meta (3,3',5,5') positions. Poland and Glover (1977) proposed two structural requirements for 3-MC type of microsomal enzyme induction by halogenated biphenyls. These authors found the presence of at least two adjacent halogen atoms in the lateral position of each benzene ring (positions 3,3',4,4',5,5') and the absence of ortho (positions 2,2',6,6') halogenation allowed coplanarity of these molecules which is important in receptor binding and induction of aryl hydrocarbon hydroxylase (AHH), an enzyme associated with 3-MC type of microsomal enzyme induction and toxicity. Congeners of PBB that are 3-MC type microsomal enzyme inducers increase AHH activity and are toxic as evidenced by light and electron microscopic and functional alterations in various tissues (Millis et al., 1985a; Render et al., 1982; Robertson et al., 1982). The interrelationship between 3-MC type of microsomal enzyme

induction, receptor binding, increased AHH activity and toxicity will be discussed in the section "Toxicity and Hepatic Microsomal Enzyme Induction - Effects of PBB".

2,4,5,3',4'-Pentabromobiphenyl, 2,3,4,5,2',4'-HBB, 2,4,5,2',4',5'-HBB and 2,3,4,5,3',4'-HBB are classified as mixed-type of microsomal enzyme inducers (Aust et al., 1981; Dannan et al., 1978b, 1982a,b). These congeners of PBB are present in FM BP-6 and have structural configurations that allow induction of both P450 and P448 microsomal enzymes. They have also been reported to cause toxic histologic and ultrastructural changes in various organ systems in rats (Akoso et al., 1982a; Dannan et al., 1978a, 1982c,d).

Hepatic Monooxygenase Metabolism of PBB

Congeners of PBB differ from one another in the number and position of bromines present on the biphenyl rings and these structural differences tend to dictate the type of microsomal enzyme induction which in turn determines the metabolic fate of these compounds. It has been proposed that in vitro and in vivo metabolism of PBB by cytochrome P450-dependent monooxygenases occurs when there are adjacent non-halogenated carbon atoms on at least one of the biphenyl rings (Millis et al., 1985a; Mills et al., 1985). Hepatic microsomes isolated from immature male rats and pretreated with 3-methylcholanthrene (3-MC) were found to increase in

vitro NADPH-dependent microsomal metabolism of 4,4'-dibromobiphenyl (DBB), 3,4,4'-tribromobiphenyl, 3,4,3',4'-tetrabromobiphenyl (TBB), 2,3,3',4'-TBB, 2,5,3',4'-TBB and 2,4,2',5'-TBB in decreasing order (Mills *et al.*, 1985). All of the above listed congeners have nonhalogenated *ortho* and *meta* carbon atoms on at least one biphenyl ring, induce P448 type microsomal enzymes and are metabolized. Phenobarbital pretreatment of isolated microsomes increased *in vitro* metabolism of congeners of PBB possessing nonhalogenated *meta* and *para* carbon atoms on at least one biphenyl ring. Examples of congeners found to have this type of structural configuration are 2,2'-DBB, 2,4,2',5-TBB, 2,5,2',5'-TBB, 2,3,3',4'-TBB, 2,5,3',4'-TBB and 2,4,5,2',5'-pentabromobiphenyl. All of these congeners have been shown to induce P450 type of microsomal enzymes and are metabolized (Mills *et al.*, 1985).

The importance of adjacent nonsubstituted carbon atoms as a determinant of halogenated biphenyl metabolism has been shown by many investigators in a series of studies using congeners of either PBB or PCB (Kato *et al.*, 1980; Kohli *et al.*, 1978; Matthews and Anderson, 1975; Mills *et al.*, 1985; Preston *et al.*, 1983; Tuey and Matthews, 1980; Van Miller *et al.*, 1975). It is postulated that aromatic hydrocarbons can be metabolized to an unstable arene oxide by the hepatic monooxygenase system (Jerina and Daly, 1974; Kohli *et al.*, 1978; Matthews, 1981). Arene oxide formation requires the availability of two adjacent unsubstituted carbon atoms.

Aryl halides having this type of structural arrangement are more readily metabolized than highly halogenated congeners lacking adjacent nonhalogenated carbon atoms. Mills et al. (1985) found that increased bromination of congeners of PBB inhibited metabolism even though they may possess adjacent nonhalogenated ortho and meta carbons. It is thought that halogenation tends to inhibit oxidative metabolism of the aromatic rings because halogen atoms are large, electronegative atoms which sterically hinders the microsomal enzymes and electronically hinders the electron deficient oxygen involved in the oxidation (Matthews, 1981). Once formed, the arene oxide may spontaneously isomerize to a monohydroxylated product, react with epoxide hydrase to yield a dihydroxylated product or possibly conjugate with reduced GSH (Jerina and Daly, 1974; Matthews, 1981; Preston et al., 1983).

An arene oxide has been identified as the intermediate metabolite of 4-bromobiphenyl (Kohli et al., 1978). This metabolite is very reactive and could possibly bind to cellular macromolecules such as cytosolic proteins, membrane lipids and/or structural components of DNA resulting in cellular damage and toxicity. Adduct formation with cellular DNA is thought to be one mechanism of tumor initiation in chemical carcinogenesis and congeners capable of being metabolized to electrophilic intermediates may act as initiators of cancer (Conney and Burns, 1972; Miller and Miller, 1977, 1981). Preston et al. (1983) have shown aryl

halide metabolism does not necessarily occur via an arene oxide. The major intermediate metabolite of 2,3',5,5'-tetrachlorobiphenyl (TCB) was reported to be a non-arene oxide, 3-hydroxy-TCB.

Dannan et al. (1982c) found congeners of PBB to be good substrates for induction of hepatic microsomal enzymes. This is important in that induction of the microsomal monooxygenases by nonmetabolized congeners of PBB may increase the rate of metabolism of metabolizable congeners. 3,4,5,3',4',5'-Hexabromobiphenyl (3,4,5-HBB), a nonmetabolized congener of PBB (not present in the FM BP-6) with 3-MC type of microsomal enzyme induction has been shown to increase the rate of metabolism of 3,4-TBB in vitro (Mills et al., 1985). If congeners of PBB are indeed metabolized to an arene oxide, metabolism of these compounds could result in formation of electrophiles with subsequent toxicity or possibly tumor initiation. On the other hand, if the parent form of the congener is important in receptor binding and that in turn is responsible for mediating toxicity, increased metabolism would decrease the toxicity of the metabolizable congener (Greenlee and Poland, 1979; Millis et al., 1985; Poland et al., 1976; Poland and Glover, 1980). It has also been shown that metabolizable congeners can induce their own metabolism, thereby, decreasing their toxicologic effects (Millis et al., 1985a).

Inhibition of metabolism can occur if nonmetabolized and metabolized congeners capable of inducing similar

isozymes of cytochrome P450 are coadministered. When isolated rat hepatic microsomes were treated with 3-MC followed by coadministration of 3,4,5-HBB and 3,4-TBB, 3,4,5-HBB appeared to have an inhibitory effect on the metabolism of 3,4-TBB (Mills et al., 1985). This is thought to have occurred as a result of 3,4,5-HBB interacting with microsomal enzymes at their binding sites and preventing the normal metabolism of 3,4-TBB. It is also suggested that nonmetabolized congeners such as 3,4,5-HBB may have a higher affinity for these binding sites, since equimolar administration 3,4,5-HBB and 3,4-TBB almost completely inhibited the metabolism of 3,4-TBB (Mills et al., 1985).

Toxicity and Hepatic Microsomal Enzyme Induction - Effects of PBB

Pathotoxicologic Effects of PBB

In the initial assessment of PBB, crude mixtures of FM BP-6 were used to evaluate the potential toxicologic effects of this mixture on various organ systems in different laboratory and domestic animals. Upon the identification and purification of individual congeners within FM, and with the correlation of toxicity and microsomal enzyme induction, research in this area veered toward assessing the pathotoxicologic effects of the individual congeners. In this section of this dissertation, I will attempt to give a broad overview of the significant toxicologic effects associated with the administration of FM BP-6, 3,4-TBB, 3,4,5-HBB and 2,4,5-HBB as evidenced by light and electron

microscopic changes seen in the rat. Also, I will review some of the current theories on the mechanism(s) of PBB toxicosis.

Hepatotoxicosis

Initially, the liver is the primary site of PBB distribution (Matthews, 1981). Understandably, this is also one of the major target organs affected in PBB ~~toxicosis~~ in several mammalian species including the rat, mouse, chick, cattle, pig, monkey and guinea pig (Allen et al., 1978; Cook et al., 1978; Gupta et al., 1983a,b; Gupta and Moore, 1979; Kimbrough et al., 1980; Render et al., 1982; Ringer, 1978; Sleight and Sanger, 1976; Werner and Sleight, 1981). Acute and chronic administration of various doses of FM (BP-6 or FF-1) has been reported to cause increased liver weights, hepatomegaly and hepatic microscopic changes characterized by centrolobular to midzonal hepatocellular vacuolation, intracytoplasmic fatty change of hepatocytes, hepatocellular hypertrophy, bile ductule proliferation and necrosis (Gupta and Moore, 1979; Kimbrough et al., 1980; Render et al., 1982; Sleight and Sanger, 1976). Hepatic ultrastructural changes associated with dietary administration of 10 ppm or 100 ppm of FM BP-6 to rats for 9 days have been characterized by increased proliferation and dilation of smooth endoplasmic reticulum (SER) and increased cytoplasmic lipid droplet accumulation (Render et al., 1982). Sleight and Sanger (1976) found dietary administration of 1 ppm or

10 ppm of FM BP-6 for 30 days caused an increase in size of hepatocyte mitochondria as evidenced by electron microscopic evaluation. These authors also reported rats fed 100 ppm or 500 ppm of FM for 30 days had ultrastructural hepatic lesions characterized by a marked increase in SER proliferation accompanied by hepatocellular vacuolation, indistinct mitochondrial morphology and concentric whorling of proliferated SER or myelin body formation. The severity of the lesions seen in this study were dose-dependent. In conclusion, FM is toxic and its toxicologic effects are attributed to the individual toxic congeners of PBB within the mixture capable of inducing 3-MC type microsomal enzymes.

2,4,5-HBB, the major congener in FM BP-6, has been reported to cause increased liver weight and hepatomegaly when administered in the diet to rats for 9 days (Render et al., 1982). These authors found that rats fed 10 ppm of 2,4,5-HBB had histologic hepatic lesions characterized by centrolobular to midzonal hepatocellular enlargement and vacuolation. Similar but more severe histologic hepatic changes were observed when rats were fed 100 ppm of 2,4,5-HBB. Ultrastructural changes observed with dietary administration of 100 ppm of 2,4,5-HBB included extensive dilation and proliferation of hepatocyte SER along with accumulation of lipid droplets in these cells (Render et al., 1982). 2,4,5-HBB is a strictly PB type of microsomal enzyme inducer, is not metabolized and does not appear to be

toxic. The increase in liver size and weight and microscopic and ultrastructural changes seen with the administration of 2,4,5-HBB are thought to result from stimulation of endoplasmic reticulum and are indicative of enhanced enzyme activity (Hansell and Ecobichon, 1974) and not true toxicity.

3,4,5-HBB, a nonmetabolizable congener not present in FM, has been reported to cause increased liver weight, hepatomegaly and microscopic hepatic lesions in rats characterized by hepatocellular hypertrophy, increased prominence of hepatocyte nucleoli, centrolobular to midzonal hepatocellular vacuolation, and bile duct epithelial hyperplasia when given at 100 ppm in the diet for 20 days (Render et al., 1982). Ultrastructural hepatic lesions seen in these rats consisted of increased lipid droplets within hepatocytes, SER proliferation with myelin body formation, swollen mitochondria and disorganization of rough endoplasmic reticulum. 3,4,5-HBB induces strictly 3-MC type of microsomal enzymes and is considered a toxic congener of PBB.

Another strictly 3-MC type of microsomal enzyme inducer is 3,4-TBB. This congener has been tentatively identified in FM and is metabolized (Mills et al., 1985; Robertson et al., 1982). 3,4-TBB is toxic, but less toxic than 3,4,5-HBB (Millis et al., 1985a). Oral administration of 21 μmoles/kg of 3,4-TBB to rats caused microscopic hepatic lesions characterized by mild, diffuse hepatocellular swelling with

decreased sinusoidal spaces in the midzonal regions of the hepatic lobule (Millis et al., 1985a). These microscopic changes were consistent with findings of Robertson et al. (1983) in which rats given a single ip dose (150 μmoles/kg) of 3,4-TBB had similar hepatic alterations. The histopathologic and ultrastructural hepatic changes associated with chronic dietary administration of 3,4-TBB will be discussed in Chapter I of this dissertation.

Immunotoxicosis

PBB have been reported to alter immune responses in pigs (Howard et al., 1980), dogs (Farber et al., 1978), mice (Fraker, 1980), rats (Luster et al., 1978), non-human primates (Allen et al., 1978), chickens (Ringer, 1978), guinea pigs (Vos and van Genderon, 1973) and humans (Bekesi et al., 1978). The results obtained from the immune function tests are not consistent within or between species in many instances. These variations may be related to PBB acting indirectly to alter the immune responses. The exact mechanism of action or cellular predilection for the immunotoxic effects observed with PBB are not known.

Many reports describe lymphoid cell depletion in the spleen and thymus after administration of FM or congeners of PBB to rats, but few studies have been performed to assess the significance of the alterations seen. Microscopically, splenic lesions seen with the oral administration of 100 mg, 300 mg or 1,000 mg of FM FF-1/kg/day (5 days/wk, 22 doses) consisted of depletion of the periarterial lymphoid cells of

the malpighian corpuscles, necrosis of lymphoblastic cells in germinal centers of lymphoid follicles and hypocellularity of the red pulp (Gupta and Moore, 1979). Thymic lesions were characterized by loss of demarcation between the cortical and medullary regions and disappearance of cortical lymphocytes. Render et al. (1982) found lymphoid cell depletion in the cortex of the thymus and follicles of the spleen in rats fed 10 ppm or 100 ppm of 3,4,5-HBB for 10 days. Millis et al. (1985a) reported severe thymic involution in rats which was characterized histologically by loss of thymocytes from both the medullary and cortical regions in rats given 21 μmoles of 3,4-TBB or 3,4,5-HBB orally. Robertson et al. (1983) found that 150 μmoles of 3,4-TBB given ip also resulted in thymic involution characterized histologically by marked depletion of lymphoid cells in the cortex of the thymus in rats.

Thyroid Gland Toxicosis

Histologic changes in the thyroid gland have been observed in male rats exposed to various doses of PBB (FM BP-6 or FF-1) (Gupta and Moore, 1979; Gupta et al., 1982a) or PBB congeners (Akoso et al., 1982b). Gupta et al. (1983a) observed thyroid changes primarily in male rats exposed to 10 mg/kg of PBB (FF-1) for 6 months. These histologic alterations consisted of thyroid follicles lined by columnar epithelium containing a few areas of epithelial papillary projections. The follicular colloid was sparse,

or bluish and had a stippled appearance. These authors also found a significant decrease in serum thyroxine (T_4) and triiodothyronine (T_3) concentrations and concluded that PBB may interfere with thyroid hormone secretion. Akoso et al. (1982b) also found decreased serum T_4 concentrations in male rats fed 100 ppm of FM or 10 ppm of 3,4,5-HBB.

Male rats fed 100 ppm of FM, 100 ppm 2,4,5-HBB or 10 ppm of 3,4,5-HBB had thyroid lesions characterized microscopically by extensive hyperplasia and hypertrophy of follicular cells and loss of follicular colloid. Rats given 100 ppm of FM or 10 ppm of 3,4,5-HBB in the diet for 30 days were found to have ultrastructural thyroid gland lesions characterized by increased lysosomal bodies, increased cytoplasmic vacuoles and dilated cisternae of the endoplasmic reticulum (Akoso et al., 1982b). These authors also found similar ultrastructural lesions in the thyroid gland of rats fed 100 ppm of 2,4,5-HBB for 30 days, but the changes were less severe. Diets containing 100 ppm of FM and 1 ppm or 10 ppm of 3,4,5-HBB in this study caused increased thyroid gland weights. Sleight et al. (1978) reported similar histologic changes in male rats given 100 mg of FM in the diet for 30 or 60 days.

Mechanism(s) of PBB Toxicosis

Congeners of PBB and other PHAH such as TCDD and PCB have been found to produce a similar pattern of toxic responses in mammalian species (Poland and Knutson, 1982). Many of these aromatic hydrocarbons are stereoisomers and

can stereotypically bind to a protein receptor (Ah or TCDD receptor) that mediates 3-MC type microsomal enzyme induction ($P_1$450), increased AHH activity and toxicity. The exact relationship between this particular type of microsomal enzyme induction and the toxicologic effects observed in mammalian species is unknown.

2,3,7,8-TCDD is the prototypical halogenated aromatic hydrocarbon for Ah or TCDD receptor binding and is the most potent known inducer of the AHH system. The current model proposed for AHH induction by TCDD and other halogenated hydrocarbons is similar to the molecular mechanism of steroid hormone action. This model postulates a two-step mechanism whereby the inducer (ligand) binds to a "cytosolic" receptor, followed by a temperature dependent "translocation" of the ligand-receptor complex from the cytoplasm to the nucleus (Greenlee and Poland, 1979; Nebert et al., 1981). This cytoplasmic to nuclear translocation results in an accumulation of ligand-receptor complexes within the nucleus with subsequent activation of structural genes including $P_1$450 specific mRNA and associated AHH activity (Nebert et al., 1981).

Recently, it has been proposed that the protein receptor is not a "cytosolic" receptor, but is nuclear in location in the intact cell (Whitlock and Galeazzi, 1984). These authors found that dilution factors contributed to the redistribution of unoccupied receptors and ligand-receptor complexes between the nuclear and cytoplasmic fractions of

broken cells and this phenomenon could account for the appearance of receptors (and complexes) in cytosolic preparations. It was further concluded by these authors that minimization of this dilution effect caused 80-90% of the receptors to associate with the nuclear fraction and this was indicative of a nuclear location for the receptor. These authors proposed a modified two-step model for the mechanism of TCDD and other PHAH mechanism of action. The first event is the binding of the ligand to the receptor which is primarily a nuclear event. The second event is a temperature dependent strengthening of the ligand-receptor complex to the nucleus and not a translocation event as proposed in the current theory, since the ligand-receptor binding occurs in the nucleus. The binding of receptors (or complexes) is thought to be primarily to chromatin. This results in the accumulation of ligand-receptor complexes within the nucleus with transcription of cytochrome P_1450 gene.

The protein receptor, be it nuclear or cytoplasmic in location, is thought to regulate genes at the Ah locus as seen in certain strains of mice (Greenlee and Poland, 1979; Nebert et al., 1981). The murine Ah locus regulates the induction by polyhalogenated and polycyclic compounds of numerous drug-metabolizing enzymes in virtually all tissues (Nebert et al., 1979). Following the proposed preceding events leading to accumulation of ligand-receptor complexes within the nucleus, there is a pleiotypic response resulting

in activation of numerous structural genes at the Ah locus (Nebert et al., 1981). AHH is one of the several drug metabolizing enzymes (structural gene products) induced upon activation of structural genes at the Ah locus, and in many mammalian systems its persistent induction is associated with toxicity.

The ability of congeners of PHAH to bind the TCDD receptor has been assessed by determining its ability to induce 3-MC type of microsomal enzyme induction and increase AHH activity. Congeners of PBB possessing this type of microsomal enzyme induction also have been shown to be toxic (Akoso et al., 1982a; Dannan, 1982a,c,d; Millis et al., 1985a; Moore et al., 1979; Render et al., 1982). It is proposed that enzyme induction is an early event and that toxicity occurs later as a result of persistent ligand occupation of the receptor and subsequent gene expression (Poland and Knutson, 1982). Congeners of PBB capable of inducing increased AHH activity have structural configurations which allow coplanarity, a prerequisite for ligand-receptor binding and enzyme induction (Poland and Glover, 1977).

