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STUDIES ON THE *IN VITRO* METABOLISM OF POLYBROMINATED BIPHENYLS AND THE INDUCTION OF RAT-NEONATAL DRUG METABOLIZING ENZYMES

By

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ABSTRACT

STUDIES ON THE IN VITRO METABOLISM OF POLYBROMINATED BIPHENYLS AND THE INDUCTION OF RAT-NEONATAL DRUG METABOLIZING ENZYMES

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Firemaster is a mixture of several brominated biphenyls which altogether are known to cause mixed-type induction of rat liver microsomal drug-metabolizing enzymes (similar to induction caused by both phenobarbital (PB) and 3-methylcholanthrene (MC) treatment). Their ability to induce the microsomal enzymes of neonates nursed by rats fed 10, 1.0, 0.1 and 0 ppm Firemaster for the eighteen days post-parturition was studied. Both the nursing pups and their dams fed 10 ppm Firemaster had significant increases in cytochrome P450, aminopyrine demethylase, benzo[α]pyrene hydroxylase and p-nitrophenol-UDP-glucuronyltransferase. Liver weight was increased in the pups only. The first three activities mentioned were also increased in the pups nursed by the 1.0 ppm PBBs-fed dams, but were lower than the pups nursing dams fed 10 ppm Firemaster. Therefore, mixed-type induction in a dose dependent manner was seen in the lactating rats and their pups, with the latter being more sensitive. Several pups from each of 0, 0.1 and 1.0 ppm groups were saved and raised on their mothers'

diets, and allowed to mate. They and their pups showed similar responses to PBBs as the original dams and pups. Therefore, no cumulative effects were evident by PBBs at the 0.1 or 1.0 ppm in the diet and the rat lifetime exposure to the lowest level tested does not seem to result in significant changes in the microsomal enzymes.

The susceptibility of PBBs to biotransformation was investigated by aerobically incubating Firemaster PBBs with rat liver microsomes and NADPH. Only peaks 1 and 3 of the twelve major PBB components were metabolized by PB- or PBBs-induced microsomes (but not by control or MC-induced microsomes). Of two model compounds tested, the 2,2'-dibromobiphenyl was rapidly metabolized by PB-induced microsomes while its 4,4'-isomer was not. The results suggest that a free _para_ position is required for the metabolism of brominated biphenyls. Of lesser importance appears to be the number of bromines or the availability of two adjacent unsubstituted carbons. _In vivo_ evidence for the metabolism of peaks 1 and 3 was also provided by their drastically diminished levels in liver and milk extracts.

TO MY PARENTS

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LIST OF ABBREVIATIONS

DNA	deoxyribonucleic acid
i.p.	intraperitoneal
MC	3-methylcholanthrene
NADH	β-nicotinamide adenine dinucleotide, reduced form
$NADP^+$	β-nicotinamide adenine dinucleotide phosphate
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced form
PB	phenobarbital
PBBs	polybrominated biphenyls
PCBs	polychlorinated biphenyls
PEG	polyethylene glycol
ppm	parts per million
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
Tris	tris(hydroxymethyl)amino-methane
UDP	uridine 5'-diphosphate

LITERATURE REVIEW

General Aspects of Drug Metabolizing Enzymes

Drugs and other foreign compounds are metabolized in the liver by a rather small number of reactions: oxidations, reductions, hydrolyses and conjugations (1,2). The essential effect of these reactions is to convert lipophilic compounds into hydrophilic ones. The hydrophilic compounds are more readily removed from the blood to be excreted by the kidneys and/or in the bile (3,4). Oxidation accounts for most of the transformations, largely because there are many ways in which a compound can be oxidized: side chain oxidation, aromatic-hydroxylation, N- and O-dealkylations, deamination and sulfoxide formation (1,2). The basic physiological role of these enzymes responsible for such reactions was presumably to metabolize endogenous naturally occurring compounds such as heme, lipids and steroids (2,5,6,7). For most xenobiotics (a collective term for compounds foreign to life), as well as endogenous substrates, conjugation is the next step after metabolism by oxidation, reduction or hydrolysis. Conjugation is achieved by enzymatic combination of a chemical with some natural constituent of the body such as glucuronic acid, sulfate, glycine or glutathione (4,8). In almost all cases the conjugated compound loses its pharmacological activity (1). The rate and extent of biliary excretion of conjugated foreign compounds seem to be dependent on

their molecular weight and polarity (4,8). From studying the biliary excretion of several different foreign compounds, including biphenyl and its hydroxylated and conjugated derivatives, Millbury et al. (4) concluded that appreciable excretion (5-10% of the dose) in the rat bile requires the compound to possess a strong polar anionic group and a molecular weight not less than about 300. Above this limit the higher the molecular weight the better is the biliary excretion. Moreover, it was suggested (4) that the mechanism of the biliary excretion of foreign compounds might be similar to that of conjugated bile acids, which are highly polar and whose molecular weights exceed 400.

Normal body constituents (2,5,6) as well as xenobiotics (1,9,10) are metabolized by the same hepatic enzymes of the endoplasmic reticulum. There are several hundred xenobiotics that can be metabolized by the hepatic endoplasmic reticulum. These include halogenated hydrocarbons, insecticides, urea-herbicides, volatile oils, polycyclic aromatic hydrocarbons, dyes, nicotine and other alkaloids, food preservatives, and substances such as safrole, xanthines, flavones, and organic peroxides that occur in food (1,9,10).

Another important aspect of drug metabolism is the capability of these compounds to induce the proliferation of the endoplasmic reticulum (microsomal membrane). Induction results in an increased ability to metabolize xenobiotics and drugs (9,11,12) as well as normal body constituents (9). On the other hand, some substances such as lead

and other heavy metals (1), organophosphorous insecticides, carbon tetrachloride, ozone and carbon monoxide (9) inhibit the microsomal function in animals.

The induction of liver microsomal enzymes was first observed by Brown, Miller and Miller (13) who studied factors that influence the activity of hepatic aminoazo dye N-demethylase. They noticed that rats fed commercial chow diets, some of which turned out to contain peroxides and cyclic pyrene derivatives, had greater hepatic N-demethylase activity than did animals fed purified diets. Mueller and Miller (14) were the first to indicate that the microsomal enzymes required molecular oxygen and the coenzyme NADPH for the oxidative N-demethylation of aminoazo dyes. The oxidative metabolism of drugs in general was later (15) proposed to be catalyzed by "mixed-function oxidases", enzyme complexes that require O_2 and NADPH, are nonspecific in terms of catalyzing the oxidation of different kinds of substrates, and catalyze the consumption of a molecule of oxygen per molecule of substrate, with one atom of the oxygen molecule appearing in the product and the other atom usually combining with two hydrogen atoms to form water. The specific incorporation of molecular oxygen, rather than the oxygen of water, was demonstrated by Posner et al. (16) by the specific incorporation of O_2^{18} in the hydroxylated products of acetanilide. The terminal enzyme of the mixed-function oxidases is cytochrome P450, a hemoprotein that acquired its name (17) because

in its reduced state it binds carbon monoxide to form a complex which shifts its absorption spectrum maximally at 450 nm. Evidence for the existence of this cytochrome, however, was first obtained by Klingenberg (18) and Garfinkel (19) in 1958. The amplitude of the difference spectra is the basis for quantitating the enzyme. In its oxidized state cytochrome P450 binds the substrate (20). The iron is then reduced and oxygen then binds to the reduced enzyme--substrate complex to form an activated oxygen complex that gets further reduced before it finally dissociates into hydroxylated product, water and oxidized cytochrome.