Competitive receptor binding studies with 3,4,5-HBB and 3,4-TBB for the specific binding of (^{3}H)TCDD to the mouse or rat hepatic receptor showed 3,4-TBB to be a more potent competitor for the TCDD receptor than 3,4,5-HBB (Millis et al., 1985a). These authors also found significantly elevated AHH activity in rats given an equimolar dose (21.3

μmoles/kg) of 3,4,5-HBB or 3,4-TBB as compared to controls, and on day 6 this enzyme activity decreased in rats given 3,4-TBB. It was concluded that 3,4-TBB is metabolized and induces its own metabolism *in vivo* as evidenced by decreased adipose tissue and liver concentrations of this congener. These authors also suggested that the metabolism of 3,4-TBB by hepatic microsomal enzymes could possibly explain the decreased AHH activity on day 6.

An equimolar dose of 3,4,5-HBB was found to be more toxic than 3,4-TBB as evidenced by histologic changes in the thymus and liver of rats (Millis *et al.*, 1985a). However, competitive binding studies performed by these authors indicated 3,4-TBB had a 10 times greater affinity than 3,4,5-HBB for the TCDD receptor and if toxicity is mediated through ligand receptor binding and induction of cytochrome P_1450 microsomal enzymes, in particular AHH, then 3,4-TBB should be considerably more toxic than 3,4,5-HBB. It was suggested that metabolism of 3,4-TBB decreases its intracellular concentration resulting in dissociation of the congener from the TCDD receptor with subsequent decreased gene expression and hence decreased toxicity. These findings support the hypothesis of Poland and Knutson (1983), in that persistent occupation, or "hyperinduction" is necessary for gene expression and toxicity. 3,4,5-HBB is a nonmetabolizable congener of PBB and is toxic due to its ability to persistently bind to the TCDD receptor which results in gene expression and toxicity.

Alternatively, formation of toxic intermediates (i.e. epoxides, arene oxides) during microsomal enzyme metabolism has been proposed as a possible mechanism of toxicity by metabolizable congeners of PBB and other PHAH (Jerina and Daly, 1974; Kato et al., 1981; Kohli et al., 1978; Sweeney et al., 1979). It is postulated that the toxicologic effects observed with PHAH administration may occur through the formation of electrophilic (reactive) intermediates capable of binding DNA (Miller and Miller, 1977), cytosolic proteins, or membrane and cytosolic lipids. This latter event could possibly result in initiation of lipid peroxidation, a proposed mechanism of cellular toxicity by PCB (Kato et al., 1981), and TCDD (Sweeney et al., 1979). Robertson et al. (1983) found that 3,4-TBB, a metabolizable congener of PBB, does not exert its toxicologic effect via a lipid peroxidation mechanism in rats given a single (150 μmoles) ip dose of this congener, since antioxidants such as butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA) or vitamin E did not prevent histologic changes in the liver.

Glutathione: Role in Detoxification and PBB Toxicity

Glutathione (γ-glu-cys-gly) is a thiol containing tripeptide consisting of L-glutamate, L-cysteine and glycine. Its gamma (γ) bond makes it resistant to normal peptidase activity and permits its presence at high concentrations in a variety of different cells (Kosower and

Kosower, 1978). Most of the cellular glutathione exists in thiol form (reduced glutathione [GSH]), although various disulfides (mainly G-SS-protein) and to a lesser extent, glutathione disulfide (GSSG) contribute to the total cellular pool of glutathione. GSH and GSSG are present in various body fluids including plasma and bile, but at lower concentrations than found intracellularly (Orrenius et al., 1983). The liver is probably the major source of plasma GSH that appears to originate from a steady efflux of GSH from hepatocytes (Orrenius et al., 1983).

Studies on the biosynthesis and degradation of glutathione have led to the characterization of a series of enzymes known as the γ-glutamyl cycle (Reed and Beatty, 1980). Glutathione is assembled from its constituent amino acids in two separate reactions (Snoke and Bloch, 1952), each requiring one molecule of ATP. The first step involves the formation of a γ-glutamyl linkage between L-glutamate and L-cysteine catalyzed by γ-glutamyl-cysteine synthetase. In the second step, glutathione synthetase catalyzes the addition of glycine to γ-glutamyl-cysteinyl to form reduced GSH. Reduced GSH is catabolized by γ-glutamyl transpeptidase (GGT) (Reed and Beatty, 1980). It is the only known enzyme capable of hydrolyzing the γ-glutamyl peptide bond (Meister, 1975). GGT cleaves the γ-glutamyl group, releasing glutamate and leaving the remaining cysteinyl-glycine peptide susceptible to cleavage by aminopeptidase (Hanigan and Pitot, 1985).

The most widely known biological role of reduced GSH is that of enzymatic or nonenzymatic conjugation with foreign compounds or their metabolites. Many chemicals that conjugate with GSH are excreted as mercapturic acids (Booth et al., 1961). Enzymatic conjugation of GSH with several aromatic compounds is catalyzed by a series of distinct enzymes known as GSH-S-transferases (Kaplowitz et al., 1975; Ketterer et al., 1984). GSH-S-transferases (Baars et al., 1978), along with epoxide hydrase (Bresnick et al., 1977) and UDP-glucuronyl transferase (Bock et al., 1973) have been reported to be induced in rats after administration of xenobiotics. Conjugation with GSH does not only facilitate the excretion of foreign compounds or their metabolites, but serves as well to intercept highly reactive electrophilic compounds before they bind to cellular macromolecules leading to a toxic or mutagenic event (Conney and Burns, 1972; Reed and Beatty, 1980).

Conjugation with GSH does not always decrease the reactivity of a compound resulting in decreased toxicity. The compound 1,2 dichloroethane, a waste product derived from vinyl chloride, has been shown to be activated through conjugation with GSH to a reactive intermediate that is mutagenic in the Ames Assay (Rannug et al., 1978). These authors proposed that 1,2 dichloroethane is activated to a mutagenic intermediate in the presence of GSH and glutathione-S-transferase A and C when incubated with the post mitochondrial liver fraction (S-9).

Glutathione also has a protective role in oxidative processes (Mills, 1957). GSH peroxidase, linked to glutathione reductase, and glucose-6-phosphate dehydrogenase as a source of NADPH was found to be the actual physiological detoxification mechanism for hydrogen peroxide in red blood cells (Cohen and Hochstein, 1963). There are two forms of GSH peroxidase found in red blood cells; one is a selenium (Se)-dependent enzyme whereas the other is Se-independent (Lawrence and Burk, 1976; Mazzella et al., 1983).

Glutathione peroxidase is also highly specific for GSH as a sulfhydryl substrate in protection against nonspecific hydroperoxides formed during lipid peroxidation of membranes. It is suggested in vivo GSH peroxidase inhibits the initiation of lipid peroxide formation rather than catalyzing the reduction of these peroxides (McCay et al., 1976). Although the in vivo mechanism of GSH peroxidase activity against lipid peroxidation is unclear, it is thought to be a critical factor in inhibiting lipid peroxidation in biological membranes.

Many xenobiotics including acetaminophen, bromobenzene, mycotoxins and benzo(a)pyrene are known to deplete liver GSH levels after in vivo exposure (Mgbodile et al., 1975; Mitchell et al., 1974; Raheja et al., 1983; Thor et al., 1979; White, 1976; Zampaglione et al., 1973). Depletion of intracellular GSH concentrations has also been proposed as a mechanism for hepatotoxicity observed in many mammalian

species exposed to PHAH. It is postulated that in the event of intracellular glutathione depletion, a reactive electrophile, such as an epoxide, would no longer be detoxified by conjugation with GSH, and could exert its toxic effects through macromolecule binding and possible initiation of lipid peroxidation. Rifkind et al. (1984) found 5 to 500 nanomoles/egg of 3,4,5-hexachlorobiphenyl or 3,4-tetrachlorobiphenyl had no significant effects on chick embryo hepatic GSH levels, and concluded that GSH depletion does not have a significant role in PCB hepatotoxicity.

Initiation and Promotion of Carcinogenesis: Pitot's Model of Experimental Hepatocarcinogenesis

It is postulated that carcinogenesis in animal test systems is a multistage process consisting of two major phases: initiation and promotion (Berenblum and Shubik, 1947; Boutwell, 1974; Farber, 1981; Pitot and Sirica, 1980; Rous and Kidd, 1941). Initiation is defined as a single irreversible event that occurs rapidly after treatment with a subcarcinogenic dose of a chemical, physical or biological agent capable of directly or indirectly altering cellular DNA (genotoxic) (Farber, 1981; Pitot et al., 1981; Pitot and Sirica, 1980; Scribner and Süss, 1978). The altered DNA may be a result of electrophilic binding (a mutational event) of the initiators to purine and pyrimidine bases on the DNA molecule (Farber, 1981) or as a result of damaged DNA-repair enzyme systems (a non-mutational event) (Boutwell, 1974;

Farber, 1981; Pitot and Sirica, 1980; Scribner and Süss, 1978). Initiation results in modified cellular DNA that is heritable and phenotypically unexpressed (Boutwell, 1974; Farber, 1981; Pitot et al., 1981; Pitot and Sirica, 1980). The initiated cell and its immediate progeny can only be identified by production of the promoted neoplasm (Boutwell, 1974; Pitot and Sirica, 1980). Initiation also requires cell replication for "fixation" and perpetuation of the altered, unexpressed genetic information (Farber, 1981; Peraino, 1971; Pitot et al., 1981; Pitot and Sirica, 1980). In Pitot's model of experimental hepatocarcinogenesis, rapid cellular proliferation is achieved by a partial hepatectomy (Higgins and Anderson, 1931), and this induces rapid hepatocyte proliferation and increased DNA synthesis which may increase the error rate in normal DNA replication and DNA repair after initiator administration.

Promotion is defined as a reversible event that occurs after repeated treatments with an agent that alters the phenotypic expression of genetic information of a cell (Boutwell, 1974; Pitot et al., 1981; Pitot and Sirica, 1980). The agent does not directly interfere with cellular DNA (epigenetic), but may combine with target cell membrane or cytoplasmic receptors (Boutwell; 1974; Pitot et al., 1981; Pitot and Sirica, 1980) resulting in altered genetic expression. Changes in cell-cell communication have been proposed as a mechanism of in vitro tumor promotion whereby neighboring initiated cells become unresponsive to contact

inhibition and begin to clonally proliferate (Newbold and Amos, 1981; Trosko et al., 1981; Tsushimoto et al., 1982). This may result from interference with gap junction function (Trosko et al., 1981). The exact mechanisms of promotion are not completely understood and may include a combination of processes such as activation of oncogenes or other regulatory proteins or necrosis with subsequent compensatory hyperplasia (Berenblum, 1944; Bishop, 1982; Jensen et al., 1983b).

In experimental hepatocarcinogenesis, once the initiated cell is promoted, the genetic alteration is phenotypically expressed by production of preneoplastic and neoplastic lesions (Pitot et al., 1981; Pitot and Sirica, 1980; Williams, 1980). The preneoplastic lesions are believed to be the initial stages of cancer formation and may be clonal in origin (Pitot et al., 1981; Pitot and Sirica, 1980; Rabes et al., 1972; Williams, 1980). These preneoplastic lesions or enzyme-altered foci have been shown microscopically to regress to a normal phenotype upon cessation of administration of a tumor promoter (Rabes et al., 1972). These lesions are resistant to iron uptake (Williams, 1980), have increased GGT activity (Fiala and Fiala, 1973; Kalengayi et al., 1975) and are deficient in ATPase and glucose-6-phosphatase (Rabes et al., 1972). These characteristics shared by enzyme-altered foci and hyperplastic nodules may be used to assess the initiating or promoting abilities of an agent in experimental

hepatocarcinogenesis. In Pitot's model of experimental hepatocarcinogenesis, increased fetal enzyme GGT is used as one criterion for assessing initiators and promoters. GGT is seen in actively proliferating and undifferentiated fetal hepatocytes of rats and is a marker of cellular dedifferentiation, signaling a reversion of the hepatocyte to a fetal phenotype (Fiala and Fiala, 1973; Hanigan and Pitot, 1985; Kalengayi et al., 1975). In the adult rat, the enzyme has decreased activity and is usually present in bile ducts and canalicular regions of the liver, the convoluted tubules of the kidney and the pancreas (Albert et al., 1961; Fiala and Fiala, 1973; Goldbarg et al., 1960; Rutenberg et al., 1969).

Hanigan and Pitot (1985) suggested that compounds used during the promotion phase of many hepatocarcinogenic protocols utilize glutathione (GSH) and interfere with the oxidation and reduction of GSH. These authors postulate that increased GGT on membranes of altered hepatocytes during hepatocarcinogenesis allows the replenishment of intracellular GSH and gives these cells a selective growth advantage during tumor promotion.

Pitot's model of experimental hepatocarcinogenesis is a two-stage model consisting of an initiation and a promotion phase (Pitot et al., 1978). The initiation phase consists of a partial hepatectomy (PH) followed 24 hours later by a subcarcinogenic dose of diethylnitrosamine (DEN), a known tumor initiator. The promotion phase in this model consists

of dietary administration of a test chemical beginning approximately 30 days after initiation. The length of the promotion phase is approximately 180 days. PB at 500 mg/kg of feed is the standard promoting agent used in this protocol.

The Pitot assay may be used to assess both initiators and promoters of experimental hepatocarcinogenesis. One of the advantages of this initiation/promotion protocol is that it probably offers one of the best methods for promotion of foci of altered hepatocytes, since interpretation is uncompromised by inclusion of a complete carcinogenic dose of DEN during the initiation phase (Leonard et al., 1982). Also the Pitot assay can detect initiation by carcinogens that do not significantly enhance hepatocyte replication, which is required to fix initiating events, due to the incorporation of PH as a mitotic stimulus during initiation. An obvious disadvantage of this model is the length of the promotion phase needed for the development of enzyme-altered foci. An additional limitation is that the Pitot model is not the most effective protocol with respect to the number of foci produced at the end of 180 days (Leonard et al., 1982).

PBB as Promoters in Experimental Hepatocarcinogenesis

Jensen et al. (1982) have demonstrated that FM BP-6 and 2,4,5-HBB are promoters of experimental hepatocarcinogenesis in vivo. The results indicated that there appeared to be a dose-dependent response associated with 2,4,5-HBB tumor

promotion. This dose-dependent tumor promoting effect was not seen with FM BP-6. The congener 3,4,5-HBB has also been reported to possess *in vivo* tumor promoting ability in Pitot's two-stage hepatocarcinogenesis assay (Jensen *et al.*, 1983b). Dietary administration of 10 mg 3,4,5-HBB/kg for 140 days caused enhancement of enzyme-altered foci in rats. Short term exposure to FM BP-6 has also been reported to be as effective as long term exposure in enhancing the development of enzyme-altered foci in Pitot's model of hepatocarcinogenesis (Jensen *et al.*, 1983a).

FM BP-6 (Trosko *et al.*, 1981), 2,4,5-HBB and 2,4,5,3',4',5'-HBB (Tsushimoto *et al.*, 1982) at nontoxic doses are capable of inhibiting cell-cell communication *in vitro* using Chinese hamster V79 cells, and this appears to be a property of known tumor promoters (Newbold and Amos, 1981). Cytotoxic congeners of PBB such as 3,4,5-HBB tested in this system at subtoxic doses were less effective inhibitors of cell-cell communication than non-cytotoxic congeners of PBB (Tsushimoto *et al.*, 1982). This suggests that toxic PBB congeners possibly promote tumorogenesis through other mechanisms such as cytotoxicity with subsequent compensatory hyperplasia and neoplasia (Boutwell, 1944; Jensen *et al.*, 1983b).

Reports on the assessment of the *in vitro* mutagenic properties of congeners of PBB have varied (Kavanagh *et al.*, 1985; Kohli *et al.*, 1978). Kohli *et al.* (1978) found the congener 4-bromobiphenyl to have mutagenic activity in the

Ames Assay, whereas Kavanagh et al. (1985) reported that FM BP-6, 2,4,5-HBB, 3,4,5-HBB and 3,4-TBB failed to induce mutations in Chinese hamster V79 cells or WB rat liver cells. More research is needed to assess the in vitro and in vivo mutagenic effects of PBB-congeners and also to define a possible mechanism(s) of tumor promotion by these compounds.

CHAPTER I

ASSESSMENT OF 3,4,3',4'-TETRABROMOBIPHENYL (3,4-TBB) AS A PROMOTER OR INITIATOR IN EXPERIMENTAL HEPATOCARCINOGENESIS IN RATS

CHAPTER I

ASSESSMENT OF 3,4,3',4'-TETRABROMOBIPHENYL (3,4-TBB) AS A PROMOTER OR INITIATOR IN EXPERIMENTAL HEPATOCARCINOGENESIS IN RATS

Introduction

To date, there is no conclusive evidence that PBB cause acute or short term illnesses in humans (Kay, 1977; Stross et al., 1981), but it is possible they may cause long term or chronic effects, such as cancer, due to their persistence in the body and ubiquitousness in the environment (Brinkman and deKok, 1981; Tuey and Matthews, 1980). Short term exposure to FM BP-6 has been found to be as effective as long term exposure in enhancing the development of preneoplastic lesions in livers of rats; therefore, continuous exposure to PBB may not be necessary for enhancement of tumorogenesis in humans (Jensen et al., 1983a).

Carcinogenesis in animal test systems is a multistage process consisting of two major phases, initiation and promotion (Berenblum and Shubik, 1947; Boutwell, 1974; Farber, 1981; Pitot and Sirica, 1980; Rous and Kidd, 1941). Initiation is defined as a single irreversible, genetic event that occurs rapidly after treatment with a subcarcinogenic dose of a chemical, physical or biological

agent that is capable of indirectly or directly altering cellular DNA (Farber, 1981; Pitot et al., 1981; Pitot and Sirica, 1980; Scribner and Süss, 1978). Promotion is a reversible, epigenetic event that occurs after repeated treatment with an agent that alters phenotypic expression of the genetic information of a cell (Boutwell, 1974; Pitot et al., 1981; Pitot and Sirica, 1980; Scribner and Süss, 1978; Stott et al., 1981). Promotion occurs subsequent to initiation and promoters of carcinogenesis appear to cause selective proliferation of initiated cells (Pugh and Goldfarb, 1978; Shulte-Hermann et al., 1981).

In chronic carcinogenicity testing, single or multiple doses of FM have been reported to cause hepatocellular carcinomas in rats after 2 years (Kimbrough et al., 1981). For FM to be a complete carcinogen, it should contain congeners capable of tumor initiation and promotion (Pitot et al., 1981; Pitot and Sirica, 1980; Scribner and Süss, 1978); however, the major congener found in FM BP-6 (2,4,5-HBB) acts as a promoter in experimental hepatocarcinogenesis in rats (Jensen et al., 1982). Also, PBB do not have properties of known initiators (Dannan et al., 1978a; Trosko et al., 1981; Tsushimoto et al., 1981) and it is speculated that the carcinogenic effects observed in rats given FM alone, may have resulted from promotion of environmentally initiated cells (Jensen et al., 1982; Pitot et al., 1980; Pitot and Sirica, 1980). Conversely, a metabolizable congener of PBB, 4-bromobiphenyl has been reported to be

metabolized to a 4-bromobiphenyl arene oxide and is highly mutagenic in the Ames Assay (Kohli et al., 1978). Arene oxides or epoxides formed during metabolism of aromatic hydrocarbons can electrophilically bind to DNA with subsequent adduct formation (Hemminki, 1983), and this is thought to be one mechanism of initiation by chemical carcinogens (Miller and Miller, 1977).

The congener 3,4-TBB is a minor component tentatively identified in FM BP-6 (Robertson et al., 1982). 3,4-TBB differs from the other congeners of PBB tested for their carcinogenic effects in rats, in that it is metabolized (in vitro and in vivo), and therefore does not accumulate in tissues of the body (Millis et al., 1985a; Mills et al., 1985). This characteristic of 3,4-TBB allowed us to evaluate the congener as both an initiator and promoter in a two-stage initiation/promotion assay of hepatocarcinogenesis in rats (Pitot et al., 1978). It is impossible to assess the initiating ability of other congeners of PBB tested thus far due to their lipid solubility and persistence within fat and liver parenchymal cells of mammals (Matthews, 1981; Tuey and Matthews, 1980). It is hypothesized that if congeners capable of tumor promotion and a metabolizable congener capable of tumor initiation through an intermediate epoxide formed during metabolism are identified within FM, this mixture can act as a complete carcinogen. This may have significance in the development of cancer in Michigan residents exposed to PBB.

3,4-TBB is also a photoproduct of 2,4,5,2',4',5'-HBB (245-HBB), the major congener in FM BP-6 (Millis et al., 1985b), binds to the TCDD receptor and is a potent inducer of aryl hydrocarbon hydroxylase (AHH) activity (Millis et al., 1985a; Robertson et al., 1982). 3,4-TBB has a binding affinity for the TCDD receptor 10 times greater than that of 3,4,5,3',4',5'-HBB (345-HBB), a slowly or nonmetabolized and very toxic congener of PBB not present in FM BP-6. However, 3,4-TBB is less toxic than 3,4,5-HBB as evidenced by histologic changes in the liver and thymus of rats (Millis et al., 1985a). Toxic effects associated with short term administration of 3,4-TBB include a dose dependent decrease in thymic and splenic weight and increased liver weight (Millis et al., 1985a; Robertson et al., 1982). Thymic and hepatic alterations were characterized microscopically by Millis et al. (1985a) and consisted of lymphoid cell depletion in the cortex and medulla of the thymus and midzonal to periportal hepatocellular hypertrophy.

The mechanism of toxicity by PHAH is unknown, although good evidence supports the hypothesis that toxicity of these compounds is mediated through binding of a congener to a polypeptide receptor, the TCDD receptor (Greenlee and Poland, 1979; Poland and Glover, 1980; Poland et al., 1976). Persistent ligand receptor binding is thought to result in induction of cytochrome P_1450 microsomal enzymes namely AHH, an enzyme associated with PHAH toxicity. Congeners capable of inducing increased hepatic AHH activity have been shown

to be toxic to various organ systems in rats (Akoso et al., 1982a; Andres et al., 1983; Millis et al., 1985a; Render et al., 1982; Robertson et al., 1982, 1983). However, the exact correlation between increased hepatic AHH activity and toxicity are unknown.

The objectives of this study were to determine if 3,4-TBB acts as an initiator or promoter of hepatocarcinogenesis in rats using Pitot's two-stage model of experimental hepatocarcinogenesis and to characterize the histologic and ultrastructural tissue changes associated with chronic dietary administration of 3,4-TBB.

Materials and Methods

Experimental Design

Treatment groups for the initiation and promotion assays consisted of 6 or 3 rats placed in groups as shown in Table 1-1.

The promoting or initiating ability of 3,4-TBB was assessed and compared to a standard promoter, phenobarbital (PB), or to a standard initiator, diethylnitrosamine (DEN), using Pitot's two-stage model of experimental hepatocarcinogenesis as outlined in Figure 1-1.

Figure 1-1. Pitot's two-stage model of experimental hepatocarcinogenesis.

Day:	0	1	31	211
Tx:	PH	Initiator	Promoter	Kill

Table 1-1. Experimental design for 3,4,3',4'-tetrabromobiphenyl (3,4-TBB) initiation and promotion assays.

Group	Tx	Initiator	Dose (mg/kg)	Promoter	Dose (mg/kg) of diet	No. of rats
Study I: Initiation assay						
1	PH[a]	DEN[b]	10	0	0	6
2	PH	DEN	10	PB[c]	500	6
3	PH	3,4-TBB	1	0	0	6
4	PH	3,4-TBB	1	PB	500	6
5	PH	3,4-TBB	5	0	0	6
6	PH	3,4-TBB	5	PB	500	6
7	PH	3,4-TBB	10	0	0	6
8	PH	3,4-TBB	10	PB	500	6
Study II: Promotion assay						
9	0	0	0	3,4-TBB	0.1	3
10	PH	DEN	10	3,4-TBB	0.1	6
11	0	0	0	3,4-TBB	1	3
12	PH	DEN	10	3,4-TBB	1	6
13	0	0	0	3,4-TBB	5	3
14	PH	DEN	10	3,4-TBB	5	6
15	0	0	0	PB	500	3

[a]Partial hepatectomy.
[b]Diethylnitrosamine.
[c]Phenobarbital.

In the initiation assay, rats were 2/3 partially hepatectomized (PH) 24 hr prior to initiation with 10 mg of DEN/kg given ip or 1, 5 or 10 mg of 3,4-TBB/kg given orally. Promotion was with 500 mg of PB/kg diet fed from day 31 to day 211. In the promotion assay, rats were 2/3 PH 24 hr prior to initiation with 10 mg of DEN/kg given ip. Rats used as controls were not PH or initiated. Promotion was with 500 mg of PB/kg diet or 0.1, 1, or 5 mg of 3,4-TBB/kg diet fed from day 31 to day 211.

Rats

Female Sprague-Dawley (C/D) rats weighing 180-200 g were obtained from Charles River Laboratories, Inc., Portage, MI. Rats were randomized and housed according to groups in clear polypropylene cages, 3 rats per cage. Rats were acclimated for 5 days. The room temperature was maintained at 22^{o} C with a 12 hr light/dark cycle. Cages containing rats for the tumor promotion assay were placed in filtered laminar flow units (Contamination Control Inc., Lansdale, PA).

Surgical Procedure for Partial Hepatectomy

Rats were partially hepatectomized by using the method described by Higgins and Anderson (1931). Rats were anesthetized by using ether and asepsis was maintained throughout the surgery. A median-line incision was made just prior to the xiphoid process of the sternum, that extended 3 or 4 cm caudally to expose the underlying

abdominal muscles. The abdominal muscles and peritoneum were bluntly dissected away to expose the median (right and left) and left liver lobes. These lobes were lifted from the abdominal cavity, ligated using sterile and absorbable 3 chromic gut (Davis-Geck, American Cyanamid Company, Pearl River, NY) and excised. The abdominal muscles and peritoneum were sutured by using 3-0 coated vicryl*, absorbable suture (Ethicon, Inc., Johnson and Johnson Company, Somerville, NJ). The skin incision was closed by using metal clamps (Michel Clamps [18/8], George Tiemann and Co., Plainview, NJ). There was no special postoperative care, and rats had fully recovered by 24 h post surgery.

Chemicals

3,4-TBB was synthesized and purified by recrystallization and alumina chromatography (Millis, 1984). Diethylnitrosamine (DEN) was purchased from Eastman Kodak Company, Rochester, NY, and phenobarbital was purchased from Sigma Chemical Co., St. Louis, MO.

Diet Preparation

The diets were prepared by adding appropriate amounts of 3,4-TBB or PB in corn oil to a ground commercial feed (Certified Rodent Chow #5002^{R}, Ralston Purina Co., St. Louis, MO). A premix containing 50 mg of 3,4-TBB/kg was prepared by dissolving 100 mg of 3,4-TBB in corn oil and adding it to 2.0 kg of feed. Diets containing 1 or 5 mg/kg of 3,4-TBB/kg were prepared from the premix, and diet

containing 0.1 mg of 3,4-TBB/kg was prepared from the mixture containing 1 mg of 3,4-TBB/kg. Rats were observed daily for clinical signs of toxicity and body weights were recorded once a week throughout the study.

Necropsy Procedure

Rats were taken off feed 16 h before necropsy and on day 211 rats were weighed, anesthetized with ether, and killed by decapitation. All organs were routinely examined for gross lesions. The liver, spleen, thymus, kidneys, adrenal glands and brain were removed from the carcass and weighed.

Collection and Preparation of Tissue Samples for Histopathologic Evaluation

Tissue samples of liver, thymus, spleen, kidney, adrenal gland, thyroid gland, heart, lung, stomach, small intestine, pancreas, colon, urinary bladder, brain and pituitary gland were collected and fixed in 10% neutral buffered formalin. Formalin-fixed tissue samples were processed, embedded in paraffin, cut by a microtome into 6 µm sections and stained with hematoxylin and eosin for histologic examination.

Collection, Preparation and Histochemical Staining for Liver Samples

At necropsy, representative liver samples were mounted on corks by using O.C.T. (Tissue-Tek^{R}, Miles Scientific, Division of Miles Laboratories, Inc., Naperville, IL). Liver samples were frozen in isopentane cooled in liquid

nitrogen. Five liver sections taken from the same lobe of each rat were cut at 8 μm (IEC Microtome-Cryostat Model/Minot Custom Microtome, International Equipment Co., Needham Heights, MA) and were stained for gamma glutamyl transpeptidase (GGT) activity by the method of Rutenberg et al. (1969). Each section of frozen liver was placed onto a glass slide, fixed in 100% acetone (4° C) for 10 to 15 minutes and air dried. Each section was incubated for 15 to 20 minutes in a freshly mixed and filtered substrate solution (25° C) containing 1 ml of gamma-glutamyl-4-methoxy-β-naphthylamide, 2.5 mg/ml (Vega Biotechnologies, Inc., Tucson, AZ), 5 ml of 0.1 M Tris buffer, pH 7.5 (Sigma Chemical Co., St. Louis, MO), 10 mg of Fast Blue BB salt (Sigma Chemical Co., St. Louis, MO), 10 mg of glycylglycine (Aldrich Chemical Co., Milwaukee, WI) and 14 ml of 0.85% saline. Each liver section was then placed in 0.85% saline for 2 minutes, followed by a 2 minute incubation in 0.1 M cupric sulfate. Each section was again placed in 0.85% saline for 2 minutes, immersed in deionized water, allowed to drain and counterstained with hematoxylin using three 1 second dips. Each section was rinsed with tap water, air dried and mounted onto a glass slide and refrigerated. Slides were evaluated within 8 h after histochemical staining.

Histochemically stained liver sections were projected and enzyme altered foci (EAF) were traced using a Leitz Prado Projector (Ernst Leitz Wetzlar GMBH, Wetzlar, West

Germany) at a magnification of 90X. Approximately 40 fields for each liver were evaluated, which was equivalent to an area of 1.5-2.0 cm^2 per rat. The area of each EAF/cm^2 of liver was determined with a planimeter (Lasico L-30, Los Angeles Scientific Co., Inc., Los Angeles, CA) and the methods of Scherer (1981) were used to compute the number of EAF/cm^3 of liver.

Chemical Analysis of Liver and Adipose Tissue

Samples of liver and adipose tissue were taken from 2 or 3 rats from each group of rats fed diets containing 0, 0.1, 1 or 5 mg of 3,4-TBB/kg. Liver and adipose samples were also taken from 3 rats initiated with 3,4-TBB and promoted with PB. Tissue concentrations of 3,4-TBB were determined by the following procedure. An electronic analytical balance (Mettler AE 163, Mettler Instrument Corporation, Highstown, NJ) was used to weigh 0.5 g of liver and abdominal adipose tissue. Weighed samples were washed in petroleum ether and ground with washed ignited sand (Mallinckrodt, Inc., Paris, KY). The ground mixture was dehydrated by the addition of 10 to 20 g of granular anhydrous sodium sulfate (Mallinckrodt, Inc., Paris, KY). 3,4-TBB was extracted from the mixture by adding 15 ml of glass distilled hexane (J.T. Baker Chemical Co., Phillipsburg, NJ) and bringing the mixture to a boil over a water bath (100° C). This mixture was filtered into a 100 ml volumetric flask and the hexane washes and subsequent

filtrations were repeated until a total of 4 extractions were completed. After the fourth extraction, the 3,4-TBB extract was brought to a volume of 100 ml by the addition of hexane. Aliquots of 20 ml were taken from the 3,4-TBB extract and evaporated with nitrogen.

The aliquot used for fat content determinations was evaporated (N-EVAP, model III, Meyer Oranomation Assoc., Inc., Shrewsberry, MA) to 2 ml and rinsed with petroleum ether into preweighed foil pans. A heated water bath was used to evaporate the solvent in the foil pans. The pans were then put in a vacuum desiccator for 24 h to promote drying of the sample. The tissue fat content was determined by subtracting the weight of the pan without the fat from the weight of the pan with the fat.

The aliquot used for chemical determinations was evaporated to 2 ml and put through a column (Chromaflex, 200 mm x 7 mm ID) that was prewashed with acetone and plugged at the tapered end with glass wool. The column was filled with 1.6 g of activated magnesium silicate (Forisil, 60-100 mesh, Fisher Scientific Co., Cleveland, OH) and 2 cm of granular anhydrous sodium sulfate was added to the top of the column. The column was washed with 5 ml of hexane and this was discarded as the first 5 ml of hexane. The 2 ml evaporated sample was added to the column and repeatedly rinsed with hexane. The sample eluant was evaporated to 0.5 ml and reconstituted to a total volume of 2 ml by adding iso-octane (2,2,4-trimethyl-pentane) (Burdick and Jackson Laboratories,

Inc., Muskegon, MI). 3,4-TBB tissue concentrations were then determined by injecting 2 µl of the sample eluant onto a gas chromatograph equipped with an electron capture detector (G.C. Model 3709, Varian Instrument Division, Palo Alto, CA) utilizing an injector temperature of 280° C, a column temperature of 250° C and a detector temperature of 300° C. Nitrogen was used as the carrier gas at a flow rate of 30 ml/min.

Gas chromatographic readings were recorded and tissue concentrations of 3,4-TBB were compared to known standard amounts of 3,4-TBB.

Collection and Preparation of Liver Samples for Ultrastructural Examination

Liver sections were fixed in Karnovsky's fixative (Karnovsky, 1965). These sections were trimmed, placed in Zetterqvist's wash (Pease, 1964) at pH 7.4 and postfixed in 1% osmium tetroxide in Zetterqvist's wash. Liver sections were then dehydrated in graded alcohol (50, 70, 95, and 100%), placed in 2,2-dimethoxypropane (Baic and Baic, 1984) and fixed in 100% acetone. An Araldite 502 plus DDSA (dodecanyl succinic anhydride) mixture was used for plastic embedding. Embedded liver sections were cured in a 100° C oven for 24 h.

Plastic embedded samples were thick sectioned (1 µm), stained with toluidine blue and examined with a light microscope. Representative areas were thin sectioned (900 Å) and were stained with uranyl acetate and lead citrate.

The thin sections were viewed by using an electron microscope (EM 9S2, Carl Zeiss, Germany).

Statistical Analysis of Data

Data were statistically analyzed after logarithmic transformation by the one-way analysis of variance (ANOVA) and differences in group means were analyzed by the Student-Newman-Keul's multiple comparison test at $p<0.05$, or the comparison of percentages arcsin $\sqrt{}$ test at $t_{infinity}$ (Steel and Torrie, 1980). Organ and body weight data were statistically analyzed by the one way ANOVA and the Student-Newman-Keul's multiple comparison test at $p<0.05$.

Results

Enzyme-Altered Foci

The mean number of EAF/cm^3 of liver for PH rats initiated with 10 mg of DEN/kg given ip and promoted with 500 mg of PB/kg diet or 0.1, 1, or 5 mg of 3,4-TBB/kg diet is given in Table 1-2. Dietary administration of 5 mg of 3,4-TBB produced significantly greater numbers of hepatic EAF/cm^3 of liver than 500 mg of PB/kg diet or 0.1 or 1 mg of 3,4-TBB/kg. There appeared to be a dose dependent increase in the number of hepatic EAF/cm^3 of liver in rats fed 0.1, 1, or 5 mg of 3,4-TBB/kg diet, although there was no statistically significant difference in the number of EAF/cm^3 of liver between rats fed 0.1 or 1 mg of 3,4-TBB/kg diet. There was no significant difference in the number of

Table 1-2. Number of enzyme-altered foci (EAF)/cm^3 of liver in rats initiated with DEN and promoted with PB or 3,4-TBB for 180 days.

Day 0 — Tx	Day 1 — Initiator	Dose (mg/kg given ip)	Day 31-211 — Promoter	Dose (mg/kg of diet)	Number of EAF/cm^3 of liver
PH	DEN	10	0	0	0±0
PH	DEN	10	PB	500	465±355
PH	DEN	10	3,4-TBB	0.1	131±121
PH	DEN	10	3,4-TBB	1.0	217±158
PH	DEN	10	3,4-TBB	5.0	1488±919[a]

Data are expressed as $\bar{x}$±SD for groups of 6 rats.
[a]Significantly different from rats promoted with PB or 0.1 or 1.0 mg of 3,4-TBB/kg or fed basal diets ($p<0.05$).

EAF/cm^3 of liver in rats initiated with DEN and promoted with diets containing PB or 0.1 or 1 mg of 3,4-TBB/kg.

The mean number of EAF/cm^3 of liver for PH rats initiated with 10 mg of DEN/kg given ip or 1, 5, or 10 mg of 3,4-TBB given orally and promoted with 500 mg of PB/kg diet or fed a basal diet for 180 days is given in Table 1-3. 3,4-TBB may have initiation potential as suggested by increased numbers of hepatic EAF in rats initiated with 3,4-TBB and fed PB, compared to rats initiated with 3,4-TBB and fed a basal diet. A single oral dose of 1, 5, or 10 mg of 3,4-TBB/kg was less effective in initiating the formation of hepatic EAF than 10 mg of DEN/kg given ip. in rats promoted with PB for 180 days. The mean number of hepatic EAF/cm^3 of liver for rats initiated with 3,4-TBB or DEN and fed a basal diet for 180 days was slightly greater than zero.

Body and Organ Weights

Data for body and organ weights in rats initiated with DEN or 3,4-TBB and promoted with PB or 3,4-TBB are given in Table 1-4. There was no significant difference in body or splenic weights of rats in the various treatment groups. Rats initiated with 5 or 10 mg of 3,4-TBB and promoted with 500 mg of PB/kg diet had significantly increased liver weights compared to rats initiated with 10 mg of DEN and fed a basal diet. Rats initiated with 10 mg of 3,4-TBB/kg and promoted with 500 mg of PB/kg diet and rats initiated with 10 mg of DEN/kg and promoted with 5 mg of 3,4-TBB/kg diet had significantly decreased thymic weights compared to rats

Table 1-3. Number of EAF/cm^3 of liver in rats initiated with DEN or 3,4-TBB and promoted with PB for 180 days.

Day 0 — Tx	Day 1 — Initiator	Dose (mg/kg)	Day 31-211 — Promoter	Dose (mg/kg) of diet	Number of EAF/cm^3 of liver
PH	DEN	10	0	0	0±0
PH	DEN	10	PB	500	465±355[a]
PH	3,4-TBB	1	0	0	0.83±2.84
PH	3,4-TBB	1	PB	500	22±32[a]
PH	3,4-TBB	5	0	0	2±4.9
PH	3,4-TBB	5	PB	500	36±21[a]
PH	3,4-TBB	10	0	0	0±0
PH	3,4-TBB	10	PB	500	111±70[a]

Data are expressed as $\bar{x}$±SD for groups of 6 rats.
[a]Significantly different from rats initiated with DEN and fed a basal diet using comparison of percentages arcsin $\sqrt{}$ at $t_{infinity}$ (∞) ($p<0.05$).

Table 1-4. Body and organ weights in rats initiated with DEN or 3,4-TBB and promoted with PB or 3,4-TBB for 180 days.