Lu and Coon were the first to succeed in preparing a soluble enzymatically active rabbit liver mixed-function oxidase system for the hydroxylation of fatty acids (21) as well as drugs (22). The system consisted of three components: cytochrome P450, NADPH-cytochrome P450 reductase and a heat stable factor that was later shown to be a phosphatidylcholine (23). The lipid-fraction could be substituted for by certain nonionic-detergents (24); and it was suggested that the lipid or detergent might function by enhancing the interaction between cytochrome P450 and the reductase (24). The second major component of the mixed-function oxidase system is the NADPH-cytochrome P450 reductase which was shown to catalyze electron transfer from NADPH to cytochrome P450 (25).

Inducers of drug metabolizing enzymes in liver have been categorized into at least two groups (11). One group, of which phenobarbital (PB) is a prototype, enhances the metabolism of a large variety of substrates. The second group stimulates the metabolism of only a few substrates and is characterized by polycyclic aromatic carcinogens such as 3-methylcholanthrene (MC) or benzo[*a*]pyrene. In addition the effects of these two groups of inducers on cytochrome P450 differ. While PB and MC both increase the concentration of the hemoprotein present in liver microsomes (1), MC does so much less than PB. MC also induces the formation of a hemoprotein that differs in spectral and catalytic activities from cytochrome P450 present in untreated rats or rats treated with PB (1). However, certain compounds like polychlorinated biphenyls (PCBs) share the properties of both the MC and the PB types of induction (26), most probably because of the heterogeneity of PCBs. The cytochrome P450 being directly and solely responsible for the difference in specificity between MC- and PB-induction was first shown by Lu et al. (27). They were able to fractionate the microsomal hydroxylation system from rats pretreated with PB or MC, and to show that the specificity for hydroxylation resides in the cytochrome fraction, rather than in the reductase or lipid fractions and that the cytochromes were different hemoproteins. However, earlier evidence existed (10,11) for the presence of spectrally different forms of the

cytochrome which are inducible in liver microsomes by pretreatment of animals with PB or MC. Kinetic studies (28,29) on the demethylation of aminopyrine by the microsomal enzymes from control, MC-, or PB-pretreated rats have also added early support for the existence of multiple drug metabolizing activities. And while PB appeared to stimulate the microsomal aminopyrine demethylase activity, MC did not (28). An earlier observation was also made on the presence of two separate enzyme systems for biphenyl hydroxylation in the 2- and 4-positions which seem to be under separate control (30). In rats and mice, benzo[*a*] - pyrene was found to stimulate the 2-hydroxylation of biphenyl but not its 4-hydroxylation, while PB causes a large increase in the 4-hydroxylation but only a minor one in the 2-hydroxylation (30). Further evidence in favor of the existence and specific inducibility of multiple cytochrome P450 hemoproteins was provided by Welton and Aust (31) who were able to resolve three hemoproteins of different molecular weights by SDS polyacrylamide gel electrophoresis of liver microsomes from control, MC-, or PB-pretreated rats. Using fixed amounts of lipid and the reductase and varying both the source and the concentration of the hemoprotein, Lu et al. (32) studied the metabolism of a number of substrates by the reconstituted system. Their results also suggested multiple forms of hemoproteins each with a different substrate specificity. More recently, evidence has been accumulating on the existence of more

numerous forms of cytochrome P450 than it was originally thought. Thomas et al. (33) provided immunochemical evidence for the existence of a total of six different but immunochemically related forms of rat liver cytochrome P450, four of which were present in the purified P450 preparation from PB-treated animals. Moreover, 6-10 distinct peaks of cytochrome P450 were obtained by Warner et al. from untreated male rat liver microsomes by preparative isoelectric focusing column, with retention of native spectra in those peaks (34). A total of at least 8 different P450s in microsomes from untreated, PB and MC treated rats has also recently been claimed (35).

Inducers seem to increase the maximal enzymatic activity without altering the Michaelis constants (36,37). The molecular mechanism of induction of hepatic microsomal enzymes seems to result from increased DNA-directed synthesis of RNA required for protein synthesis (11). Phenobarbital is thought to increase the total liver microsomal protein in rats by increasing the rate of microsomal protein synthesis while decreasing its rate of breakdown at the same time (11). In contrast, polycyclic hydrocarbons cause little or no increase in the amount of microsomal protein per gram liver, but do stimulate liver growth and total liver protein synthesis (11).

The hepatic microsomal drug metabolizing enzyme activities were found to be species, strain, as well as sex-dependent (38,39). Quinn et al. (38) using hexobarbital

as well as other drugs were able to show that the biological half life of each drug is inversely related to the ability of the microsomes to metabolize the drug in different species and in opposite sexes in the rat. Kato and Gillett (39) showed marked sex differences among rats for the metabolism of certain drugs like hexobarbital and aminopyrine, but no sex differences existed in the metabolism of others. On the other hand, the microsomal drug metabolizing enzyme activities have been known to be age-dependent as well. For example, new born guinea pigs and mice (40) as well as new born rabbits (41) are markedly deficient in their ability to metabolize several drugs. A gradual increase in drug-enzyme activities starts, however, after birth and reaches adult activities at 3-8 weeks of age (40,41). Similarly, a very low drug metabolizing activity was found in one day old rats, but it increased to a maximum at about 30 days of age and tended to decline thereafter (42). The specific activity of rat hepatic aminopyrine demethylase was found to be age-dependent where it increased 3-4 folds in the first 30 days. Sex differences in drug metabolsim were observed to develop concurrent with sexual maturation (38,39), with mature males having approximately twice the specific activity of females. Basu et al. (44) found that cytochrome P450 content, biphenyl hydroxylase, glucuronyl transferase and *p*-nitrobenzoate reductase activities peaked between 3-5 weeks. And in all cases after peaking, the activities

decreased with age. In this study (44) male and female rats were not separated until 21 days after birth when only males were used. On the other hand, Gram et al. (45) found only a slight change of microsomal cytochrome P450 between 1 and 12 week old male rats. Sex differences in cytochrome P450 content or in Vmax or Km for either ethylmorphine demethylation or aniline hydroxylation were not detectable in two week old rats (45). Finally, the data (45) indicated no temporal correlation in the developmental changes between the activities of ethylmorphine demethylase and aniline hydroxylase on one hand, and between either of these two oxidases and cytochrome P450 content on the other. Fouts and Devereux (46) found two general patterns of age-related changes in rabbits hepatic microsomes. Cytochrome P450 reductase showed a gradual increase from 3 days to one month (46). Whereas, cytochrome P450 content, benzphetamine metabolism, and benzo[α]pyrene hydroxylase activity showed a discontinuous increase with a sharp jump in level between two weeks and one month of age (46). A control mechanism seems to operate in fetal rat liver which selectively suppresses the induction of cytochrome P450 by PB, but permits the induction of cytochrome P448 by MC (47). After parturition, however, elimination of the control mechanism allows the expected enzymatic inductions associated with either inducer (47). A similar phenomenon to that observed in the fetal liver appeared to be present in pregnant rats starting 3 days after conception and disappearing on parturition (48).

Although induction of the microsomal drug metabolizing enzymes is supposed to facilitate the elimination of foreign inducing compounds from the body, it can also result in various physiological, clinical, as well as toxicological and carcinogenic consequences. Some of the immediate physiological consequences of inducing agents are related to changes in the normal body constituents. PB enhances both the biosynthesis and breakdown of cholesterol, as well as the hydroxylation of cholic acids and several other steroid hormones including vitamin D (49). Epileptics given long term barbiturate treatments have been known to develop osteomalacia probably due to the conversion of 25-hydroxycholecalciferol, an essential product of vitamin D, into more polar metabolites that can be excreted into the bile. This is thought to lead to the ultimate depletion of vitamin D body pools (50). Other physiological side effects resulting from barbiturates include increased bile and hepatic blood flows and increased bilirubin conjugation.

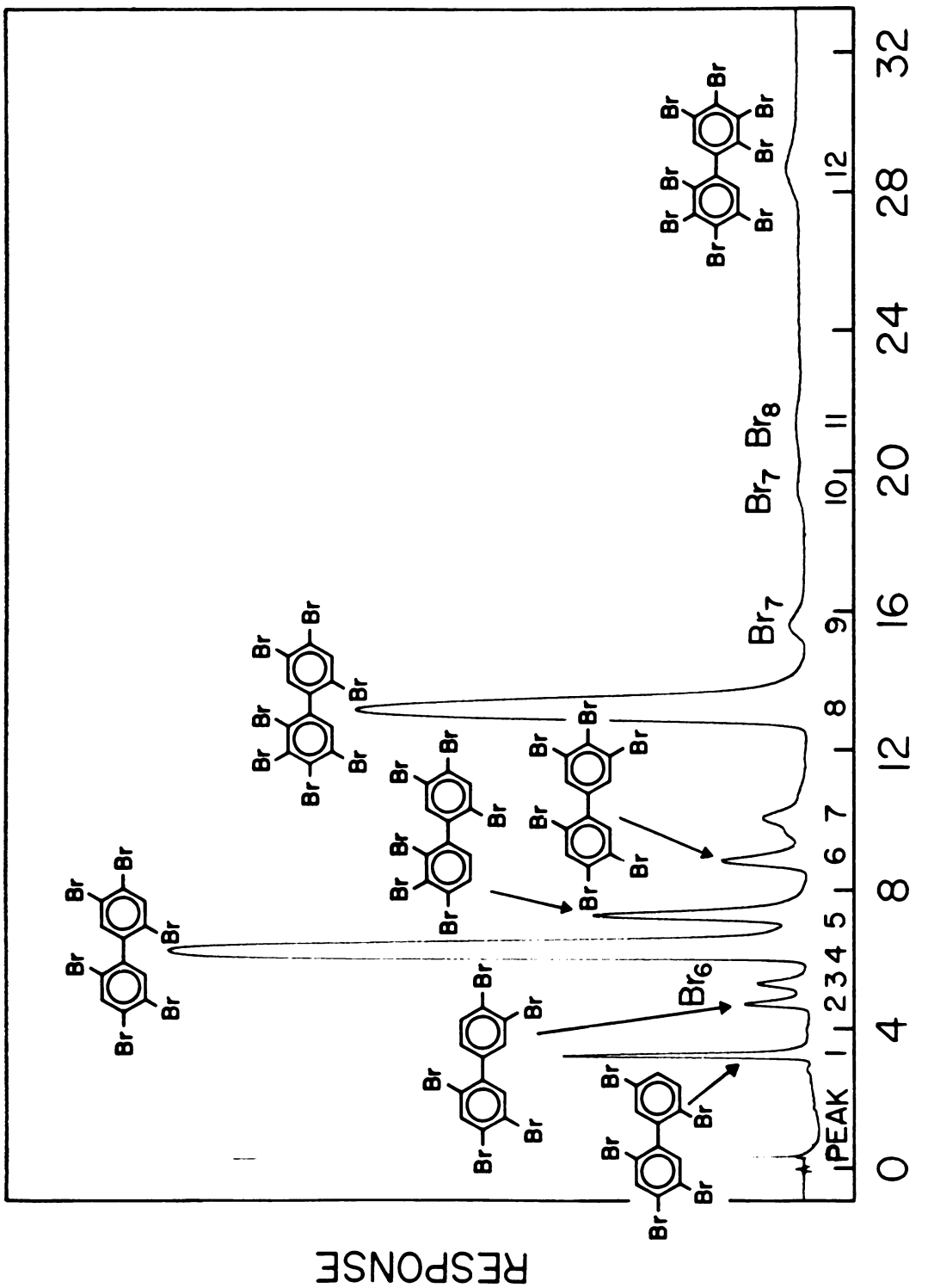
Another important aspect of microsomal enzyme induction is the observed clinical and pharmacological interactions between some drugs administered simultaneously. For example, enzyme induction is believed to result in shortening the half life of oral anticoagulants. Other hypnotic and sedative drugs, such as antipyrene, can also enhance the oxidation of the anticoagulant, warfarin (50).

Induction of the microsomal drug metabolizing enzymes can also alter the toxicity and carcinogenicity of certain drugs and foreign compounds through the formation of metabolites that have an increased or lowered toxicity. The increase in hepatotoxicity due to induction is best exemplified by carbon tetrachloride toxicity. Pretreatment with PB results in dramatic potentiation of CCl_4 hepatotoxicity, probably through the enhanced formation of its free radical metabolite $.CCl_3$ (49) that may be involved in the peroxidative decomposition of lipids in membranes. Also the nature of induced microsomal enzymes determines the carcinogenicity of many chemicals. While MC-induction causes a significant decrease in the hepatocarcinogenicity of 3'-methyl-4-dimethylaminoazobenzene, PB tends to increase the bladder carcinogenicity of 2-Naphthylamine through its N-hydroxylation (49).