Initiator	Dose (mg/kg)	Promoter	Dose (mg/kg) of diet	Absolute body weight (g)	Absolute liver weight (g)	Absolute thymic weight (g)	Absolute splenic weight (g)	Absolute thyroid gland weight (g)
DEN	10	0	0	319±34	7.70±1.11	0.386±0.027	0.508±0.092	0.039±0.015
DEN	10	PB	500	300±30	10.90±1.12	0.351±0.052	0.502±0.077	0.033±0.005
3,4-TBB	1	0	0	316±32	10.25±2.87	0.348±0.086	0.551±0.119	0.030±0.008
3,4-TBB	1	PB	500	298±15	10.15±1.58	0.393±0.089	0.530±0.099	0.029±0.002
3,4-TBB	5	0	0	344±42	9.01±1.13	0.416±0.064	0.482±0.053	0.028±0.006
3,4-TBB	5	PB	500	297±16	12.14±2.55[a]	0.326±0.064	0.581±0.071	0.029±0.006
3,4-TBB	10	0	0	310±40	9.33±2.05	0.293±0.070	0.520±0.033	0.027±0.004
3,4-TBB	10	PB	500	312±36	11.12±1.79[a]	0.276±0.104[b]	0.623±0.071	0.035±0.009
DEN	10	3,4-TBB	0.1	309±41	8.96±1.79	0.302±0.042	0.570±0.075	0.024±0.006[a]
DEN	10	3,4-TBB	1	301±26	9.38±1.27	0.312±0.031	0.506±0.045	0.023±0.004[a]
DEN	10	3,4-TBB	5	289±41	8.93±0.81	0.278±0.082[b]	0.514±0.072	0.026±0.004[a]

Data are expressed as group $\bar{x}$±SD for 6 rats.
All rats were 2/3 partially hepatectomized.
[a]Significantly different ($p<0.05$) from values for rats initiated with 10 mg of DEN/kg and fed a basal diet.
[b]Significantly different ($p<0.05$) from values for rats initiated with 5 mg of 3,4-TBB/kg and fed a basal diet.

initiated with 5 mg of 3,4-TBB and fed a basal diet. Rats initiated with 10 mg of DEN and promoted with 0.1, 1, or 5 mg of 3,4-TBB had decreased thyroid gland weights compared to rats initiated with 10 mg of DEN/kg and fed a basal diet.

There were no significant differences in body or organ weights in non-PH or noninitiated rats fed diets containing 0.1, 1, or 5 mg of 3,4-TBB/kg or 500 mg of PB/kg for 180 days.

Histologic Evaluation of Liver

PH rats initiated with DEN or 3,4-TBB and fed a diet containing PB had hepatic histologic changes characterized by multifocal areas of moderate to severe centrolobular to midzonal hepatocellular hypertrophy. The hepatocytes in these regions had abundant acidophilic cytoplasm that caused obliteration of the adjacent hepatic sinusoidal spaces as shown in Figure 1-2. Similar changes were observed in non-PH and non-initiated rats promoted with PB. Liver sections from PH rats initiated with DEN and fed diets containing 3,4-TBB had mild periportal to midzonal hepatocellular intracytoplasmic vacuolation and hypertrophy with obliteration of the adjacent hepatic sinusoidal spaces as shown in Figure 1-3. Similar changes were observed in groups of non-PH and noninitiated rats that were promoted with diets containing 3,4-TBB. The severity of the histologic lesions in rats fed diets containing 3,4-TBB appeared to be dose-dependent. There were no significant

Figure 1-2. Photomicrograph of a liver section from a rat fed a diet containing 500 mg of PB/kg for 180 days. Notice areas of centrolobular hepatocellular hypertrophy with obliteration of adjacent hepatic sinusoidal spaces (H & E stain, 108X).

Figure 1-3. Photomicrograph of a liver section from a rat fed a diet containing 5 mg of 3,4-TBB/kg for 180 days. Notice areas of periportal to midzonal hepatocellular intracytoplasmic vacuolation and obliteration of adjacent hepatic sinusoidal spaces (H & E stain, 200X).

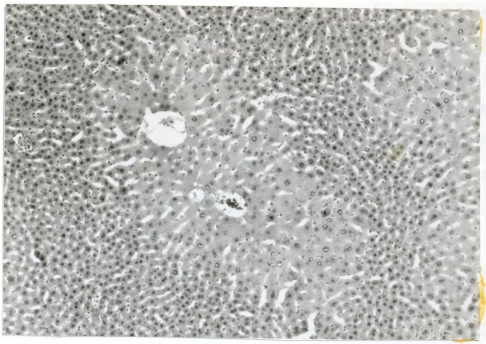

Figure 1-2

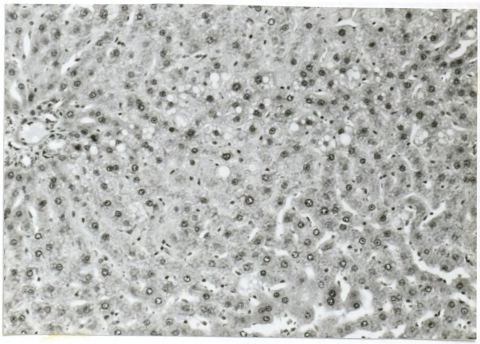

Figure 1-3

changes observed in sections of thymus, spleen and thyroid gland from rats in the various treatment groups.

The categories of preneoplastic lesions observed in liver sections taken from PH rats initiated with DEN or 3,4-TBB and promoted with diets containing 3,4-TBB or PB consisted of EAF and neoplastic nodules (Institute of Laboratory Animal Resources, National Research Council, 1980). The foci of altered cells were composed of circumscribed regions of enlarged hepatocytes containing abundant acidophilic to pale cytoplasm (Figure 1-4). These cells had single to multiple vesicular nuclei containing multiple prominent nucleoli (Figure 1-5). These EAF were positive for GGT (Figure 1-6). The neoplastic nodules consisted of large circumscribed regions occupying 2 or more hepatic lobules. The nodules were composed of enlarged hepatocytes having abundant acidophilic cytoplasm and enlarged nuclei containing single or multiple prominent nucleoli. There was a sharp demarcation of the periphery of nodules from the surrounding liver tissue (Figure 1-7). There were no preneoplastic changes observed in non-PH or noninitiated rats fed diets containing 3,4-TBB. Rats initiated with DEN or 3,4-TBB and fed a basal diet had no apparent histologic or preneoplastic hepatic lesions (Figure 1-8). There were no hepatocellular carcinomas in any of the livers from rats in the initiation or promotion assay.

Figure 1-4. Photomicrograph of an EAF within a liver section from a rat initiated with 5 mg of 3,4-TBB/kg given orally and promoted with 500 mg of PB/kg for 180 days. Notice enlarged hepatocytes with abundant amounts of pale cytoplasm (H & E stain, 200X).

Figure 1-5. Photomicrograph of an EAF within a liver section from a rat initiated with 10 mg of DEN/kg given ip and promoted with 5 mg of 3,4-TBB/kg for 180 days. Notice enlarged hepatocytes with single to multiple vesicular nuclei containing multiple prominent nucleoli (H & E stain, 180X).

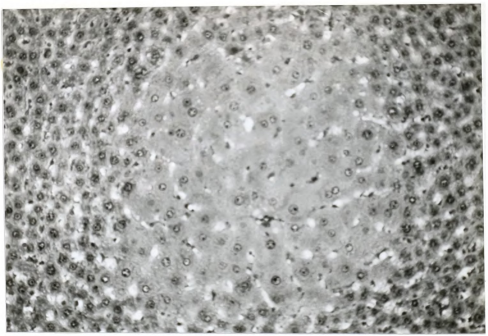

Figure 1-4

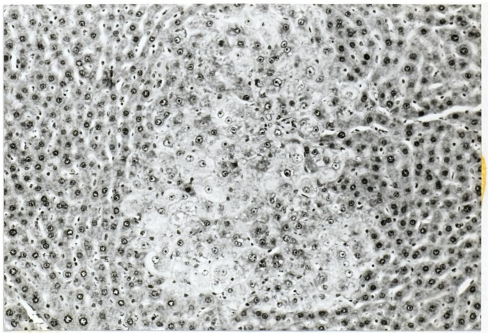

Figure 1-5

Figure 1-6. Photomicrograph of a histochemically-stained EAF within a liver section from a rat initiated with 10 mg of DEN/kg given ip and promoted with 500 mg of PB/kg for 30 days (Gamma-glutamyl transpeptidase stain, 170X).

Figure 1-7. Photomicrograph of a neoplastic nodule within a liver section from a rat initiated with 10 mg of DEN/kg given ip and promoted with 500 mg of PB/kg for 180 days. Notice sharp demarcation of periphery of nodule from the surrounding liver tissue (H & E stain, 76X).

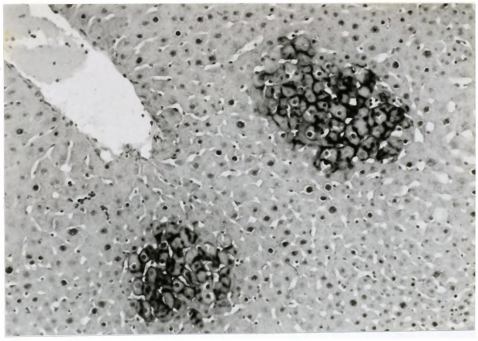

Figure 1-6

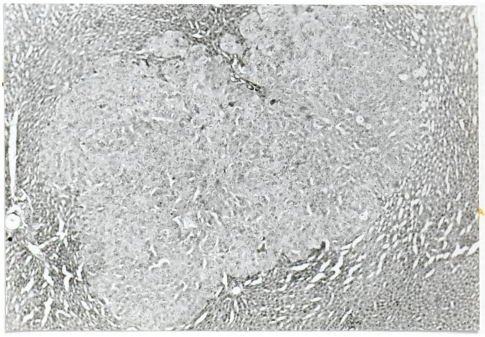

Figure 1-7

Figure 1-8. Photomicrograph of a liver section from a rat initiated with 10 mg of DEN/kg given ip and fed a basal diet for 180 days. Notice normal appearing hepatocytes (H & E stain, 210X).

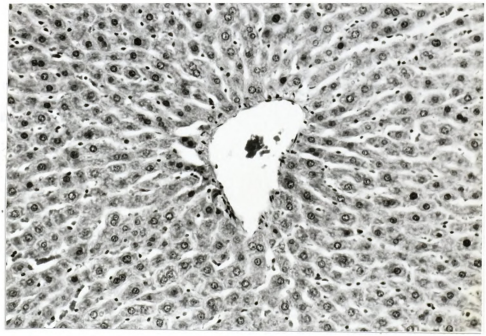

Figure 1-8

Ultrastructural Evaluation of Liver

Hepatocytes from non-PH and noninitiated rats given 5 mg of 3,4-TBB/kg for 180 days had abundant amounts of smooth endoplasmic reticulum (SER) and mild intracytoplasmic vacuolation. There was no evidence of cytotoxicity (Figures 1-9 and 1-10).

Chemical Analysis

Concentrations of 3,4-TBB in liver and adipose tissue are given in Table 1-5. Results indicate that 3,4-TBB, when administered to rats in the diet for 180 days, does not persist in body tissues and is readily metabolized and excreted from the body.

Figure 1-9. Electron micrograph of a hepatocyte from a nonpartially hepatectomized, noninitiated rat fed a diet containing 5 mg of 3,4-TBB/kg for 180 days. Notice the abundant amounts of smooth endoplasmic reticulum (arrow) and mild intracytoplasmic vacuolation and lack of cytotoxic changes (Uranyl acetate-lead citrate stain, 6400X).

Figure 1-10. Higher magnification of electronmicrograph of hepatocyte in Figure 1-10. Notice abundant amounts of smooth endoplasmic reticulum (arrow) (Uranyl acetate-lead citrate stain, 12,800X).

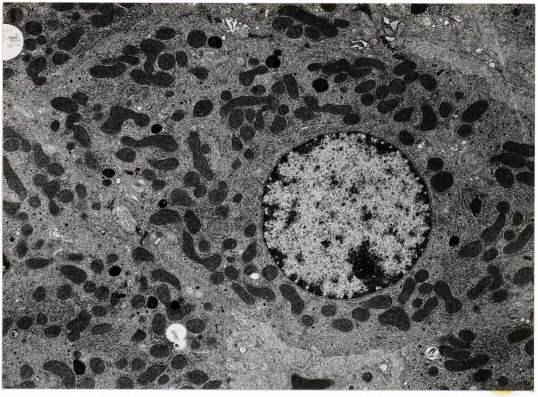

Figure 1-9

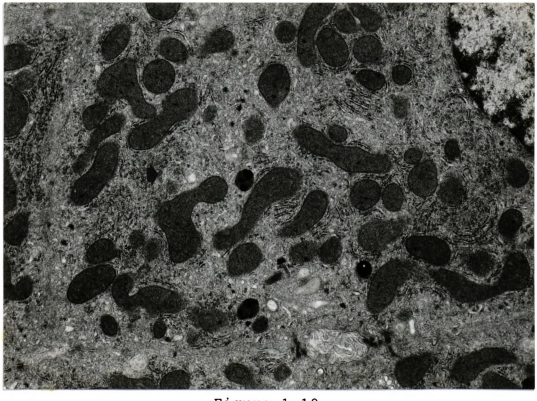

Figure 1-10

Table 1-5. Liver and adipose tissue concentration of 3,4-TBB.

Initiation	Dose (mg/kg)	Promoter	Dose (mg/kg) of diet	No. of tissue samples	Tissue concentration of 3,4-TBB (mg/kg)[a]	
					Liver	Adipose tissue
DEN	10	0	0	3	0	0.006±0.005 (84.9±1.8)
DEN	10	PB	500	3	0.0000±0 (4.3±0.20)	0.012±0.008 (81.7±1.7)
3,4-TBB	10	PB	500	3	0.003±0.003 (3.9±0.15)	0.010±0.006 (82.3±2.1)
DEN	10	3,4-TBB	0.1	3	0	0.023±0.022 (76.3±3.9)
0	0	3,4-TBB	0.1	2	0.003±0.004 (3.2±0.56)	0.012±0 (86.0±2.1)
DEN	10	3,4-TBB	1	3	0.004±0.002 (3.7±0.56)	0.033±0.010 (81.7±1.6)
0	0	3,4-TBB	1	2	0.003±0 (4.8±0)	0.065±0.39 (79.1±4.8)
DEN	10	3,4-TBB	5	3	0.017±0.016 (4.2±1.00)	0.163±0.044 (75.9±2.7)
0	0	3,4-TBB	5	2	0.016±0.008 (3.9±0.99)	0.045±0.008 (86.0±0.6)

Percent lipid is given in parentheses.

[a]Data are expressed on a percent lipid basis and represent $\bar{x}$±SD for 2 or 3 rats.

Discussion

The results of this study indicate that 0.1, 1, or 5 mg of 3,4-TBB/kg when given in the diet for 180 days, increases the number of GGT-positive EAF and at these doses appears to act as a promoter in experimental hepatocarcinogenesis in rats. Enhancement of hepatic EAF formation after initiation with a subcarcinogenic dose of an initiator is a known property of tumor promoters (Peraino et al., 1971, 1981; Pitot, 1979; Watanabe and Williams, 1978). The mechanism whereby 3,4-TBB promotes hepatocarcinogenesis in rats is unknown, but it appears to be more effective in enhancing the number of hepatic EAF than PB, a known tumor promoter, at a dose 100 times less than that of PB. It is suggested that congeners of PBB may act as promoters in hepatocarcinogenesis by inhibiting cell to cell communication when given at noncytotoxic doses (Trosko et al., 1981; Tsushimoto et al., 1982), or by chronic or recurrent toxicity resulting in necrosis, cellular regeneration and subsequent neoplasia (Berenblum, 1944) when given in cytotoxic doses (Jensen et al., 1983b).

3,4-TBB did not cause hepatocellular necrosis and regeneration in non-PH and noninitiated rats given 0.1, 1 or 5 mg/kg in the diet for 180 days, therefore it can be concluded that 3,4-TBB probably does not promote by a cytotoxic mechanism. To date, there is no conclusive evidence to support the hypothesis that 3,4-TBB can inhibit cell to cell communication in vitro at noncytotoxic doses.

Speculatively, 3,4-TBB may promote tumorogenesis through gene activation (Boutwell, 1974). It is suggested that treatment with an initiator results in formation of permanent and heritable unexpressed alteration in the cell genome, and if promoters regulate nuclear gene transcription, then treatment with a promoter would result in increased synthesis of RNA and protein and these may in part come from regions of the genome not normally expressed (derepression). Gene activation (Boutwell, 1974) is, as is toxicity of PHAH, thought to be regulated by a receptor (Poland et al., 1976). The theory of gene activation in part is invoked in the oncogene theory of carcinogenesis (Cooper and Lane, 1984) and may be a credible theory of promotion by 3,4-TBB and other congeners of PBB. Increased expression of hepatic GGT in the adult rat liver is thought to occur by derepression of a gene involved in the GGT synthesis in the fetal liver that is normally repressed in the adult state (Fiala and Fiala, 1970). Congeners of PBB have been shown to enhance the expression of this fetal enzyme in adult rat liver after initiation with DEN.

The results of this study also indicate that 3,4-TBB may have weak initiation potential as suggested by increased numbers of GGT-positive hepatic EAF in rats initiated with 3,4-TBB and fed PB, compared to rats initiated with 3,4-TBB and fed a basal diet. However, a single oral dose of 1, 5, or 10 mg of 3,4-TBB was less effective in initiation of the formation of hepatic EAF than 10 mg of DEN given ip. It is

suggested that metabolizable congeners of PBB such as 3,4-TBB or 4-bromobiphenyl are metabolized by the hepatic microsomal monooxygenase system to a toxic intermediate (i.e. epoxide) that may electrophilically bind to cytosolic macromolecules or structural components of DNA (Kohli et al., 1978). The latter event is thought to be one mechanism of tumor initiation by chemical carcinogens (Conney and Burns, 1972; Hemminki, 1983; Miller and Miller, 1977).

There have been conflicting reports as to the mutagenic effects of metabolizable congeners of PBB. The congener 3,4-TBB has been reported to be nonmutagenic in Chinese hamster V79 cells (Kavanagh et al., 1985), whereas 4-bromobiphenyl has been reported to be highly mutagenic in the Ames Assay (Kohli et al., 1978). Further in vitro and in vivo studies are needed to assess the tumor initiating potential and mechanism(s) of tumor promotion by 3,4-TBB.

Dietary administration of 1 mg 3,4,5-HBB/kg has been reported to be hepatotoxic in rats (Jensen et al., 1983b), whereas in this study chronic dietary administration of as much as 5 mg of 3,4-TBB appeared to be relatively nontoxic in rats as evidenced by histologic changes in the liver, spleen, thymus and thyroid gland and ultrastructural changes in the liver. It is postulated that enzyme induction is an early event and that toxicity occurs later as a result of persistent receptor occupation and gene expression (Poland and Knutson, 1982). 3,4-TBB is a metabolizable congener and the metabolism of congeners such as 3,4-TBB decreases the

toxicologic effects of these compounds in mammalian systems (Mills et al., 1985). The decreased toxicologic effects of metabolizable congeners is thought to be due to their inability to exist in high intracellular concentrations to allow persistent occupation of the TCDD receptor (Millis et al., 1985a). Nonmetabolizable congeners, such as 3,4,5-HBB, are not readily excreted from the tissues of rats, and if toxicity is mediated through persistent receptor binding with subsequent gene expression, then 3,4,5-HBB, a persistent congener, would be predicted to be more toxic than the metabolizable congener 3,4-TBB when administered to rats at lower or equimolar doses even though 3,4-TBB is a better ligand for the TCDD receptor.

Liver and adipose tissue from rats fed diets containing 0.1, 1 or 5 mg of 3,4-TBB did not have appreciable concentrations of this compound at the end of 180 days. This indicates that chemical analysis of tissue is not a good indicator of levels of exposure to metabolizable congeners of PBB.

Chronic administration of low dietary levels of 3,4-TBB to non-PH and noninitiated rats did not cause a significant increase in liver weights or decrease in thymic or splenic weights as observed in rats given a single oral dose (21.3 μmol/kg) (Millis et al., 1985a) or a single ip injection (150 μmol/kg) (Robertson et al., 1982) of 3,4-TBB. These authors gave rats single exposures to relatively high doses of this compound, whereas in this study, 3,4-TBB was given

at lower doses over a longer period of time. 3,4-TBB induces its own metabolism and during chronic dietary administration, intracytoplasmic concentrations may never be high enough at one time period to evoke a toxic response. It is suggested that chronic administration of relatively high doses of 3,4-TBB would increase intracytoplasmic concentrations of this congener and produce toxic changes analogous to those seen after acute administration.