Both PCBs and PBBs have been widely used as industrial chemicals in a variety of commercial and domestic products and as a consequence both have contaminated the environment (51,52) and have been found to accumulate in human and animal tissues. Concern over the contamination and persistence of these compounds have resulted in a vast literature on their toxicological and pharmacological effects, especially those caused by PCBs. Some of the studies that are done on both of these mixtures and are related to the theme of this thesis are discussed below.

Figure 1. GAS CHROMATOGRAPHIC PROFILE OF FIREMASTER PBBs AND STRUCTURES OF IDENTIFIED COMPONENTS.

The unidentified components are designated with their bromine content. All twelve main components are numbered according to their gas chromatographic chronological order.


RESPONSE
PEAK
1
2
3
4
5
6
7
8
9
10
11
12
0
4
8
12
16
20
24
28
32
RETENTION TIME (min.)
Br6
Br7
Br7 Br8

The Toxicology and Biochemical Pharmacology of Polyhalogenated Biphenyls

Crude mixtures of polybrominated biphenyls (PBBs) are used in the synthesis of products in which fire resistance is desired (52). One such mixture is Firemaster which contains an average of six bromine atoms per biphenyl and which is composed of twelve major components (as shown by the GLC, see Fig. 1) ranging in their bromine content between 5 and 8 per biphenyl molecule. There are 2 penta-, 4 hexa-, 3 hepta-, and 2 octa- bromobiphenyls. In addition there is one component (peak number 7) with an unknown number of bromines (53). Also, 2,2'-dibromobiphenyl is suspected to be a trace component of the mixture (53). In 1973, Firemaster, manufactured by Michigan Chemical Corporation, was mistakenly mixed with cattle feed as it was thought to be a magnesium oxide supplement that was named "Nutrimaster" (52). In the state of Michigan, thousands of cattle and other farm animals have died or were destroyed due to their contamination by PBBs (52). However, PBBs-contaminated meat and dairy products made their way to the tables of Michigan residents due to a lag of several months in the discovery of the contamination (52). The economic losses and health effects resulting from this incident have been reviewed by Dunkel (54), Mercer *et al.* (55) and Kay (56). Some of the toxicity signs in cattle associated with the PBBs contamination were also reported by Jackson and Halbert (57).

So far, biochemical data related to the toxicological effects of PBBs is still far from being complete. In a relatively early study on a highly brominated biphenyl mixture [octabromobiphenyls (OBBs)], 10 ppm feeding to rats for 28 days (58), was found to be the minimal level to cause bromine accumulation in fat, liver and muscle with bromine being retained in body fat for long periods after OBBs feeding was stopped. However, 100 ppm was the effective level in increasing liver to body weight ratio and causing histopathologic changes in tissues. And feeding up to 10,000 ppm OBBs over ten days to pregnant rats resulted in little or no effects on the mothers as well as their fetuses even though a dose related level of bromine was found in the fetuses. In another study (59), a single oral dose of 1000 mg/kg of OBBs or two consecutive oral doses of 3000 mg/kg of the same material to rats resulted in no abnormal clinical signs or deaths over 28 days after treatment. However, enlarged livers resulted, and glycogen depletion and inclusion bodies were seen in the liver cells under the electron microscope.

The effects of Firemaster PBBs on the hepatic microsomal drug metabolizing enzymes in male rats were investigated by Catherine Troisi in Dr. Aust's lab (60). She found a single i.p. injection (90 mg/kg body weight) more effective than a single MC injection (20 mg/kg body weight) or five daily PB injections (50 mg/kg body weight, each) in inducing liver weight, microsomal protein, NADPH-cytochrome

P450 reductase, cytochrome P450 content, aminopyrine demethylase, and UDP-glucuronyltransferase activities. However, benzo[*a*]pyrene hydroxylation was slightly higher in MC-treated rats than those treated with PBBs. Moreover, PBBs were even more effective than the combined treatment of PB plus MC. Still, five daily PBBs injections did not result in any significant increases in all parameters over those caused by a single injection. An assessment of the effects of a chronic exposure of male rats to 10 ppm PBBs in the feed was also done. Every three days, and up till sixteen days after feeding 10 ppm PBBs in the diet, microsomes were isolated and studied. Significant increases were seen in all hepatic drug metabolizing parameters after the first three days, and almost all such parameters peaked within the first six to nine days of the experiment. Moreover, such chronic PBBs feeding was more effective than the single PBBs injection (90 mg/kg body weight). The results of all of these studies indicated that PBBs share effects similar to both PB and MC, yet distinct from either alone.

Another series of studies on PBBs toxicity to female rats (61-64) also suggested that PBBs are mixed-type inducers sharing properties of PB and MC. In one such study (61) PBBs feeding as low as 18.75 ppm for two weeks to female rats resulted in significant increases in cytochrome P450, its reductase, and the drug metabolizing enzymes that are inducible by MC and PB, including epoxide

hydratase which was the only enzyme induced at the lower level of PBBs, namely 4.69 ppm. Moreover, a dose-response relationship was established. In another report (62) the time course of enzyme-induction following a single dose of PBBs (25 or 150 mg/kg body weight) was studied and compared to the profile of induction produced by maximally effective doses of PB and MC given alone or together. While most liver drug metabolizing enzymes reached their maximal activities only 48 hours after 150 mg/kg, 25 mg/kg dose needed 192 hours to show any significant increase in any of the parameters studied. Moreover, the maximum level of induction was higher at the higher PBBs dose. However, in none of the parameters observed was PBBs (at 150 mg/kg) more effective than the combined treatment of PB plus MC, except for epoxide hydratase activity. From the enzyme activities and the wavelength of absorption maximum for the cytochrome P450-CO complex the authors concluded that PBBs have a broad range of induction effects that could resemble those produced by both MC and PB. A more recent work by Dent et al. (63) showed that feeding 50 ppm of PBBs for 4 weeks to pregnant rats from day 8 of gestation until 14 days postpartum resulted in induction in aryl hydrocarbon hydroxylase (AHH) and epoxide-hydratase (EH) activities in liver, kidney and mammary glands of the dams in the order mentioned (liver>kidney>mammary gland). A recent work (64) concentrated on the pattern of induction of hepatic and

renal mixed-function oxidase in young rats due to a single i.p. injection of 150 mg/kg of PBBs. Such a treatment resulted in several fold increases in different oxidative enzymic activities both in the kidneys and livers within the first week of treatment. The enzymic inductions were shown to belong to cytochrome P448 dependent enzymes which lasted up to four weeks posttreatment. The authors were led to conclude that PBBs are not only long-lasting inducers of the mixed-function oxidases, but also can significantly modify the normal developmental pattern of these enzymes in young rats.

All of these studies have shown that PBBs are potent inducers capable of causing a mixed-type induction similar but not identical to that caused by PB and MC together. But since PBBs are a mixture of at least two dozen different components, it is likely that some components are responsible for PB-type induction while others may cause the MC-like induction. Each of the two major components in the PBBs mixture, namely, 2,4,5,2',4',5'-hexa- and 2,3,4,5,2',4',5'-hepta-bromobiphenyls (peaks 4 and 8 respectively), has been shown to be a strictly PB-type inducer in the rat (65,66). 2,2'-Dibromobiphenyl, a suspected trace component of PBBs, did not cause either type of induction (66). The effect of other PBB congeners on drug metabolizing enzymes are not known.

The teratogenic effects of PBBs were looked at through different experiments. Pregnant rats and mice were subjected to dietary levels of 50, 100, and 1000 ppm of PBBs during the last two weeks of gestation (67). No significant fetal deaths were caused at any level. However, PBBs seemed to be weakly teratogenic in mice where excencephaly and cleft palate were observed. Also, there seemed to be a decreasing birth weight as the dosage of PBBs increased in both species. In another teratology study rats were given a single treatment of PBBs between days six and fourteen of gestation at a dose ranging from 40-800 mg/kg maternal body weight. On day twenty, the fetuses were examined. Resorptions resulted from all treatments, and the number of resorbed fetuses was dose-dependent. No skeletal malformations resulted although cleft palate and diaphragmatic hernia were observed among fetuses from mothers given the 800 mg/kg dose. Another study on the embryotoxicity of PBBs was done by giving an oral dose of 0, 0.25, 0.5, 1, 5, or 10 mg PBBs to pregnant rats each day from day 7 through day 15 of gestation (69). On day 20, the mothers were killed and examined along with their fetuses. The only effect seen in mothers was an increased liver weight among those treated with 1, 5, or 10 mg PBBs. No external abnormalities were seen among any of the fetuses. In the same study, young rats, which were exposed to PBBs through their maternal blood during their embryonic life, were compared with young

rats which nursed from mothers treated with PBBs. In either case, livers of young rats tended to increase in weight and there was a delay in female reproductive maturity. It was also concluded that exposure of young rats to PBBs through mothers milk causes a decrease in their growth. The tissue distribution of PBBs in pregnant and lactating rats as well as in their offspring was investigated by feeding 50 ppm PBBs in the diet to female rats during the last two weeks of their pregnancy, during the first two weeks after delivery, or during a 4 week period starting one week after pregnancy and ending two weeks after delivery (70). In mothers, PBBs were highest in tissues that have the highest lipid content, such as adipose and mammary tissues. Also there was an increased accumulation of PBBs in mammary tissue of pregnant animals as compared to the same tissue of lactating mothers. An interesting finding was the higher level of PBBs in livers of neonates exposed via the milk as compared to the level of PBBs in their mothers' livers. Also important was the finding that PBBs-transfer to the young via the milk is far more important than placental transfer.

Another class of compounds closely related to PBBs is polychlorinated biphenyls (PCBs) with chlorine substituents, instead of bromines, on the biphenyl nucleus. PCBs have experienced a widespread industrial use, and as a consequence they widely contaminate the environment (51,71). They have not only been identified in animals

but also in human adipose tissue (72) as well as human milk (73). In rats, PCBs (Aroclor 1254 or 1260 - Aroclor is a commercial name, and the last two digits of each number refer to the percent of chlorine in the mixture) were found to be primarily stored in the adipose tissue followed by the liver (74). Moreover, the proportions of the different isomers were not the same in the fat and urine, and in both it was different from the standard parent mixture; possible metabolism or differential absorption was suggested to be the reason. On the other hand, Aroclor was unaltered in the feces which could be indicative of uniform lack of absorption.

Induction of the microsomal enzymatic activities was found to be dependent on the dose as well as the chlorine content of PCBs. A feeding study of Aroclor 1260 to weanling male and female rats at 1, 5, 25, and 50 ppm over different periods lasting up to 13 weeks (75) showed induction of both N- and O-demethylase drug metabolizing enzymes (the first is characteristic of PB-like induction, while the second is MC-type) and the level of induction was dose-dependent. Moreover, the N-demethylase activity was found to be proportional to the chlorine content of the different PCB-mixtures tested (Aroclors 1260, 1254, and 1221). On the other hand, young animals showed more response to enzyme-induction than adults (75). Bickers _et al_. (76) also found Aroclor 1254 to be superior to Aroclor 1016 in inducing cytochrome P450, as well as

the associated drug metabolizing activities. Goldstein et al. (77) noticed that for induction of the drug metabolizing enzymes, the percentage of chlorine is not as important as the amount of highly chlorinated biphenyls. They found that Aroclor 1016 is a poorer inducer than Aroclor 1242 even though both have the same chlorine content (41 and 42%, respectively). However, the latter has a higher content (nine times) of biphenyls with five chlorines or more. They concluded that the highly chlorinated homologs are more active in inducing the drug metabolizing enzymes than the less chlorinated ones. In the same study (77) there was a shift in the absorption maximum of the reduced cytochrome P450-CO complex from 450 nm to 449 nm upon induction by Aroclor 1242 but not by Aroclor 1016. However, a similar observation had already been reported upon induction by a different PCBs mixture, namely Aroclor 1254 (26) which caused a shift in the difference spectrum of cytochrome P450 to 448nm. The cytochrome content was tripled, and both benzo[*a*]pyrene hydroxylase (inducible by MC) as well as ethylmorphine N-demethylase (inducible by PB) were increased in activity. Also, like MC treatment, Aroclor 1254 increased the ratio of 455 to 430 nm peaks for the ligand ethylisocyanide (26). It was concluded that Aroclor 1254 might induce a mixture of both cytochromes P448 and P450 which result in catalytic properties pertaining to both hemoproteins. Support for this was recently provided (78) when purified liver microsomal

hemoprotein from rats pretreated with the same mixture showed spectral, catalytic, electrophoretic and immunochemical properties indicative of a mixture of cytochromes induced by both PB and MC treatments.