Summary

Pitot's bioassay for hepatocarcinogenesis was used to determine if 3,4,3',4'-tetrabromobiphenyl (3,4-TBB) can promote or initiate the development of gamma-glutamyl transpeptidase (GGT) positive enzyme-altered foci (EAF). 3,4-TBB is a minor component of Firemaster BP-6. It is metabolized, binds to the TCDD receptor, and induces AHH activity. Groups of 6 female, 180-200 g rats were used for initiation and promotion assays. To test for initiation, rats were partially hepatectomized (PH) and given 3,4-TBB or diethylnitrosamine (DEN) as an initiator. Thirty days later, rats were fed 500 ppm of phenobarbital (PB) as a promoter for 180 days. The mean number of EAF/cm^3 of liver was: DEN (10 mg/kg), 465; 3,4-TBB: (1 mg/kg), 22; (5 mg/kg), 36; (10 mg/kg), 111. The mean number of EAF/cm^3 of liver for PH rats initiated with 3,4-TBB or DEN and fed a basal diet for 180 days was slightly greater than zero. To test for promotion, PH rats were initiated with 10 mg of

DEN/kg and 30 days later fed 3,4-TBB or PB for 180 days. The mean number of EAF/cm^3 of liver was: PB (500 ppm), 465; 3,4-TBB: (0.1 ppm), 131; (1 ppm), 217; (5 ppm), 1488. 3,4-TBB fed continually in the diet increased the number of EAF and thus appears to act as a hepatic tumor promoter. Also, 3,4-TBB may have initiating potential as suggested by increased numbers of EAF in rats initiated with 3,4-TBB and promoted by PB.

Non-PH and noninitiated female Sprague-Dawley rats weighing 180-200 g were used as controls to assess the histologic and ultrastructural tissue changes associated with chronic administration of 3,4-TBB. Dietary administration of 0.1, 1 or 5 mg of 3,4-TBB/kg does not appear to be severely toxic in rats as evidenced by light and electron microscopic changes and alterations in organ and body weights. 3,4-TBB is metabolized and does not accumulate in the liver and adipose tissue of rats.

CHAPTER II

CHRONIC DIETARY ADMINISTRATION OF 3,4,3',4'-TETRABROMOBIPHENYL (3,4-TBB) TO RATS: EFFECTS ON SERUM AND HEPATIC VITAMIN A HOMEOSTASIS AND SERUM TRIIODOTHYRONINE (T_3) AND THYROXINE (T_4) CONCENTRATIONS

CHAPTER II

CHRONIC DIETARY ADMINISTRATION OF 3,4,3',4'-TETRABROMOBIPHENYL (3,4-TBB) TO RATS: EFFECTS ON SERUM AND HEPATIC VITAMIN A HOMEOSTASIS AND SERUM TRIIODOTHYRONINE (T_3) AND THYROXINE (T_4) CONCENTRATIONS

Introduction

Polybrominated biphenyls (PBB) are compounds classified as polyhalogenated aromatic hydrocarbons (PHAH) and are components of Firemaster (FM) BP-6, the commercial mixture of PBB that contaminated much of Michigan's livestock and residents in 1973 (Carter, 1976). Also belonging to the PHAH group are the polychlorinated dibenzo-p-dioxins, classically 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polychlorinated biphenyls (PCB) and polychlorinated dibenzofurans (TCDF). These chemicals, along with PBB, are all environmental contaminants and have been shown to produce a similar toxic syndrome and biochemical responses in mammalian species (Poland and Knutson, 1982). The toxicity is characterized by a wasting syndrome, lymphoid involution, hepatotoxicity, chloracne, and gastric lesions. Some of the biochemical responses that are affected by PHAH administration include hepatic vitamin A levels, serum T_3 and T_4 concentrations and lipid metabolism (Poland and Knutson, 1982). FM and 3,4,5,3',4',5'-HBB (3,4,5-HBB) have

been found to affect hepatic and serum vitamin A homeostasis. Alterations in vitamin A status were thought to be related to 3-methylcholanthrene (3-MC) type microsomal enzyme induction (Jensen, 1983). 3,4-TBB is also a 3-MC type microsomal enzyme inducer and if there is a correlation between this type of enzyme and vitamin A status, then 3,4-TBB would be predicted to cause alterations in serum or hepatic vitamin A concentrations. 3,4,5-HBB has also been found to alter serum T_4 concentrations in rats (Akoso et al, 1982b).

The objectives of this study were to assess the effects of chronic dietary administration of 3,4-TBB on serum and hepatic vitamin A homeostasis and serum T_3 and T_4 concentrations in rats.

Materials and Methods

Experimental Design

Rats were placed in treatment groups as shown in Table 2-1. Rats had free access to 0.1, 1, or 5 mg of 3,4-TBB/kg or 500 mg of PB/kg administered in the diet for 180 days.

Chemical

3,4-TBB was synthesized and purified by recrystallization and alumina chromatography (Millis, 1984).

Rats

Female Sprague-Dawley rats weighing 180-200 g were obtained from Charles River Laboratories, Inc., Portage, MI.

Table 2-1. Experimental design for dietary administration of 3,4,3',4'-tetrabromobiphenyl (3,4-TBB) for 180 days.

Group	Tx	Concentration (mg/kg) in the diet	Number of rats
1	3,4-TBB	0.1	3
2	3,4-TBB	1	3
3	3,4-TBB	5	3
4	PB[a]	500	3

[a]Phenobarbital.

Rats were randomized and housed according to groups in clear polypropylene cages, 3 rats per cage. All rats were acclimated for 5 days. The room temperature was maintained at 22° C with a 12 hour light/dark cycle.

Diet Preparation

The diets were prepared by adding appropriate amounts of 3,4-TBB or PB dissolved in corn oil to a ground commercial feed (Certified Rodent Chow #5002®, Ralston Purina Co., St. Louis, MO). Rats were fed the diets for 180 days before necropsy.

Collection of Serum Samples for Vitamin A and Thyroid Hormone Analysis

Approximately 5 ml of blood was obtained by intracardiac puncture from anesthetized rats. The blood samples were collected in Vacutainer®, nonadditive, disposable tubes (165 x 16 mm) (Vacutainer Systems, Rutherford, NJ), placed in a test tube rack that was stored in reduced light and allowed to clot. The samples were centrifuged and approximately 2 ml of serum was removed from each tube and frozen at -20° C until vitamin A or thyroid hormone determinations were performed.

Extraction of Serum Samples for Vitamin A Analysis

Plasma extractions for vitamin A analysis were done using the method of Bieri et al. (1979). Serum samples were thawed at room temperature (approximately 25° C) and were

extracted as follows: 1) 200 µl of serum and 12 µl of 13-cis-ethylretinamide (CER) (5.07 ng/µl) standard (gift from Dr. Y.F. Shealy, Southern Research Institute, Birmingham, AL) were placed into a glass disposable tube (10 x 75 mm) and nitrogenized. 2) The contents of the tube were mixed using a Vortex-Genie^{TM} (Scientific Industries, Inc., Bohemia, NY) for 5 seconds and was set on ice for at least 5 minutes. 3) Five hundred µl of ethanol (plus 10 mg/500 ml of butylated hydroxy toluene [BHT]) was added to this mixture and the contents of the tube were nitrogenized and vortexed for 5 seconds. 4) 1 ml of hexane was added to the tube and this mixture was nitrogenized and vortexed for exactly 1 minute using several start and stop motions. 5) The contents of the tube were centrifuged for 5 minutes in a refrigerated centrifuge and by using a pasteur pipet the hexane phase was removed and placed into a conical bottom tube. 6) The hexane phase was dried with nitrogen. Steps 4-6 were repeated once.

Prior to sample analysis, the hexane phase was reconstituted with 200 µl of acetylnitrile and 100 µl of extracted serum was directly injected onto a high performance liquid chromatograph.

Collection and Extraction of Liver Samples for Vitamin A Analysis

Approximately 2 g of liver was collected from rats at necropsy, placed in aluminum foil and frozen at -20^{o} until liver extractions for vitamin A analysis were performed.

Liver extractions were done using a modified method of Olson (1979). Samples of 100 mg of liver and 500 mg of anhydrous sodium sulfate were weighed using an electronic analytical balance (Mettler AE 160, Mettler Instrument Corporation, Highstown, NJ), ground together (mortar and pestle), and packed into the bottom of a 5 ml flat-bottomed scintillation vial. Next, 6 µl of 13-CER (0.507 µg/µl) standard was injected into the contents of the vial followed by the addition of 1 ml of chloroform. The contents of the vial were nitrogenized and stored in a -20° C freezer for 8 to 12 hours. At the end of this time, 4 ml of methanol was mixed into the frozen contents of the vial and this mixture was centrifuged. Either 50 µl or 100 µl of extracted liver sample was directly injected onto a high performance liquid chromatograph.

Vitamin A Analysis of Liver or Serum Samples

Serum or liver vitamin A concentrations were determined by using high performance liquid chromatography. A Waters 590 Programmable Solvent Delivery Module (Waters Chromatography Division, Milford, MA) with a flow rate of 2 ml/minute and a Waters 440 absorbance detector utilizing a 340 nm wavelength filter was used for vitamin A analysis of liver and serum extracts. The column was a reverse phase Partisil 10 ODS-2 (Whatman, Inc., Clifton, NJ).

The chromatographic method of Cullum and Zile (unpublished) using a solvent switching system was used as follows: 88% methanol and 12% water for 13 minutes (retinol

phase); 93% methanol and 7% water for 5 minutes (retinyl acetate phase); and 85% methanol and 15% chloroform for 12 minutes (retinyl esters phase). Chromatographed peaks were integrated using a Hewlett Packard 339A integrator (Hewlett Packard Co., Avondale, PA).

Serum T_3 and T_4 Analysis

Serum T_3, T_4, free (F)T_3 and FT_4 were determined by solid phase radioimmunoassay methods (Beckers et al., 1973; Yoshida et al., 1980). Serum thyroid hormone extractions and analysis were done in the endocrinology laboratory of the Animal Health Diagnostic Laboratory (AHDL), Michigan State University, using a solid phase radioimmunoassay kit (Immunodiagnostics, Becton Dickinson and Company, Orangeburg, NY).

Statistical Analysis of Data

Data were statistically analyzed by the one-way analysis of variance (ANOVA) and the Student-Newman-Keul's multiple comparison test at $p<0.05$ (Steel and Torrie, 1980).

Results

Liver Vitamin A

Results of analysis for liver retinol (ROH) and liver retinyl esters (RE) are given in Table 2-2. Rats fed 5 mg of 3,4-TBB/kg had significantly decreased concentrations of liver RE compared to rats fed 0.1 or 1.0 mg of 3,4-TBB/kg or 500 mg of PB/kg diet. There was no significant difference

Table 2-2. Liver and serum retinol (ROH) and liver retinyl esters (RE) concentrations in rats fed 3,4-TBB for 180 days.

Treatment group and concentration of test chemical in feed	Liver ROH (μg/liver)	Liver RE (μg/liver)	Serum ROH (ng/ml)
3,4-TBB (0.1 mg/kg)	741.0±172.8	10708.3±988.2	262.7±10.9
3,4-TBB (1 mg/kg)	353.5±79.7	7764.7±966.1	275.5±84.5
3,4-TBB (5 mg/kg)	241.5±12.5	2342.1±232.8[a]	176.0±18.5
PB (500 mg/kg)	568.8±166.8	5891.5±1167.5[b]	237.3±8.12

Data are expressed as $\bar{x}$±SD. N=3.
[a]Significantly different ($p<0.05$) from group $\bar{x}$ of rats fed 0.1 or 1 mg of 3,4-TBB/kg diet or 500 mg PB/kg diet.
[b]Significantly different ($p<0.05$) from group $\bar{x}$ of rats fed 0.1, 1 or 5 mg of 3,4-TBB/kg diet.

in liver concentrations of RE between groups of rats fed diets containing 0.1 or 1.0 mg of 3,4-TBB/kg diet. Rats fed 500 mg of PB/kg diet had significantly lower liver RE concentrations than groups of rats fed 0.1 or 1 mg of 3,4-TBB/kg diet. There was no significant difference in the liver ROH concentration in groups of rats fed the four treatment diets, however there appeared to be a dose dependent decrease in liver ROH concentrations in groups of rats fed 0.1, 1.0, or 5.0 mg of 3,4-TBB/kg diet.

Serum Vitamin A

Results of serum ROH determinations are given in Table 2-2. There was no significant difference in the serum ROH concentrations in rats fed 0.1, 1.0, or 5.0 mg of 3,4-TBB/kg diet or 500 mg of PB/kg diet.

Serum Thyroid Hormones

Results of serum T_3, T_4, FT_3 and FT_4 determinations are given in Table 2-3. Rats fed diets containing 0.1, 1.0, or 5.0 mg of 3,4-TBB/kg had significantly decreased serum concentrations of T_4 compared to rats fed 500 mg of PB/kg diet. The decrease in serum T_4 levels appeared to be a dose dependent response. Serum FT_4 levels were also significantly decreased in the rats given 3,4-TBB compared to rats fed PB. There was no significant difference in concentrations of serum T_3 or serum FT_3 in groups of rats given the four diets.

Table 2-3. Serum triiodothyronine (T_3), thyroxine (T_4), free (F)T_3 and FT_4 concentrations in rats fed 3,4-TBB for 180 days.

Treatment group and concentration of test chemical in feed	Serum T_4 (ng/ml)	Serum T_3 (ng/ml)	FT_4 (pg/ml)	FT_3 (pg/ml)
3,4-TBB (0.1 mg/kg)	27.4±2.8	1.74±0.20	15.8±0.75	3.4±0.5
3,4-TBB (1 mg/kg)	24.9±5.6	1.35±0.50	16.2±3.2	2.7±0.94
3,4-TBB (5 mg/kg)	20.7±2.6	2.04±0.20	16.7±3.0	3.9±0.41
PB (500 mg/kg)	37.7±0.4[a]	1.50±0.30	24.1±2.3[a]	3.4±0.83

Data are expressed $\bar{x}$±SD for 3 rats.
[a]Significantly different ($p<0.05$) from group $\bar{x}$ of rats fed 0.1, 1 or 5 mg of 3,4-TBB/kg diet.

Discussion

Serum T_4 and FT_4 were significantly decreased in groups of rats fed 0.1 mg, 1.0 mg or 5.0 mg of 3,4-TBB/kg, however there were no histologic lesions observed in sections of thyroid gland examined in rats from these groups. 3,4,5-HBB, a toxic, slowly or non-metabolized congener of PBB, was found to alter thyroid gland structure and function when fed to rats at 1.0 or 10.0 ppm of the diet for 30 days (Akoso et al., 1982b). 3,4,5-HBB is a 3-MC type microsomal enzyme inducer capable of increasing hepatic AHH activity and UDP-glucuronyl transferase (UDP-GT) activity (Aust et al., 1981), and has been found to decrease serum T_4 concentrations in rats (Akoso et al., 1982b). It is suggested that induction of increased UDP-GT activity could be related in part to increased glucuronidation of T_4 and increased biliary excretion of this thyroid hormone (Akoso et al., 1982a). 3,4-TBB is also a 3-MC type of microsomal enzyme inducer and has been found to significantly increase UDP-GT activity in rats (Millis, 1984). Increased glucuronidation and increased biliary excretion of T_4 could in part be a mechanism for the decreased serum thyroid hormone concentrations observed in rats fed 3,4-TBB in this study.

Hepatic concentrations of RE were significantly decreased in rats fed 5 mg of 3,4-TBB/kg as compared to rats fed diets containing 0.1 or 1.0 mg of 3,4-TBB or 500 mg of PB/kg. 3,4,5-HBB and FM BP-6 were found to cause decreased

liver concentrations of retinyl palmitate with concomitant increases in serum ROH concentrations when administered to rats in the diet for 140 days (Jensen, 1983). This effect on vitamin A homeostasis was observed only in rats treated with FM or a congener cable of 3-MC type microsomal enzyme induction. The mechanism(s) whereby this occurs and the significance of the level of vitamin A depletion associated with administration of PBB is unknown. It has been suggested that PHAH can decrease hepatic vitamin A levels as a result of increased conjugation with UDP-GT with subsequent increased biliary excretion (Thunberg et al., 1980) or by PHAH induction of microsomal enzymes capable of metabolizing vitamin A to inactive metabolites (Kato et al., 1978).

Summary

Female Sprague-Dawley rats weighing 180-200 g were fed diets containing 0.1, 1.0 or 5.0 mg of 3,4,3',4'-TBB (3,4-TBB)/kg diet for 180 days to assess the effects of this congener on serum and hepatic vitamin A homeostasis and serum thyroid hormone concentrations. 3,4-TBB is a minor component of FM BP-6, it is metabolized (in vitro and in vivo), binds to the TCDD receptor and induces increased AHH activity. 3,4-TBB has a binding affinity for the TCDD 10 times greater than that of 3,4,5,3',4',5'-HBB (3,4,5-HBB), a toxic, nonmetabolized congener of polybrominated biphenyls (PBB) not present in FM, but an oral equimolar dose of 3,4-

TBB is less toxic than 3,4,5-HBB as evidenced by histologic changes in the liver and thymus of rats. The results of this study indicate that chronic dietary administration of 3,4-TBB does alter hepatic vitamin A homeostasis and serum thyroxine (T_4) and free T_4 concentrations in rats. The significance of these alterations and the exact biochemical mechanism(s) whereby these changes occur are unknown.

CHAPTER III

ASSESSMENT OF THE EFFECTS OF 3,4,3',4'-TETRABROMOBIPHENYL (3,4-TBB) ON HEPATIC GLUTATHIONE (GSH) CONCENTRATIONS IN RATS

CHAPTER III

ASSESSMENT OF THE EFFECTS OF 3,4,3',4'-TETRABROMOBIPHENYL (3,4-TBB) ON HEPATIC GLUTATHIONE (GSH) CONCENTRATIONS IN RATS

Introduction

Congeners of polybrominated biphenyls (PBB) and chemically related polyhalogenated aromatic hydrocarbons (PHAH) produce a variety of toxic effects which include a wasting syndrome, thymic atrophy, chloracne, edema, hyperkeratosis, alterations in biochemical responses and hepatotoxicity (Poland and Knutson, 1982). The mechanism of toxicity of PHAH is unknown, although there is evidence that toxicity is caused by congeners of PHAH that are inducers of cytochrome P448 mediated microsomal enzymes, namely aryl hydrocarbon hydroxylase (AHH) (Poland and Knutson, 1982). Toxicity and P448 type of microsomal enzyme induction are thought to be coordinated through a protein receptor, known as the TCDD receptor, which is regulated by a single gene locus referred to as the Ah locus (Greenlee and Poland, 1979; Poland and Glover, 1980; Poland et al., 1976). To date, a definite cause and effect relationship between hepatotoxicity observed with the administration of PHAH and P448 type of microsomal enzyme induction has not been established and alternate mechanisms, such as lipid

peroxidation, have been proposed for the hepatotoxic changes observed in mammalian species exposed to congeners of PHAH (Albro et al., 1978; Sato et al., 1981; Sweeney et al., 1979).

The congener 3,4-TBB is a minor component tentatively identified in the commercial mixture of PBB, Firemaster (FM) BP-6 (Robertson et al., 1982). This congener is a good ligand for the TCDD receptor, induces P448 type microsomal enzymes, in particular hepatic AHH activity (Millis et al., 1985a; Mills et al., 1985), and is hepatotoxic in rats (Millis et al., 1985a; Robertson et al., 1982). This congener is also metabolized (in vitro and in vivo) (Millis et al., 1985a; Mills et al., 1985). It has been established that oxidative metabolism of many aromatic hydrocarbons occur primarily through an arene oxide mechanism with possible formation of reactive intermediates (i.e. epoxides) which may spontaneously isomerize to monohydroxylated products, react with epoxide hydrolase to yield dihydroxylated products or possibly conjugate with reduced GSH (Jerina and Daly, 1974; Preston et al., 1983; Matthews, 1981). In the event of hepatic GSH depletion, a reactive electrophile, such as an epoxide formed as an intermediate during the metabolism of a congener, would no longer be detoxified by conjugation with GSH and could extert its toxic effects by initiating lipid peroxidation, or by binding to nuclear, cytosolic or membrane macromolecules of cells (Conney and Burns, 1972; Kohli et al., 1978; Miller

and Miller, 1977). It is therefore of interest to assess whether the hepatotoxic effects of 3,4-TBB are solely mediated by the TCDD receptor, or whether other factors such as glutathione depletion also contribute to the histologic changes observed in rats.