Studies with pure chlorinated biphenyls have shown that even though induction increases with the degree of chlorination, the pattern of chlorine substitutions is also quite important. Johnston et al. (79) showed that the biphenyl nucleus itself and the 4-monochlorobiphenyl are non-inducers of the drug metabolizing enzymes. On the other hand, induction was caused by hexa- and octachlorobiphenyls and by di- and tetrachlorobiphenyls with chlorines substituted at the 3- and 4- positions. More recently, Goldstein et al. (80) classified a number of pure PCBs into either PB-type or MC-type based on the drug metabolizing enzymes induced and the resulting spectral properties of the monoxygenase heme. The symmetrical chlorination of biphenyls in both the meta- and para-positions (3,4,3',4'- or 3,4,5,3',4'-5'-) specifically increased the formation of cytochrome P448 and the associated drug metabolizing activities, and caused changes in the spectral properties similar to those caused by MC or benzo[*a*]pyrene. And such compounds were found to be the most toxic in terms of causing the most dramatic weight losses. Chlorination of a single ortho site to yield 2,3,4,5,3',4',5'-heptachlorobiphenyl causes loss of the MC-like activity characteristic of the parent 3,4,5,3',4',5'-

hexa molecule and renders the hepta derivative devoid of any activity in regard to induction of the drug metabolizing enzymes (80). On the other hand, chlorination in both the *para*- and *ortho*- positions, regardless of the chlorination of the *meta*- position, specifically causes the induction of cytochrome P450 and its associated drug metabolizing activities, characteristic of PB-type, with no changes in the spectral properties. These PCB components include 2,4,2',4'-tetra- and 2,4,5,2',4',5'-, 2,3,4,2',3',4'- and 2,4,6,2',4',6'-hexachlorobiphenyls. Goldstein *et al*. also found that chlorination in only one ring, or chlorination in both rings but not in the *para*- positions, renders the molecule weakly active in inducing the liver enzymes. Out of several dichlorobiphenyls tested, only 3,3'- and 4,4'-dichlorobiphenyls have very slight activity at extremely high doses. Biphenyl itself was devoid of any activity except the induction of arylhydrocarbon hydroxylase at high doses (3 daily injections of 108 mg/kg). Goldstein and co-workers concluded that chlorination of the *para*- position and the degree of chlorination appear to be the most important factors in causing enzymatic induction (80).

The effects of chronic exposure of male rats to low levels of PCBs in the diet for three months were evaluated (81). Significant increases in the liver microsomal protein, the level of cytochrome P450, as well as several drug metabolizing activities belonging to both PB- and MC-type

inductions were observed at 10 ppm PCBs feeding level but not at the 1.0 or 0.1 ppm (81).

Concern was also given to the effects of PCBs on reproduction, fetal development, and weanling animals. Daily administration of Aroclor 1221 or 1254 for the first four weeks of gestation in rabbits did not affect implantation, fetal development, fetal growth, or litter size at dose levels of 1.0 or 10 mg/kg body weight (82). However, placental transfer to fetuses of both compounds occurred. The no-effect level of Aroclor 1221 (21% Cl) in inducing enzymatic activities was shown to be higher than that of Aroclor 1254 (54% Cl) (82). On the other hand, the lower chlorinated biphenyls seem to affect reproduction more than the higher ones in birds (83). And in rats, Aroclor 1242 or 1254 at 100 ppm dietary levels caused decreased survival of pups (83). In another study, neither 1.0 nor 5.0 ppm of Aroclor 1254 had any effect on rat reproduction; however, the higher dose increased the liver to body weight ratio in weanling rats. The same compound at 20 ppm in the diet caused a decrease in the number of litters as well as the number of pups per litter (83).

Since the passage of chemicals through milk largely depends on their lipid solubility and state of ionization (84), it is not surprising to find the polyhalogenated biphenyls in the milk of mammals which have been exposed to these lipid soluble-non-ionizable compounds. PBB residues

have been found in the milk of contaminated cows (85) as well as PBBs-force fed cows (86). In the latter case, 2.22% of the total PBBs given over 15 days were excreted in the milk over 31 days. PCBs are also excreted in cows milk where their concentration in the milk fat exceeded that of the body's fat (87). The average rate of secretion of PCBs in milk was 42.3 mg/day which accounted for 21% of the daily intake of Aroclor 1254 (87). Metabolites were also found in milk from cows given 2,5,2',5'-tetra- and 2,4,5,2',5'-penta-chlorobiphenyls, Aroclor 1242, or Aroclor 1254 (88). Even though some of the metabolites were free, most of them were conjugated, and the bulk of the metabolites was in the urine rather than in the milk. The total amounts of metabolites represented about 20% of either of the two unchanged pure components present in the milk (88).

While some studies have been performed on the metabolic conversion of PCBs, no such studies have been done on PBBs yet. Metabolic products of PCBs were first reported by Hutzinger and co-workers (89) who discovered hydroxylated derivatives of di- and tetra-chlorobiphenyls in rats and pigeon excreta based on mass spectral evidence. Urine collected from rabbits treated with either 2,5,2',5'-tetrachloro, 4,4'-dichloro-, or 4,4'-dibromo-biphenyls was found to contain three hydroxylated metabolites in each case, in a manner suggestive of an arene-oxide intermediate (90,91). Aromatic compounds, in general as well

as chlorinated biphenyls (90-93) are thought to undergo metabolic hydroxylation via arene-oxide intermediates by the hepatic mixed-function oxidase system. Evidence for arene-oxide intermediates includes the occurrence of the NIH- (1,2-) shift as well as the formation of dihydrodiols (or catechols) in addition to the appearance of glutathione conjugates (92). In PCBs, it is widely accepted that the number and location of the chlorines on the biphenyl nucleus determine whether or not the halogenated biphenyl will undergo metabolism by the hepatic mixed-function oxidase, presumably through an arene-oxide type intermediate. Chlorobiphenyls with four chlorines or less were readily hydroxylated by rats, rabbits, and pigeons (89, 90, 94-96). For a rapid enzymatic hydroxylation of chlorinated benzenes, two adjacent unsubstituted carbon atoms were found to be required (97). This requirement is met by all chlorobiphenyls with four chlorines or less as well as by many penta-, hexa-, and hepta- isomers. However, certain other structural requirements also seem to be important in determining the rate of the hepatic microsomal hydroxylation. That two vicinal hydrogens is insufficient by itself as a criterion for rapid metabolism was shown in a comparative study on the levels of certain structurally known PCBs in human adipose tissue (72). Out of three hexachlorinated biphenyls, namely 2,3,6,2',4',5'-, 2,4,5,2',4',5'-, and 2,3,4,2',4',5'-, only the first, with a 2,3,6-trichloro substitution on

the first ring, seemed to be decreased in the human tissue (as compared to the components in the PCB mixture believed to have been the source of contamination) even though the third compound had two vicinal hydrogens as well. Similar results were obtained in avians where 2,3,6,3',4'-pentachlorobiphenyl was cleared faster than its isomer 2,4,5,3',4'-pentachlorobiphenyl, or its congener 2,3,4,2',3',4'-hexachlorobiphenyl (98). It was concluded that 4,4'-substitution is more important than the degree of chlorination in determining the persistence of chlorinated biphenyls in the hen (98). Avians, in another study, were shown to rapidly metabolize those PCBs that have 2,3-, 3,4-, or 2,3,6- chlorine substitution on at least one phenyl ring (99). Those PCBs that were not metabolized had a combination of 2,4,5-, 2,3,4- and 2,3,4,5- chlorine substituents on both rings (99). Another structural feature that may affect the metabolic conversions of chlorobiphenyls is the chloro substitution pattern in the ortho positions to the biphenyl bridge (72). More planar chlorobiphenyls, due to the presence of only one or two chlorines ortho to the biphenyl bridge, were present in human adipose tissue at concentrations higher than chlorobiphenyls that have more than two ortho chlorines and thus are less planar (72).