The objectives of this study were to assess the acute effects of a single oral dose of 3,4-TBB on rat hepatic GSH concentrations and to determine if induction of cytochrome P448 type microsomal enzymes by pretreatment of rats with a single oral dose of 3,4,5,3',4',5'-HBB (3,4,5-HBB) would alter the effects of 3,4-TBB on hepatic GSH concentrations. Histologic evaluation of liver sections was done to characterize the acute changes associated with the administration of 3,4-TBB at 2, 4, 8, 24, or 48 h after dosing.

Materials and Methods

Experimental Design

Rats were divided in groups as shown in Table 3-1. Rats were given a single oral dose of corn oil, 17 mg of 3,4-TBB/kg, or 1 mg of 3,4,5-HBB/kg. A single oral dose of 3,4,5-HBB/kg was given as a control to determine the effects of this congener when given alone and to compare these effects to those observed in the combination treatment groups. A combination treatment consisted of 1 mg of 3,4,5-HBB/kg given 24 h prior to administration of 17 mg of 3,4-TBB/kg; both congeners were given in a single oral dose.

Table 3-1. Experimental design for assessment of the effects of 3,4,3',4'-tetrabromobiphenyl (3,4-TBB) on hepatic glutathione (GSH) concentrations.

Group	Tx	Dose[a] (mg/kg)	Number of rats and time of killing					No. of rats
			2 hr	4 hr	8 hr	24 hr	48 hr	
I	Corn oil	1 ml	3	3	3	3	3	15
II	3,4-TBB	17	3	3	3	3	3	15
III	3,4,5-HBB[b]	1	3	3	3	3	3	15
IV	3,4,5-HBB[b]* + 3,4-TBB	1 + 17	3	3	3	3	3	15

[a]Chemical was given orally in 1 ml of corn oil.
[b]3,4,5,3',4',5'-Hexabromobiphenyl.
*3,4,5-HBB was given 24 h before administration of 3,4-TBB.

Rats were killed at 2, 4, 8, 24 or 48 h after dosing with 3,4-TBB.

Chemicals

The congener 3,4-TBB was synthesized and purified by recrystallization and alumina chromatography (Millis, 1984). 3,4,5-HBB was purchased from RFR Corporation, Hope, RI and was purified by personnel in the Department of Biochemistry, Michigan State University. The procedure used for purification of 3,4,5-HBB consisted of repeated alumina chromatography (Aust et al., 1981).

Rats

Sixty male Sprague Dawley rats (C/D) weighing 70-90 g were obtained from Charles River Laboratories, Inc., Portage, MI. Rats were randomized and housed according to groups in clear polypropylene cages, 3 rats per cage. All rats were acclimated for 5 days. The room temperature was maintained at 22^{o} C with a 12 hr light/dark cycle.

Dose Preparation

Oral doses were prepared by adding appropriate amounts of 3,4-TBB or 3,4,5-HBB to corn oil. Rats were given 1 ml of corn oil containing 17 mg of 3,4-TBB/kg or 1 mg of 3,4,5-HBB/kg by using a 16 gauge, 3" curved feeding needle (Harvard Bioscience, South Natick, MA).

Necropsy Procedure

Rats were weighed and were killed by using dry ice (CO_2). All organs were routinely examined for gross lesions. The liver was removed from the carcass and weighed.

Preparation of Liver for Histologic Evaluation

Liver samples were fixed in 10% neutral buffered formalin. Formalin-fixed liver samples were processed (Fisher Histomatic^{TM} Tissue Processor Models 165 and 166, Fisher Scientific Co., Pittsburgh, PA), embedded in paraffin, cut by a microtome into 6 m sections and stained with hematoxylin and eosin for histologic examination.

Hepatic Glutathione Determinations

Hepatic GSH levels were measured by the enzymatic recycling assay of Griffith (1980) in which GSH is sequentially oxidized by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and reduced by NADPH in the presence of glutathione reductase.

Liver samples were placed into cryotubes and immediately frozen by submersion into isopentane cooled in liquid nitrogen. Frozen liver samples were kept in a freezer at -80^{o} C until liver glutathione assays were performed.

One gram of frozen liver sample was weighed using a top-loading balance (Mettler Instrument Corp., Highstown,

NJ), placed in a 50 ml polycarbonate centrifuge tube and set on ice.

Liver samples were homogenized (Polytron, Brinkmann Instruments, Westbury, NY) in 4 volumes of 1% (W/V) picric acid and centrifuged at 9,000 x g for 15 minutes. The 9,000 x g supernatant equivalent to 0.2 to 0.4 g wet weight liver, 0.3 mM NADPH (β-NAD phosphate, reduced form, type III) 6 mM DNTB and glutathione reductase-type III (from Baker's yeast) were combined in a 1.5 ml disposable cuvette in a total volume of 1 ml. Enzymes and substrates were purchased from Sigma Chemical Co., St. Louis, MO. The change in absorbance over 2 optical density units at 412 nm at room temperature was monitored using a spectrophotometer (Beckman Model 35, Series 30 UV-Visable, Beckman Instruments, Inc., Fullerton, CA). The glutathione content of the aliquot assayed was determined by comparison of the change in adsorbance over the linear part of the curve to the change in adsorbance measured with known amounts of glutathione on the same day.

Statistical Analysis

Data from the glutathione assays were statistically analyzed by using the factorial analysis of variance (ANOVA). Organ and body weight data were statistically analyzed by using the one-way ANOVA and the Student-Newman-Keul's multiple comparison test at $p<0.05$ (Steel and Torrie, 1980).

Results

Hepatic GSH

Results for analysis of hepatic GSH levels are given in Table 3-2. Hepatic GSH concentrations in rats treated with 3,4-TBB, 3,4,5-HBB or a combination of both congeners were not significantly different from control GSH concentrations at 2, 4, 8, 24 or 48 h after dosing.

Histopathology

Liver sections taken from rats given a single oral dose of 17 mg of 3,4-TBB/kg, 1 mg of 3,4,5-HBB/kg or a combination of both congeners and killed at 2, 4, or 8 h after dosing did not differ from controls. Liver sections from rats treated with a combination of 3,4-TBB and of 3,4,5-HBB and killed at 24 or 48 h after dosing had mild to moderate intracytoplasmic vacuolation of hepatocytes in the periportal regions. Rats treated with 3,4-TBB and killed at 24 h had similar histologic changes, however they were less severe than changes observed in rats given the combination treatment and killed at 24 or 48 hr after dosing. Histologic changes observed in liver sections from rats treated with 1 mg of 3,4,5-HBB and killed at 24 or 48 h after dosing consisted of diffuse areas of mild to moderate hepatocellular vacuolation.

Body and Liver Weights

Data for body and liver weights are given in Table 3-3. Rats given a single oral dose of 3,4,5-HBB before

Table 3-2. Hepatic GSH concentrations in rats given a single oral dose of 3,4-TBB, 3,4,5,3',4',5'-hexabromobiphenyl (3,4,5-HBB), or a combination of both congeners.

Tx	Kill time (h)	Hepatic glutathione concentration (nmol/g of liver)
Corn oil	2	2.90±0.36
3,4-TBB	2	2.93±0.25
3,4,5-HBB	2	3.30±0.17
3,4,5-HBB[a] + 3,4-TBB	2	2.97±0.45
Corn oil	4	2.13±0.80
3,4-TBB	4	2.23±0.25
3,4,5-HBB	4	2.20±0.50
3,4,5-HBB[a] + 3,4-TBB	4	2.16±0.29
Corn oil	8	1.67±0.40
3,4-TBB	8	2.00±0.26
3,4,5-HBB	8	2.33±0.50
3,4,5-HBB[a] + 3,4-TBB	8	1.97±0.40
Corn oil	24	3.13±0.83
3,4-TBB	24	2.40±0.20
3,4,5-HBB	24	2.20±0.35
3,4,5-HBB[a] + 3,4-TBB	24	2.73±1.30
Corn oil	48	2.90±0.60
3,4-TBB	48	2.63±0.83
3,4,5-HBB	48	2.27±0.60
3,4,5-HBB[a] + 3,4-TBB	48	2.57±0.29

Data are expressed as group $\bar{x}$ and SD for 3 rats.
All rats were given either a single oral dose of 1 ml of corn oil, 17 mg 3,4-TBB/kg, 1 mg of 3,4,5-HBB/kg or a combination of 1 mg of 3,4,5-HBB/kg and 17 mg 3,4-TBB/kg in 1 ml of corn oil.
[a]3,4,5-HBB was given 24 h before administration of 3,4-TBB.

Table 3-3. Body and liver weights in rats given a single oral dose of 3,4-TBB, 3,4,5-HBB, or a combination of both congeners.

Tx	Kill time (h)	Absolute body weight (g)	Absolute liver weight (g)
Corn oil	2	194.0±1.0	9.81±1.21
3,4-TBB	2	189.0±4.4	10.60±0.86
3,4,5-HBB	2	194.7±8.3	9.94±1.03
3,4,5-HBB[a] + 3,4-TBB	2	185.6±9.0	9.54±0.37
Corn oil	4	197.7±16.3	8.90±0.71
3,4-TBB	4	184.3±13.8	8.88±1.06
3,4,5-HBB	4	187.3±6.5	8.80±0.52
3,4,5-HBB[a] + 3,4-TBB	4	182.7±2.5	9.67±0.99
Corn oil	8	183.7±6.5	8.93±0.64
3,4-TBB	8	184.3±18.9	9.79±1.03
3,4,5-HBB	8	182.7±10.1	9.46±0.80
3,4,5-HBB[a] + 3,4-TBB	8	165.7±18.2	8.83±1.18
Corn oil	24	174.0±10.4	10.22±0.35
3,4-TBB	24	188.7±10.4	10.66±0.28[b]
3,4,5-HBB	24	194.0±7.0	9.71±0.34
3,4,5-HBB[a] + 3,4-TBB	24	182.7±9.0	10.85±0.48[b]
Corn oil	48	208.7±8.1	11.09±0.39
3,4-TBB	48	196.7±16.8	11.26±1.72
3,4,5-HBB	48	199.3±7.6	11.09±0.81
3,4,5-HBB[a] + 3,4-TBB	48	199.0±8.7	12.54±1.50

Data are expressed as group $\bar{x}$ and SD for 3 rats.
All rats were given either a single oral dose of 1 ml of corn oil, 17 mg 3,4-TBB/kg, 1 mg of 3,4,5-HBB/kg or a combination of 1 mg of 3,4,5-HBB/kg and 17 mg 3,4-TBB/kg in 1 ml corn oil.
[a]3,4,5-HBB was given 24 h before administration of 3,4-TBB.
[b]Significantly different ($p<0.05$) from groups of rats given 3,4,5-HBB and killed at 24 h.

administration of a single oral dose of 3,4-TBB or rats given a single oral dose of 3,4-TBB alone and killed at 24 h had significantly increased liver weights compared to rats given a single oral dose of 3,4,5-HBB alone and killed at 24 h. There were no significant differences in body weights between rats killed at 24 h. Body and liver weights did not differ significantly between rats killed at 2, 4, 8 or 48 h.

Discussion

Results from this study indicate that 3,4-TBB when administered to rats in a single oral dose (17 mg/kg) had no effect on hepatic GSH concentrations compared to controls at 2, 4, 8, 24 or 48 h after dosing. Liver sections from rats given 3,4-TBB and killed 24 h after dosing had mild intracytoplasmic vacuolation and hypertrophy of hepatocytes in the periportal regions, but at 2, 4, 8 or 48 h after dosing there were no differences in liver sections from rats given 3,4-TBB compared to controls. Therefore, depletion of hepatic GSH did not appear to be a factor in causing the histologic changes observed in rats given 3,4-TBB alone, and killed at 24 h after dosing.

Dannan *et al.* (1982c) found congeners of PBB to be good substrates for inducing hepatic microsomal enzymes. This is important in that induction of the hepatic microsomal monooxygenase system (MMS) by nonmetabolizable congeners may increase the rate of metabolism of metabolizable congeners. 3,4,5-HBB, a slowly or nonmetabolizable congener of PBB capable of P448 type microsomal enzyme induction, has been found to increase the rate of metabolism of 3,4-TBB *in vitro* (Mills *et al.*, 1985). If metabolizable congeners of PBB, such as 3,4-TBB, are indeed metabolized to epoxide intermediates by the hepatic MMS, then induction of microsomal enzymes by pretreatment of rats with a nonmetabolizable congener such as 3,4,5-HBB could potentiate the toxic effects of congeners metabolized by P448 type

microsomal enzymes. Alternately, if the parent compound is important in receptor binding and that in turn mediates toxicity (Greenland and Poland, 1979; Poland and Glover, 1980; Poland et al., 1976), then increased rates of metabolism would decrease the toxicity of metabolizable congeners. In this study, pretreatment of rats with a single oral dose (1 mg/kg) of 3,4,5-HBB 24 h before oral administration of 3,4-TBB (17 mg/kg), did not potentiate the effects of 3,4-TBB on hepatic GSH concentrations compared to controls at 2, 4, 8, 24 or 48 h after dosing.

It is suggested that enzyme induction is an early event and persistent occupation of the TCDD receptor by the ligand is necessary for gene expression and subsequent toxicity (Poland and Knutson, 1982). The congener 3,4-TBB induces its own metabolism and is rapidly removed from the liver of rats (Millis et al., 1985a; Mills et al., 1985), and if persistent ligand-receptor binding is necessary for toxicity, then single doses of 3,4-TBB should not be severely hepatotoxic. Histologic examination of liver sections from rats given 3,4-TBB alone and killed at 24 h suggest that 3,4-TBB is not extremely hepatotoxic and that the mild acute changes observed at 24 h were reversible as evidenced by lack of histologic changes in rats given 3,4-TBB and killed at 48 h compared to controls. It is suggested that metabolism of congeners such as 3,4-TBB decreases intrahepatic concentrations of the parent compound

resulting in decreased ligand-receptor binding and hence, decreased toxicity.

The congener 3,4,5-HBB is not metabolized and accumulates in the liver of rats (Jensen et al., 1982; Millis et al., 1985a; Mills et al., 1985). Again, if toxicity associated with PHAH occurs as a result of persistent ligand-receptor binding and AHH activity, then the histologic changes in livers of rats fed the combination treatment and killed at 24 h were primarily due to 3,4,5-HBB, and at 48 h can be solely attributed to the effects of 3,4,5-HBB due to rapid metabolism of 3,4-TBB by this time period. Changes observed in rats given 3,4,5-HBB and killed at 24 or 48 h after dosing were similar to those seen in the combination group at 24 or 48 h, but were less severe. Increased severity of hepatic lesions in the combination group is most likely due to the increased time of exposure to 3,4,5-HBB. Rats in this group were given 3,4,5-HBB 24 h prior to administration of 3,4-TBB, so that histologic hepatic changes observed at 24 or 48 h were representative of 48 or 72 h, respectively.

In conclusion, the results from this study indicate that hepatotoxicity associated with 3,4-TBB is not due to depletion of hepatic GSH concentrations. Similar findings have been reported for the chlorinated analog (3,4,3',4'-tetrachlorobiphenyl) of 3,4-TBB (Rifkind et al., 1984). These findings suggest that 3,4-TBB toxicity is most probably coordinated with induction of P448 type microsomal

enzymes, namely AHH which is thought to be mediated by the TCDD receptor (Poland and Glover, 1977, 1980). Results from this study also indicate that it is important to consider the manner of administration of a metabolizable PHAH, the number of observations after the compound is given, and also at what time periods the observations are made. In this study, histologic changes associated with the administration of 3,4-TBB occurred by 24 h, and by 48 h rats treated with 3,4-TBB were similar to controls.

Summary

Male Sprague-Dawley rats weighing 160-180 g were given a single oral dose of 17 mg of 3,4,3',4'-TBB (3,4-TBB)/kg, 1 mg of 3,4,5,3',4',5'-HBB (3,4,5-HBB)/kg or a combination of both congeners to assess the effect of 3,4-TBB on hepatic glutathione (GSH) concentrations and to determine if induction of cytochrome P448 type microsomal enzymes by pretreatment of rats with a single oral dose of 3,4,5-HBB would alter the effects of 3,4-TBB on hepatic GSH concentrations. Histologic evaluation of liver sections was done to characterize the acute changes associated with the administration of 3,4-TBB at 2, 4, 8, 24 or 48 h after dosing. The congener 3,4-TBB when given to rats alone or 24 h after administration of 3,4,5-HBB had no effect of hepatic GSH concentrations at 2, 4, 8, 24 or 48 h after dosing compared to controls. Histologic changes were observed only at 24 h in rats given 3,4-TBB alone and consisted of mild,

focal areas of hepatocellular hypertrophy and intracytoplasmic vacuolation. These changes were absent in rats given 3,4-TBB and killed at 48 h. In conclusion, GSH depletion does not appear to be significant in the hepatotoxicity observed with 3,4-TBB. The acute histologic changes associated with the administration of a single oral dose of 17 mg of 3,4-TBB/kg appears to be rapidly reversible.

CONCLUSIONS

The results from the research presented in this dissertation indicate that:

1) The metabolizable congener, 3,4,3',4'-tetrabromobiphenyl (3,4-TBB), when administered to rats in the diet for 180 days acts as a promoter in Pitot's model of experimental hepatocarcinogenesis as evidenced by enhancement of the number of gamma-glutamyl transpeptidase (GGT) positive enzyme altered foci (EAF) after initiation with diethylnitrosamine (DEN).

2) Dietary levels of 5 mg of 3,4-TBB/kg are more effective than 500 mg of phenobarbital (PB)/kg (standard tumor promoter) in enhancing the number of hepatic GGT positive EAF.

3) 3,4-TBB appears to have weak initiating potential in Pitot's model of experimental hepatocarcinogenesis.

4) Single oral doses of 1, 5 or 10 mg of 3,4-TBB are less effective than 10 mg of DEN (standard tumor initiator)/kg given ip in initiating the formation of hepatic GGT positive EAF.

5) Dietary administration of 0.1, 1 or 5 mg of 3,4-TBB/kg for 180 days is not severely toxic in rats as

evidenced by histologic and ultrastructural changes in the liver.

6) Chronic dietary administration of 3,4-TBB does not cause toxic changes in the thyroid gland, spleen or thymus in rats as evidenced by lack of histologic changes in these organs.

7) 3,4-TBB does not accumulate in the liver or adipose tissue of rats when administered in the diet for 180 days.

8) Dietary administration of 3,4-TBB does not cause significant changes in liver, thymic, splenic or thyroid gland weights in rats.

9) Dietary administration of 3,4-TBB/kg for 180 days causes decreased liver retinyl esters concentrations in rats, but has no effect on serum retinol concentrations.

10) Dietary administration 3,4-TBB for 180 days causes significantly decreased serum thyroxine (T_4) and free T_4 concentrations in rats.

11) A single oral dose of 17 mg of 3,4-TBB/kg does not cause alterations in hepatic reduced glutathione (GSH) concentrations in rats, and GSH depletion does not appear to play a significant role in 3,4-TBB hepatotoxicosis.

12) Pretreatment of rats with a single oral dose of 1 mg of 3,4,5,3',4',5'-hexabromobiphenyl (3,4,5-HBB)/kg 24 h before a single oral dose of 17 mg of 3,4-TBB/kg does not potentiate the effects of 3,4-TBB on hepatic GSH concentrations in rats.

13) 3,4-TBB causes mild hepatic lesions that appear to be rapidly reversible when administered to rats in a single oral dose.

LIST OF REFERENCES

LIST OF REFERENCES

Akoso, B.T., Sleight, S.D., Aust, S.D., and Stowe, H.D. (1982a). Pathologic effects of purified polybrominated biphenyl congeners in rats. J. Am. Coll. Toxicol. 1, 1-21.

Akoso, B.T., Sleight, S.D., Nachreiner, R.F., and Aust, S.D. (1982b). Effects of purified polybrominated biphenyl congeners on the thyroid and pituitary glands in rats. J. Am. Coll. Toxicol. 1, 23-36.

Albert, Z., Orlowski, M., and Szewczuk, A. (1961). Histochemical demonstration of gamma-glutamyl transpeptidase. Nature 191, 767-768.

Allen, J.R., Lambrecht, L.K., Barsotti, D.A. (1978). Effects of polybrominated biphenyls in non-human primates. J. Am. Vet. Med. Assoc. 173, 1485-1489.

Alvares, A.P., Schilling, G., Levin, W., and Kuntzman, R. (1967). Studies on the induction of CO-binding pigments in liver microsomes by phenobarbital and 3-methylcholanthrene. Biochem. Biophys. Res. Commun. 29, 521-526.

Andres, J., Lambert, L., Robertson, L., Bandiera, S., Sawyer, T., Lovering, S., and Safe, S. (1983). The comparative biologic and toxic potencies of polychlorinated and polybrominated biphenyls. Toxicol. Appl. Pharmacol. 70, 204-215.

Aust, S.D., Dannan, G.A., Sleight, S.D., Fraker, P.J., Ringer, R.K., and Polin, D. (1981). Toxicology of polybrominated biphenyls. In Toxicity of Halogenated Hydrocarbons: Health and Ecological Effects (M.A.Q. Khan, R.H. Stanton, eds.). Pergamon Press, New York.

Baars, A.J., Jansen, M., and Breimer, D.D. (1978). The influence of phenobarbital, 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin on glutathione S-transferase activity of rat liver cytosol. Biochem. Pharmacol. 27, 2487-2494.

Baic, D., and Baic, B. (1984). A fast method for processing biopsy material for electron microscopy. Ultrastructural Pathol. 6, 347-349.

Beckers, C., Corrette, C., and Thalasso, M. (1973). Evaluation of serum thyroxine by radioimmunoassay. J. Nucl. Med. 14, 317-320.

Bekesi, J.G., Holland, J.F., Anderson, H.A., Fischbein, A.S., Rona, W., Wolff, M.S., and Selikoff, I.J. (1978). Lymphocytic function of Michigan farmers exposed to polybrominated biphenyls. Science 199, 1208-1209.

Berenblum, I. (1944). Irritation and carcinogenesis. Arch. Pathol. 38, 233-244.

Berenblum, I., and Shubik, P. (1947). A new quantitative approach to the study of the stages of chemical carcinogenesis in mouse skin. Br. J. Cancer 1, 383-390.

Besaw, L.C., Moore, R.W., Dannan, G.A., and Aust, S.D. (1978). Effect of 2,2',3,3',4,4',5,5'-octabromobiphenyl on microsomal drug metabolism enzymes. Pharmacologist 20, 251.

Bieri, J.G., Tolliver, T.J., and Catiynani, G.L. (1979). Simultaneous determination of α-tocopherol and retinol in plasma or red cells by high pressure liquid chromatography. Am. J. Clin. Nutri. 32, 2143-2149.

Bishop, J.M. (1982). Oncogenes. Scientific American 246, 80-90.

Bock, K.W., Fröhling, W., Hemmer, H., and Rexer, B. (1973). Effects of phenobarbital and 3-methylcholanthrene on substrate specificity of rat liver microsomal UDP-glucuronyltransferase. Biochim. Biophys. Acta 327, 46-56.

Booth, J., Boyland, E., and Sims, P. (1961). An enzyme from rat liver catalyzing conjugations with glutathione. Biochem. J. 79, 516-523.

Boutwell, R.K. (1974). The function and mechanism of promoters of carcinogenesis. CRC Crit. Rev. Toxicol. 2, 419-443.

Bresnick, E., Mukhtar, H., Stoming, T.A., Dansette, P.M., and Jerina, D.M. (1977). Effect of phenobarbital and 3-methylcholanthrene administration on epoxide hydrase levels in liver microsomes. Biochem. Pharmacol. 26, 891-892.

Brinkman, U.A.Th., and deKok, A. (1980). Production, properties, and usage. In Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins, and Related Products (R.D. Kimbrough, ed.). Pp. 1-40, Elsevier/North Holland, New York.

Carter, L.J. (1976). Michigan's PBB incident: Chemical mix-up leads to disaster. Science 192, 240-243.

Cohen, G., and Hochstein, P. (1963). Glutathione peroxidase: The primary agent for the elimination of hydrogen peroxide in erythrocytes. Biochem. 2, 1420-1428.

Conney, A.H. (1967). Pharmacological implications of microsomal enzyme induction. Pharmacol. Rev. 19, 317-366.

Conney, A.H., and Burns, J.J. (1972). Metabolic interactions among environmental chemicals and drugs. Science 178, 576-586.

Cook, R.M., Prewitt, L.R., and Fries, G.F. (1978). Effects of activated carbon, phenobarbital, and vitamin A, D and E on polybrominated biphenyl excretion in cows. J. Dairy Sci. 61, 414-419.

Cooper, D.Y., Levin, S., Narashimhulu, S., Rosenthal, O., and Estabrook, R.W. (1965). Photochemical action spectrum of the terminal oxidase of mixed-function oxidase systems. Science 147, 400-402.

Cooper, G.M., and Lane, M.-A. (1984). Cellular transforming genes and oncogenesis. Biochim. Biophys. Acta 738, 9-20.

Culliton, B.J. (1977). Widespread PBB contamination can affect immune system. Science 197, 849.

Dannan, G.A., Guengerich, F.P., Kaminsky, L.S., and Aust, S.D. (1983). Regulation of cytochrome P-450 immunological quantitation of eight isozymes in liver microsomes of rats treated with polybrominated biphenyl congeners. J. Biol. Chem. 258, 1282-1288.

Dannan, G.A., Mileski, G.J., and Aust, S.D. (1982a). Reconstitution of some biochemical and toxicological effects of commercial mixtures of polybrominated biphenyls. Fund. Appl. Toxicol. 2, 322-326.

Dannan, G.A., Mileski, G.J., and Aust, S.D. (1982b). Purification of polybrominated biphenyl congeners. J. Toxicol. Environ. Health 9, 423-438.

Dannan, G.A., Moore, R.W., and Aust, S.D. (1978a). Studies on the microsomal metabolism and binding of polybrominated biphenyls (PBB's). Environ. Health Perspect. 37, 179-182.

Dannan, G.A., Moore, R.W., Besaw, L.C., and Aust, S.D. (1978b). 2,4,5,3',4',5'-Hexabromobiphenyl is both a 3-methylcholanthrene- and a phenobarbital-type inducer of microsomal drug metabolizing enzymes. Biochem. Biophys. Res. Comm. 85, 450-458.

Dannan, G.A., Sleight, S.D., and Aust, S.D. (1982c). Toxicity and microsomal enzyme induction effects of several polybrominated biphenyls of Firemaster. Fund. Appl. Toxicol. 2, 313-321.

Dannan, G.A., Sleight, S.D., Fraker, P.J., Krehbiel, J.D., and Aust, S.D. (1982d). Liver microsomal enzyme induction and toxicity studies with 2,4,5,3',4'-pentabromobiphenyl. Toxicol. Appl. Pharmacol. 64, 187-203.

Dent, J.G., Graichen, M.E., Schnell, S., and Lasker, J. (1980). Constitutive and induced hepatic microsomal cytochrome P-450 monooxygenase activities in male Fischer-344 and CD rats. A comparative study. Toxicol. Appl. Pharmacol. 52, 45-53.

Dent, J.G., Netter, K.J., and Gibson, J.E. (1976a). Effect of chronic administration of polybrominated biphenyls on parameters associated with hepatic drug metabolism. Res. Commun. Chem. Pathol. Pharmacol. 13, 75-82.

Dent, J.G., Netter, K.J., and Gibson, J.E. (1976b). The induction of hepatic microsomal metabolism in rats following acute administration of a mixture of polybrominated biphenyls. Toxicol. Appl. Pharmacol. 38, 237-249.

Dunckel, A.E. (1975). An updating on the polybrominated biphenyl disaster in Michigan. J. Am. Vet. Med. Assoc. 167, 838-841.

Farber, E.. (1981). Chemical carcinogenesis. New Engl. J. Med. 305, 1379-1389.

Farber, T., Kasza, L., Giovetti, A., Carter, C., Earl, F.L., and Balazs, T. (1978). Effect of polybrominated biphenyls (Firemaster BP-6) on the immunologic system of the beagle dog. Toxicol. Appl. Pharmacol. 45, 343.

Fiala, S., and Fiala, A.E. (1970). Aquisition of an embryonal biochemical feature by rat hepatomas. Experientia 26, 889-890.

Fiala, S., and Fiala, E.S. (1973). Activation by chemical carcinogens of gamma-glutamyl transpeptidase in rat and mouse liver. J. Natl. Cancer Inst. 51, 151-158.

Fraker, P.J. (1980). Antibody mediated and delayed type hypersensitivity responses in mice to polybrominated biphenyls. Toxicol. Appl. Pharmacol. 43, 1-7.

Fries, G.F. (1978). Distribution and kinetics of polybrominated biphenyls and selected chlorinated hydrocarbons in farm animals. J. Am. Vet. Med. Assoc. 173, 1479-1484.

Garfinkel, D. (1957). Isolation and properties of cytochrome b_5 from pig liver. Arch. Biochem. Biophys. 71, 111-120.

Goldbarg, J.A., Friedman, O.M., Pineda, E.P., Smith, E.E., Chatterji, T., Stein, E.H., and Rutenberg, A.M. (1960). The colorimetric determination of gamma-glutamyl transpeptidase with a synthetic substrate. Arch. Biochem. Biophys. 91, 61-70.

Greenlee, W.F., and Poland, A. (1979). Nuclear uptake of 2,3,7,8-tetrachlorodibenzo-p-dioxin by hepatic cytosol. J. Biol. Chem. 254, 9814-9821.

Griffith, O.W. (1980). Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal. Biochem. 106, 207-212.

Guengerich, F.P. (1977). Separation and purification of multiple forms of microsomal cytochrome P-450. J. Biol. Chem. 252, 3970-3979.

Guengerich, F.P., Wang, P., Mason, P.S., and Mitchell, M.B. (1981). Immunological comparison of rat, rabbit and human microsomal cytochrome P-450. Biochem. 20, 2370-2378.

Gupta, B.N., McConnell, E.E., Goldstein, J.A., Harris, M.W., and Moore, J.A. (1983a). Effects of a polybrominated biphenyl mixture in the rat and mouse. I. Six-month exposure. Toxicol. Appl. Pharmacol. 68, 1-18.

Gupta, B.N., McConnell, E.E., Moore, J.A., and Haseman, J.K. (1983b). Effects of a polybrominated biphenyl mixture in the rat and mouse. II. Lifetime study. Toxicol. Appl. Pharmacol. 68, 19-35.

Gupta, B.N., and Moore, J.A. (1979). Toxicologic assessments of a commercial polybrominated biphenyl mixture in the rat. Am. J. Vet. Res. 40, 1458-1468.

Hanigan, M.H., and Pitot, H.C. (1985). Gamma-glutamyl transpeptidase - its role in hepatocarcinogenesis. Carcinogenesis 6, 186-202.

Hansell, M.M., and Ecobichon, D.J. (1974). Effects of chemically pure chlorobiphenyls on morphology of rat liver. Toxicol. Appl. Pharmacol. 28, 418-427.

Hemminki, K. (1983). Nucleic acid adducts of chemical carcinogens and mutagens. Arch. Toxicol. 52, 249-285.

Higgins, G.M., and Anderson, R.M. (1931). Experimental pathology of the liver: Restoration of the liver of the white rat following partial surgical removal. Arch. Pathol. 12, 186-202.

Howard, S.K., Werner, P.R., and Sleight, S.D. (1980). PBB toxicosis in swine. Effects on some aspects of the immune system in lactating sows and their offspring. Toxicol. Appl. Pharmacol. 55, 146-153.

Ingelman-Sundberg, M. (1980). Bioactivation or inactivation of toxic compounds? Trends in Pharmaceutical Sciences 1, 176-179.

Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences. (1980). Histologic typing of liver tumors of the rat. J. Natl. Cancer Inst. 64, 178-190.

Jacobs, L.W., Chou, S.-F., and Tiedje, J.M. (1976). Fate of polybrominated biphenyls (PBB's) in soils. Persistence and plant uptake. J. Agric. Food Chem. 24, 1198-1201.

Jensen, R.K. (1983). Pathologic effects and hepatic tumor promoting ability of Firemaster BP-6, 3,3',4,4',5,5'-hexabromobiphenyl and 2,2',4,4',5,5'-hexabromobiphenyl in the rat. Ph.D. Dissertation, Michigan State University.

Jensen, R.K., Sleight, S.D., and Aust, S.D. (1983a). Effect of varying the length of exposure to polybrominated biphenyls in the development of gamma-glutamyl transpeptidase enzyme-altered foci. Carcinogenesis 5, 63-66.

Jensen, R.K., Sleight, S.D., Aust, S.D., Goodman, J.I., and Trosko, J.E. (1983b). Hepatic tumor-promoting ability of 3,3',4,4',5,5'-hexabromobiphenyl: The interrelationship between toxicity, induction of hepatic microsomal drug metabolizing enzymes, and tumor-promoting ability. Toxicol. Appl. Pharmacol. 71, 163-176.

Jensen, R.K., Sleight, S.D., Goodman, J.I., Aust, S.D., and Trosko, J.E. (1982). Polybrominated biphenyls as promoters in experimental hepatocarcinogenesis in rats. Carcinogenesis 3, 1183-1186.

Jerina, D.M., and Daly, J.W. (1974). Arene oxides: A new aspect of drug metabolism. Science 185, 573-582.

Kalengayi, M.M.R., Ronchi, G., and Desmet, V.J. (1975). Histochemistry of gamma-glutamyl transpeptidase in rat liver during aflatoxin B_1-induced carcinogenesis. J. Natl. Cancer Inst. 55, 579-588.

Kamataki, T., Maeda, K., Yamzoe, Y., Nagai, T., and Kato, R. (1983). Sex difference of cytochrome P-450 in the rat: Purification, characterization, and quantitation of constitutive forms of cytochrome P-450 from liver microsomes of male and female rats. Arch. Biochem. Biophys. 225, 758-770.

Kaplowitz, N., Kuhlenkamp, J., and Clifton, G. (1975). Drug induction of hepatic glutathione S-transferase in male and female rats. Biochem. J. 146, 351-356.

Kappas, A., and Alvares, A.P. (1975). How the liver metabolizes foreign substances. Sci. Amer. 232, 22-31.

Karnovsky, M.J. (1965). A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. Abstr. J. Cell Biol. 27, 137A-138A.

Kato, N., Kato, M., Kimura, T., and Yoshida, A. (1978). Effect of dietary addition of PCB, DDT, or BHT and dietary protein on vitamin A and cholesterol metabolism. Nutr. Report Int. 18, 437-445.

Kato, N., Kawai, K., and Yoshida, A. (1981). Effects of dietary level of ascorbic acid on the growth, hepatic lipid peroxidation, and serum lipids in guinea pigs fed polychlorinated biphenyls. J. Nutr. 111, 1727-1733.

Kato, S., McKinney, J.D., and Matthews, H.B. (1980). Metabolism of symmetrical hexachlorobiphenyl isomers in the rat. Toxicol. Appl. Pharmacol. 53, 389-398.

Kavanagh, T.J., Rubinstein, C., Liu, P., Chang, C.-C., Trosko, J.E., and Sleight, S.D. (1985). Failure to induce mutations in Chinese hamster V79 cells and WB rat liver cells by the polybrominated biphenyls Firemaster BP-6, 2,2',4,4',5,5'-hexabromobiphenyl, 3,3',4,4',5,5'-hexabromobiphenyl, and 3,3',4,4'-tetrabromobiphenyl. Toxicol. Appl. Pharmacol. 79, 91-98.

Kay, K. (1977). Polybrominated biphenyls (PBB) environmental contamination in Michigan, 1973-1976. Environ. Res. 13, 74-93.

Ketterer, B., Meyer, D., Taylor, J., and Burchell, B. (1984). Glutathione transferases and their role in xenobiotic metabolism. Trends in Pharmaceutical Sciences 5, 319-320.

Kimbrough, R.D., Groce, D.F., Korver, M.P., and Burse, V.W. (1981). Induction of liver tumors in female Sherman rats by polybrominated biphenyls. J. Natl. Canc. Inst. 66, 535-542.

Kimbrough, R.D., Korver, M.P., Burse, V.W., and Groce, D.F. (1980). The effect of different diets or mineral oil on liver pathology and polybrominated biphenyl concentration in tissues. Toxicol. Appl. Pharmacol. 52, 442-453.

Klingenberg, M. (1958). Pigments of rat liver microsomes. Arch. Biochem. Biophys. 75, 376-386.

Kohli, J., Wyndham, C., Smylie, M., and Safe, S. (1978). Metabolism of bromobiphenyls. Biochem. Pharmacol. 27, 1245-1249.

Kosower, N.S., and Kosower, E.M. (1978). The glutathione status of cells. Int. Rev. Cytol. 54, 109-160.

Lawrence, R.A., and Burk, R.F. (1976). Glutathione peroxidase activity in selenium deficient rat liver. Biochem. Biophys. Res. Commun. 71, 952-958.

Leonard, T.B., Dent, J.G., Graichen, M.E., Lyght, O., and Popp, J.A. (1982). Comparison of hepatic carcinogen initiation-promotion systems. Carcinogenesis 3, 851-856.

Lu, A.Y.H., Junk, K.W., and Coon, M.J. (1969). Resolution of the cytochrome P-450 containing ω-hydroxylation system of liver microsomes. J. Biol. Chem. 244, 3714-3721.

Lu, A.Y.H., and West, S.B. (1978). Reconstituted mammalian mixed-function oxidases: Requirements, specificities and other properties. Pharmacol. Ther. Part A 2, 337-358.

Lu, A.Y.H., and West, S.B. (1980). Multiplicity of mammalian microsomal cytochrome P-450. Pharmacol. Rev. 31, 277-295.

Luster, M., Faith, R., and Moore, J.A. (1978). Effects of PBB on the immune response in rodents. Environ. Health Perspect. 23, 227-232.

Lutz, R.J., Dedrick, R.L., Matthews, H.B., Eling, T.E., and Anderson, M.E. (1977). A preliminary pharmacokinetic model for several chlorinated biphenyls in the rat. Drug Metab. Dispos. 5, 386-396.

Mason, H.S. (1957). Mechanisms of oxygen metabolism. Science 125, 1185.

Matthews, H.B. (1981). Disposition of persistent halogenated hydrocarbons in higher animals. In Toxicology of Halogenated Hydrocarbons: Health and Ecological Effects (M.A.Q. Khan and R.H. Stanton, eds.). Pp. 289-297, Pergamon Press, New York, pp. 289-297.

Matthews, H.B., and Anderson, M.W. (1975). Effect of chlorination on the distribution and excretion of polychlorinated biphenyls. Drug Metab. Dispos. 3, 371-380.

Matthews, H.B., Surles, J.R., Carver, J.E., and Anderson, M.W. (1977). Polychlorinated biphenyl transport by blood components. Toxicol. Appl. Pharmacol. Abstract 41, 201.

Mazzella, G.L., Sinforiani, E., Savoldi, F., Allegrini, M., Lanzola, E., and Scelsi, R. (1983). Blood cells glutathione peroxidase activity and selenium in multiple sclerosis. Eur. Neurol. 22, 442-446.

McCay, P.B., Gibson, D.D., Fong, K.-L., and Hornbrook, K.R. (1976). Effect of glutathione peroxidase activity on lipid peroxidation in biological membranes. Biochim. Biophys. Acta 431, 459-468.