In vitro studies on the hepatic microsomal metabolism of different chlorobiphenyls in presence of NADPH were also done. ^{14}C-labeled 2,5,2'-tri-, 2,5,2',5'-tetra-,

and 2,4,5,2',5'-pentachlorobiphenyls were metabolized by microsomes from PB-pretreated rats in a manner where the number and quantity of the metabolites formed decreased with the increasing number of chlorines (100). No metabolites were formed by microsomes from non-pretreated rats. Related work on the directing influence of Cl-substitution on the position of hydroxylation was done by Greb et al. (101). Mono- and dihydroxylated metabolites were formed from ^{14}C-labeled 2,2'-di-, 2,4'-di-, and 2,5,2'-trichlorobiphenyls in the presence of microsomes from PB-pretreated female rats. It was clear (101) that the lower chlorinated biphenyls are readily metabolized by the rat hepatic mixed-function oxidases and the yields and profiles of the hydroxylated derivatives seemed to be dependent on the nature of chlorine substitution. A study on the capacity of rat liver and lung microsomal fractions to metabolize ^{14}C-2,4,5,2',5'-pentachlorobiphenyl showed that the lung microsomes were ten times more active than liver microsomes in producing metabolites (102). Whereas, the lung microsomes continued to produce the metabolites for 12 hrs before reaching a plateau, production of metabolites by liver microsomes plateaued after the first hour. Although the identity of the metabolites was not disclosed, they seemed to be hydroxy derivatives. More recently (103) microsomes from PB-pretreated rabbits metabolized 2,2'-di, 2,5,2',5'-tetra-, and 2,4,6,2',4',6'-hexachlorobiphenyls at rates ranging from fast, to slow, to non-detectable, respectively.

Since PBBs are potent mixed type inducers of the hepatic mixed-function oxidases, and since they are thought to be transmitted through the mammalian milk, it was necessary to look into the induction pattern they might produce in rat neonates nursed by mothers maintained on diets containing different low levels of PBBs throughout the nursing period. The first part of my thesis is, therefore, devoted to a study on the effects of feeding 10, 1.0, 0.1 or 0 ppm PBBs in the diet on the hepatic drug metabolizing enzymes of lactating rats and their neonates.

The persistence of PBBs in the body, and their unknown biochemical fate, prompted me to investigate their susceptibility to *in vitro* metabolism by microsomes from rats which had undergone different pretreatments. Results showing evidence for the *in vitro* metabolism of certain PBB-components are provided in the second chapter and the effect of bromine substitution on the susceptibility of PBBs to metabolism is also discussed.

CHAPTER ONE

INDUCTION OF DRUG METABOLIZING ENZYMES IN POLYBROMINATED BIPHENYLS-FED LACTATING RATS AND THEIR PUPS

ABSTRACT

Polybrominated biphenyls (PBBS) are known to cause mixedtype inductions (similar to those caused by both phenobarbital (PB) and 3-methylcholanthrene (MC) treatment) in rat liver microsomal drug-metabolizing enzymes. The potential for PBBs transmitted through the milk to exert similar effects on neonatal liver enzymes was investigated. Lactating rats were fed Firemaster PBBs at 0, 0.1, 1.0 or 10 ppm for a period of eighteen days following parturition. Both mothers and pups in the 10 ppm group had significant increases in cytochrome P450, aminopyrine demethylase, benzo[*a*]pyrene hydroxylase, and *p*-nitrophenol-UDP-glucuronyl transferase; and in addition, the pups had significantly increased liver weights. Lower, but still significant increases were also seen in cytochrome P450, aminopyrine demethylation, and benzo[*a*]pyrene hydroxylation in pups nursing from 1.0 ppm PBBs-fed rats, which unlike their pups were unaffected. Several pups from the 0, 0.1 and 1.0 ppm groups were raised on their mothers' diets and allowed to mate. The grown adult females and their pups showed comparable responses to PBBs as the original dams and pups.

Therefore, PBB components similar to both PB and MC-type inducers can be transmitted through the milk to cause mixed-type inductions in the nursing pups. The nursing rats seemed to be at least 10 times more sensitive to PBBs than their mothers; the 0.1 ppm PBBs in the diet

of the adult seemed to be the lowest no-effect level on the microsomal enzymes of nursing rats.

INTRODUCTION

The liver microsomal drug metabolizing enzymes can be induced by a wide variety of xenobiotics including drugs, pesticides, and carcinogens (9,11,12). These inducers, in general, belong to either of two classes of compounds based on their induction properties that usually tend to resemble either one of the two classic inducers, phenobarbital (PB) or 3-methylcholanthrene (MC). While either PB or MC increases the concentration of the microsomal hemoprotein(s), PB doing so more than MC, MC causes the formation of cytochrome P448 which differs in spectral and catalytic properties from cytochrome P450 present in untreated rats or rats treated with PB. Specific increases in liver weight, microsomal protein, NADPH-cytochrome P450 reductase, aminopyrine demethylase and epoxide hydratase are all caused by PB. However, MC causes specific induction in the activities of benzo[*a*] pyrene hydroxylase and UDP-glucuronyltransferase when *p*-nitrophenol is used as an acceptor for the second enzyme. Therefore, by screening the microsomal drug metabolizing enzymes, it is possible to characterize the type and extent of induction that may result from treatment of lab animals with a certain drug.

Certain compounds like PCBs (26,78,81) or PBBs (60-64), however, can cause the induction of both types of microsomal mixed-function oxidases in a manner similar to treatment by both MC and PB together. These compounds share mixed-type induction properties because they are a mixture of several polyhalogenated biphenyl congeners each of which being like one or the other of the two classical inducers (26,65,66,80). However, the possibility also exists that one or more of the PBB components could by itself be a mixed-type inducer similar to hexachlorobenzene, 2,3,4,2',3',4'- or 2,3,4,2',4',5'-hexachlorobiphenyls (104,105). Also possible, is the capability of the parent compound to cause one type of induction while its in vivo metabolites cause the other. In animals as well as humans, the polyhalogenated biphenyls are deposited in fatty tissues and are excreted in their milk (58,70,72-74,86-88). However, their distribution in tissues or milk was quite different mainly because of the preferential metabolism of certain components over others (72, also see chapter two of this thesis). Since significant mixed-type inductions of the drug metabolizing enzymes resulted from PBBs feeding to male rats at 10 ppm in the diet (60) it was interesting to see if similar inductions could result in neonates nursing from rats fed PBBs in their diets at 10 ppm or less (1.0, 0.1 or 0 ppm) over the nursing period. The pattern of induction in the neonatal enzymes was to be used as an indicator for the presence or absence

of PBBs belonging to PB- and MC-type inducers in the milk. Also it was hoped to establish a dietary limit for the lactating rat and its nursing offspring below which the drug metabolizing enzymes are insensitive to PBBs in the diet or the milk. In a further study, the effects of life-time exposure to low levels of PBBs at 0.1 and 1.0 ppm in the diet were also looked at in the adult male rat, the lactating female and their offspring.