Meister, A. (1975). Biochemistry of glutathione. In Metabolic Pathways (D.M. Greenberg, ed.). Vol. VII, pp. 101-188, Academic Press, New York.

Mgbodile, M.U.K., Holscher, M., and Neal, R.A. (1975). A possible protective role for reduced glutathione in aflatoxin B_1 toxicity: Effect of treatment of rats with phenobarbital and 3-methylcholanthrene on aflatoxin toxicity. Toxicol. Appl. Pharmacol. 34, 128-142.

Miller, E.C., and Miller, J.A. (1981). Mechanisms of chemical carcinogenesis. Cancer 47, 1055-1064.

Miller, J.A., and Miller, E.C. (1977). Ultimate chemical carcinogens as reactive mutagenic electrophiles. In Origins of Human Cancer (H.H. Hiatt, J.D. Watson, J.A. Winsten, eds.). Pp. 605-627, Cold Spring Harbor, New York.

Millis, C.D. (1984). Studies on the chemical and pharmacotoxicological properties of polybrominated biphenyls. M.S. Thesis, Michigan State University.

Millis, C.D, Mills, R.A., Sleight, S.D., and Aust, S.D. (1985a). Toxicity of 3,4,5,3',4',5'-hexabromobiphenyl and 3,4,3',4'-tetrabromobiphenyl. Toxicol. Appl. Pharmacol. 78, 88-95.

Millis, C.D., Mills, R.A., Sleight, S.D., and Aust, S.D. (1985b). Photolysis products of 2,4,5,2',4',5'-hexabromobiphenyl: Hepatic microsomal enzyme induction and toxicity in Sprague-Dawley rats. Fund. Appl. Toxicol. 5, 555-567.

Mills, G.C. (1957). Hemoglobin catabolism. I. Glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown. J. Biol. Chem. 229, 189-197.

Mills, R.A., Millis, C.D., Dannan, G.A., Guengerich, F.P., and Aust, S.D. (1985). Studies on the structure-activity relationships for the metabolism of polybrominated biphenyls by rat liver microsomes. Toxicol. Appl. Pharmacol. 78, 96-104.

Mitchell, J.R., Thorgeirsson, S.S., Potter, W.Z., Jollow, D.J., and Kwiser, H. (1974). Acetaminophen-induced hepatic injury: Protective role of glutathione in man and rationale for therapy. Clin. Pharmacol. Therap. 16, 676-684.

Moore, R.W., and Aust, S.D. (1978). Purification and structural characterization of polybrominated biphenyl congeners. Biochem. Biophys. Res. Commun. 84, 936-942.

Moore, R.W., Dannan, G.A., and Aust, S.D. (1980). Structure-function relationships for the pharmacological and toxicological effects and metabolism of polybrominated biphenyl congeners. In Molecular Basis of Environmental Toxicity (R.S. Bhatnagar, ed.). Pp. 173-212, Ann Arbor Science Publishers, Michigan.

Moore, R.W., O'Connor, J.V., and Aust, S.D. (1978a). Identification of a major component of polybrominated biphenyls as 2,2',3,4,4',5,5'-heptabromobiphenyl. Bull. Environ. Contam. Toxicol. 20, 478-483.

Moore, R.W., Sleight, S.D., and Aust, S.D. (1978b). Induction of liver microsomal drug metabolizing enzymes by 2,2',4,4',5,5'-hexabromobiphenyl. Toxicol. Appl. Pharmacol. 44, 309-321.

Moore, R.W., Sleight, S.D., and Aust, S.D. (1979). Effects of 2,2'-dibromobiphenyl and 2,2',3,4,4',5,5'-heptabromobiphenyl on liver microsomal drug metabolizing enzymes. Toxicol. Appl. Pharmacol. 48, 73-86.

Nebert, D.W., Eisen, H.J., Negishi, M., Lang, M.A., Hjelmeland, L.M., and Okey, A.B. (1981). Genetic mechanisms controlling the induction of polysubstrate monooxygenase (P-450) activities. Ann. Rev. Pharmacol. Toxicol. 21, 431-462.

Nebert, D.W., and Jensen, N.M. (1979). The Ah locus: Genetic regulation of the metabolism of carcinogens, drugs, and other environmental chemicals by cytochrome P-450-mediated monooxygenases. In CRC Critical Reviews in Biochemistry (G.D. Fasman, ed.). CRC Press, Cleveland, Ohio, pp. 401-437.

Newbold, R.F., and Amos, J. (1981). Inhibition of metabolic cooperation between Chinese mammalian cells in culture by tumor promoters. Carcinogenesis 2, 243-249.

Olson, J.A. (1979). A simple dual assay for vitamin A and carotenoids in human liver. Nutr. Rep. Internatl. 19, 807-813.

Orrenius, S., Ormstad, K., Thor, H., and Jewell, S. (1983). Turnover and functions of glutathione studied with isolated hepatic and renal cells. Fed. Proc. 42, 3177-3188.

Omura, T., and Sato, R. (1964a). The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. 239, 2370-2378.

Omura, T., Sato, R., Cooper, D.Y., Rosenthal, O., and Estabrook, R.W. (1965). Function of cytochrome P-450 microsomes. Fed. Proc. 24, 1181-1189.

Parke, D.V. (1968). The Biochemistry of Foreign Compounds. Pergamon Press, New York.

Pease, D.C. (1964). Histological Technique for Electron Microscopy, 2nd ed. Pp. 38-39, New York.

Peraino, C., Fry, R., and Staffeldt, E. (1971). Reduction and enhancement by phenobarbital of hepatocarcinogenesis induced in the rat by 2-acetylaminofluorene. Cancer Res. 31, 1506-1512.

Peraino, C., Staffeldt, E.F., and Ludeman, V.A. (1981). Early appearance of histochemically altered hepatocyte foci and liver tumors in female rats treated with carcinogens one day after birth. Carcinogenesis 2, 463-465.

Pitot, H.C. (1979). Drugs as promoters of carcinogenesis. In The Induction of Drug Metabolism (R.W. Estabrook and E. Lindenlaub, eds.). Pp. 471-483, F.K. Schattauer Verlag•Stuttgart, New York.

Pitot, H.C., Barsness, L., Goldsworthy, T., and Kitagawa, T. (1978). Biochemical stages of hepatocarcinogenesis after a single dose of diethylnitrosamine. Nature 271, 456-458.

Pitot, H.C., Goldsworthy, T., Campbell, H.A., and Poland, A. (1980). Quantitative evaluation of the promotion by 2,3,7,8-tetrachlorodibenzo-p-dioxin of hepatocarcinogenesis from diethylnitrosamine. Cancer Res. 40, 3616-3620.

Pitot, H.C., Goldsworthy, T., and Moran, S. (1981). The natural history of carcinogenesis: Implications of experimental carcinogenesis in the genesis of human cancer. J. Supramol. Cell Biochem. 17, 133-146.

Pitot, H.C., and Sirica, A.E. (1980). The stages of initiation and promotion in hepatocarcinogenesis. Biochim. Biophys. Acta 605, 191-215.

Poland, A., and Glover, E. (1977). Chlorinated biphenyl induction of aryl hydrocarbon hydroxylase activity: A study of the structure-activity relationship. Mol. Pharmacol. 13, 924-938.

Poland, A., and Glover, E. (1980). 2,3,7,8-tetrachlorodibenzo-p-dioxin: Segregation of toxicity with the Ah locus. Mol. Pharmacol. 17, 86-94.

Poland, A., Glover, E., and Kende, A.S. (1976). Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin by hepatic cytosol. J. Biol. Chem. 251, 4936-4946.

Poland, A., and Knutson, J. (1982). 2,3,7,8-Tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. Ann. Rev. Pharmacol. Toxicol. 22, 517-554.

Preston, B.D., Miller, J.A., and Miller, E.C. (1983). Non-arene oxide aromatic ring hydroxylation of 2,2',5,5'-tetrachlorobiphenyl as the major pathway catalyzed by phenobarbital-induced rat liver microsomes. J. Biol. Chem. 258, 8304-8311.

Pugh, T.D., and Goldfarb, S. (1978). Quantitative histochemical and autoradiographic studies of hepatocarcinogenesis in rats fed 2-acetylaminofluorene followed by phenobarbital. Cancer Res. 38, 4450-4457.

Rabes, H.M., Scholze, P., and Jantsch, B. (1972). Growth kinetics of diethylnitrosamine-induced enzyme-deficient "preneoplastic" liver cell populations in vivo and in vitro. Cancer Res. 32, 2577-2586.

Raheja, K.L., Linscheer, W.G., and Cho, C. (1983). Prevention of acetaminophen hepatotoxicity by propylthiouracil in the glutathione depleted rat. Comp. Biochem. Physiol. 76C, 9-14.

Rannug, U., Sundvall, A., and Ramel, C. (1978). The mutagenic effect of 1,2-dichloroethane on Salmonella typhimurium. I. Activation through conjugation with glutathione in vitro. Chem. Biol. Interactions 20, 1-16.

Reed, D., and Beatty, P. (1980). Biosynthesis and regulation of glutathione: Toxicological implications. In Reviews in Biochemical Toxicology (E. Hodgson, J. Bend and R. Philipot, eds.). Pp. 213-241, Elsevier/North Holland, New York.

Render, J.A., Aust, S.D., and Sleight, S.D. (1982). Acute pathologic effects of 3,3',4,4',5,5'-hexabromobiphenyl in rats: Comparison of its effects with Firemaster BP-6 and 2,2',4,4',5,5'-hexabromobiphenyl. Toxicol. Appl. Pharmacol. 62, 428-444.

Rifkind, A.B., Firpo, Jr., A., and Alonso, D.R. (1984). Coordinate induction of cytochrome P-448 mediated mixed function oxidases and histopathologic changes produced acutely in chick embryo liver by polychlorinated biphenyl congeners. Toxicol. Appl. Pharmacol. 72, 343-354.

Ringer, R.K. (1978). PBB fed to immature chickens: Its effects on organ weights and function and on the cardiovascular system. Environ. Health Perspect. 23, 247-255.

Robertson, L.W., Andres, J.L., and Safe, S.H. (1983). Toxicity of 3,3',4,4'- and 2,2',5,5'-tetrabromobiphenyl: Correlation of activity with aryl hydrocarbon hydroxylase induction and lack of protection by antioxidants. J. Toxicol. Environ. Health 11, 81-91.

Robertson, L.W., Parkinson, A., Campbell, M.A., and Safe, S. (1982). Polybrominated biphenyls as aryl hydrocarbon hydroxylase inducers: Structure-activity correlations. Chem.-Biol. Interactions 42, 53-66.

Rous, P., and Kidd, J.G. (1941). Conditional neoplasms and subthreshold neoplastic states: A study of the tar tumors of rabbits. J. Exp. Med. 73, 365-390.

Rutenberg, A.M., Kim, H., Fischbein, J.W., Hanker, J.S., Wasserkrug, H.L., and Seligman, A.M. (1969). Histochemical and ultrastructural demonstration of gamma-glutamyl transpeptidase activity. J. Histochem. Cytochem. 17, 517-526.

Sato, R., Nishibayashi, H., and Ito, A. (1969). Characterization of two hemoproteins of liver microsomes. In Microsomes and Drug Oxidations (J.R. Gillette, A.H. Conney, G.J. Cosmides, R.W. Estabrook, J.R. Fouts and G.J. Mannering, eds.). Pp. 111-128, Academic Press, New York.

Scherer, E. (1981). Use of a programmable pocket calculator for the quantitation of precancerous foci. Carcinogenesis 2, 805-807.

Schulte-Hermann, R., Ohde, G., Schuppler, J., and Timmerman-Trosiener, I. (1981). Enhanced proliferation of putative preneoplastic cells in the rat liver following treatment with the tumor promoters phenobarbital, hexachlorocyclohexane, steroid compounds, and nafenopin. Cancer Res. 41, 2556-2562.

Scribner, J.D., and Süss, R. (1978). Tumor initiation and promotion. In International Review of Experimental Pathology (G.W. Richter and M.A. Epstein, eds.). Vol. 18, pp. 137-198, Academic Press, New York.

Selikoff, I.J., and Anderson, H.A. (1979). A survey of the general population of Michigan for health effects of PBB exposure. Submitted to the Department of Health.

Sladek, N.E., and Mannering, G.J. (1966). Evidence for a new P-450 hemoprotein in hepatic microsomes from methylcholanthrene-treated rats. Biochem. Biophys. Res. Commun. 24, 668-674.

Sleight, S.D., Mangkoewidjojo, S., Akoso, B.T., and Sanger, V.L. (1978). Polybrominated biphenyl toxicosis in rats fed an iodine-deficient, iodine-adequate, or iodine-excess diet. Environ. Health Perspect. 23, 341-346.

Sleight, S.D., and Sanger, V.L. (1976). Pathologic features of polybrominated biphenyl toxicosis in the rat and guinea pig. J. Am. Vet. Med. Assoc. 169, 1231-1235.

Smith, R.L. (1973). The Excretory Function of Bile. Chapman and Hall, London.

Snoke, J.E., and Bloch, K. (1952). Formation and utilization of γ-glutamylcysteine in glutathione synthesis. J. Biol. Chem. 199, 407-414.

Steel, R.G.D., and Torrie, J.H. (1980). Principles and Procedures of Statistics. A Biomedical Approach (C. Napier and J.W. Maisel, eds.). McGraw-Hill, New York.

Stott, W.T., Reitz, R.H., Schumann, A.M., and Watanabe, P.G. (1981). Genetic and nongenetic events in neoplasm. Fd. Cosmet. Toxicol. 19, 567-576.

Strobel, H.W., Lu, A.Y.H., Heidema, J., and Coon, M.J. (1970). Phosphatidylcholine requirement in the enzymatic reduction of hemoprotein P-450 and in fatty acid, hydrocarbon, and drug hydroxylation. Biol. Chem. 245, 4851-4856.

Stross, J.K., Smokler, I.A., Isbister, J., and Wilcox, K.R. (1981). The human health effects of exposure to polybrominated biphenyls. Toxicol. Appl. Pharmacol. 58, 145-150.

Sundström, G., Hutzinger, O., and Safe, S. (1976). Identification of 2,2',4,4',5,5'-hexabromobiphenyl as the major component of flame retardant Firemaster^{R} BP-6. Chemosphere 1, 11-14.

Surak, J.G., and Bradley, R.L. (1976). Transport of organochlorine chemicals across cell membranes. Environ. Res. 11, 343-352.

Sweeney, G.D., Jones, K.G., Cole, F.M., Basford, D., and Krestynski, F. (1979). Iron deficiency prevents liver toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Science 204, 332-335.

Thor, H., Moldéus, P., and Orrenius, S. (1979). Metabolic activation and hepatotoxicity effect of cysteine, n-acetylcysteine, and methionine on glutathione biosynthesis and bromobenzene toxicity in isolated rat hepatocytes. Arch. Biochem. Biophys. 192, 405-413.

Thunberg, T., Ahlborg, U.G., Hakansson, H., Krantz, C., and Monier, M. (1980). Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the hepatic storage of retinol in rats with different dietary supplies of vitamin A (retinol). Arch. Toxicol. 45, 273-285.

Trosko, J.E., Dawson, B., and Chang, C.-C. (1981). PBB inhibits metabolic cooperation in Chinese hampster V79 cells in culture by various polybrominated biphenyl (PBB) congeners. Carcinogenesis 3, 181-186.

Tsushimoto, G., Trosko, J.E., Change, C.-C., and Aust, S.D. (1982). Inhibition of metabolic cooperation in Chinese hampster V79 cells in culture by various polybrominated biphenyl (PBB) congeners. Carcinogenesis 3, 181-186.

Tuey, D.B., and Matthews, H.B. (1980). Distribution and excretion of 2,2',4,4',5,5'-hexabromobiphenyl in rats and man: Pharmacokinetic model predictions. Toxicol. Appl. Pharmacol. 53, 420-431.

Van Miller, J.P., Hsa, I.C., and Allen, J.R. (1975). Distribution and metabolism of ^{3}H-2,5,2',5'-tetrachlorobiphenyl in rats. Proc. Soc. Exp. Biol. Med. 148, 682-687.

Vermilion, J.L., Ballou, D.P., Massey, V., and Coon, M.J. (1981). Separate roles for FMN and FAD in catalysis by liver microsomal NADPH-cytochrome P-450 reductase. J. Biol. Chem. 256, 266-277.

Vermilion, J.L., and Coon, M.J. (1978). Purified liver microsomal NADPH-cytochrome P-450 reductase. Spectral characterization of oxidation-reduction states. J. Biol. Chem. 253, 2694-2704.

Vos, J.G., and van Genderon, H. (1973). Toxicological aspects of immunosuppression. In Pesticides and the Environment. A Continuing Controversy (W.B. Diechmann, ed.). Symposia Specialists, Florida, pp. 527-546.

Watanabe, K., and Williams, G.M. (1978). Enhancement of rat hepatocellular-altered foci by the liver promoter phenobarbital: Evidence that foci are precursors of neoplasms and that the promoter acts on carcinogen-induced lesions. J. Natl. Cancer Inst. 61, 1311-1314.

Waxman, D.J., and Walsh, C. (1982). Phenobarbital-induced rat liver cytochrome P-450 purification and characterization of two closely related isozymic forms. J. Biol. Chem. 257, 10446-10457.

Welton, A.F., and Aust, S.D. (1974). Multiplicity of cytochrome P-450 hemoproteins in rat liver microsomes. Biochem. Biophys. Res. Comm. 56, 898-906.

Werner, P.R., and Sleight, S.D. (1981). Toxicosis in sows and their pigs caused by feeding diets containing polybrominated biphenyls to sows during pregnancy and lactation. Amer. J. Vet. Res. 42, 183-188.

White, I.N.H. (1976). The role of liver glutathione in the acute toxicity of retrorsine to rats. Chem.-Biol. Interactions 13, 333-342.

Whitlock, Jr., J.P., and Galeazzi, D.R. (1984). 2,3,7,8-Tetrachlorodibenzo-p-dioxin receptors in wild type and variant mouse hepatoma cells. J. Biol. Chem. 256, 980-985.

Wilkinson, C.F., and Brattsten, L.B. (1972-73). Microsomal drug metabolizing enzymes in insects. In Drug Metabolism Reviews (F.J. DiCarlo, ed.). Vol. 1, pp. 153-227, Marcel Dekker, New York.

Williams, G.M. (1980). The pathogenesis of rat liver cancer caused by chemical carcinogens. Biochim. Biophys. Acta 605, 187-189.

Yasukochi, Y., and Masters, B.S.S. (1976). Some properties of a detergent-solubolized NADPH-cytochrome c (cytochrome P-450) reductase purified by biospecific affinity chromatography. J. Biol. Chem. 251, 5337-5344.

Yoshida, K., Sakurada, T., Kitanka, H., Fukazawa, H., Kaise, N., Kaise, K., Yamamoto, M., Saito, S., and Yoshinaga, K. (1980). A solid-phase radioimmunoassay for free thyroxine in serum compared with equilibrium dialysis method. Tohoku J. Exp. Med. 132, 375-383.

Zampaglione, N.D., Jollow, J., Mitchell, J.R., Stripp, B., Hamrick, M., and Gillette, J.R. (1973). Role of detoxifying enzymes in bromobenzene-induced liver necrosis. J. Pharmacol. Exp. Ther. 187, 218-227.

VITA

The author was born in Newark, New Jersey on July 10, 1958. She received her primary and secondary education in the public and private school systems of East Orange, New Jersey. She received the Degree of Doctor of Veterinary Medicine from the School of Veterinary Medicine, Tuskegee Institute, Tuskegee Institute, Alabama.

In June of 1982 she began a M.S. program as a minority fellow in the Department of Pathology at Michigan State University. In 1984 she was admitted into the Multidisciplinary Doctoral Program in Pathology/Environmental Toxicology. In 1984 the author became a N.I.H. Pathotoxicology Fellow.

In 1985 the author was admitted into a 2-year Postdoctoral Training Program in Pathotoxicology and Laboratory Animal Medicine at Rockefeller University, New York, New York.