MATERIALS AND METHODS

Chemicals

The PBBs used in these studies was obtained from a feed mixing mill shortly after the discovery of its accidental mixing into cattle feed. It is of the Firemaster type manufactured by Michigan Chemical Corp., St. Louis, Mich. The gas chromatographic profile is shown in Figure 1. Also included in this figure is the numbering system used to identify the components of the mixture. The structures and bromine contents are shown for those components which have been identified (53). Peak 4 is the major component of the mixture (56% by weight), previously identified as 2,2',4,4',5,5'-hexabromobiphenyl (106,107). The column packing for the GLC was 3% OV-1 on Gas Chrom Q, 100-120 mesh from Supelco Inc., Bellefonte, Pa.

Phenobarbital sodium U.S.P. and dibenzidine dihydrochloride were obtained from Merck and Co., Inc., Rahway, N.J. Polyethylene glycol (PEG, approximate molecular

weight 400), 3-methylcholanthrene, 3,4-benzo[*a*]pyrene, $NADP^+$, tetrasodium NADPH (Type I), disodium NADH (Grade III), ammonium UDP-glucuronate, cytochrome c (from horse heart, type VI), bovine serum albumin, nicotinamide, butylated hydroxytoluene, Florisil (magnesium silicate activated at 1250°F), trisodium DL-isocitrate (type I), and isocitrate dehydrogenase (from pig heart, type IV, highly purified) were all obtained from Sigma Chemical Co., St. Louis, Mo. Ethyl acetate, glass distilled, suitable for pesticide analysis, was purchased from Burdick and Jackson Laboratories, Inc., Muskegon, Mich. Aminopyrine was obtained from K and K Rare and Fine Chemicals, Plainview, N.Y. *P*-Nitrophenol was from the Aldrich Chemical Co., Milwaukee, Wi. Sodium dodecyl sulfate (SDS) was from Pierce Chemical Co., Elkhart, In. N,N'-methylene bisacrylamide was from Canalco Inc., Rockville, Md. Ammonium persulfate and N,N,N',N'-tetramethylethylenediamine (TEMED) were obtained from Bio-Rad Laboratories, Richmond, Ca. Hydrogen peroxide was from Mallinckrodt, St. Louis, Mo. Pitocin (Oxytocin) was purchased from Parke-Davis & Co., Detroit, Mich. Sodium pentobarbital (Nembutal) was obtained from Abbott Laboratories, North Chicago, Ill. All other chemicals and solvents used were of reagent grade quality.

Animals and Treatments

Pregnant female Sprague-Dawley rats were obtained from Spartan Research Animals, Inc., Haslett, Mich. They were put on water and feed pellets ad libitum until the day of parturition when the normal pelleted feed was exchanged by the special PBBs diets. These diets were prepared by grinding the chow pellets in a Willey Mill, followed by a thorough mixing of the required amounts of PBBs dissolved in 2 ml corn oil/kg feed to achieve a final PBBs concentration of 0, 0.1, 1.0 and 10 ppm in the feed. The dams were fed the special diet ad libitum throughout the nursing period which lasted for 18 days after parturition. During this time, special care was practiced to ensure that the pups had no access to the feed. There were four separate mothers on each diet and the livers of the pups from each mother were pooled together. However, one of the four pooled liver samples from the 1.0 ppm group was lost while it was being homogenized. Eighteen days after parturition, on the night before sacrifice, the feed was removed, but the pups were allowed to continue nursing from their dams. On the next morning, the mothers and most of their pups were sacrificed by decapitation. Three pairs of pups from each of the control, 0.1 and 1.0 ppm groups were spared and put on the same diet their mothers were fed. All of these animals lived, except for one pair from the control group, and were allowed to mate after sexual maturation. Eighteen

days after delivery, these parents and their pups were sacrificed and their liver microsomes obtained as described.

Preparation of Microsomes

Microsomes were isolated according to previously described procedures (28,108). Rats were sacrificed by decapitation and the liver microsomes of the dams, and the adult males of the second part of the study, were isolated individually while those of the pups from each dam were isolated in one pool. Then all microsomes were washed with a 0.3 M sucrose containing 0.1 M sodium pyrophosphate to remove ribosomes and adsorbed-proteins. The microsomes were stored at -20°C in 50 mM Tris-HCl, pH 7.5, containing 50% glycerol and 0.01% butylated hydroxytoluene. The microsomal protein concentration was determined by the method of Lowry et al. (109) using bovine serum albumin as the standard according to Rutter (110).

Enzyme Assays

Cytochrome P450 concentration was determined by the method of Omura and Sato (17). The detection of specific hemoproteins was done using benzidine and hydrogen peroxide staining following SDS polyacrylamide gel electrophoresis. The method was described by Moore et al. (111). The methods dealing with preparing the gels and performing electrophoresis were based upon Fairbanks et al. (112) procedure.

Cytochrome P450 reductase was assayed by the rate of cytochrome c reduction (at 550 nm) according to Massey (113) using an extinction coefficient of $21.0 \times 10^3\ M^{-1}cm^{-1}$.

The microsomal N-demethylase activity catalyzed by the phenobarbital inducible cytochrome P450 was assayed using aminopyrine as a substrate where the amount of formaldehyde formed was measured according to the method of Nash (114). The aminopyrine metabolism assay was described by Pederson and Aust (28) and later modified by Moore et a . (65) as follows: The reaction mixture (5 ml total volume) contained 50 mM Tris-HCl, pH 7.5, 5 mM $MgCL_2$, 5 μM $MnCL_2$, 0.5 mM $NADP^+$, 4 mM sodium D,L-isocitrate, 0.44 units of isocitrate dehydrogenase, 20 mM aminopyrine (recrystallized twice from hexane before use), and 2-3 mg microsomal protein. The complete reaction mixture was prepared, on ice, in a 20 ml beaker and aminopyrine was added last. The enzymic reaction was started by transferring the beaker to a 37°C Dubnoff metabolic shaker. Formaldehyde production was measured in 1.0 ml aliquots taken from the reaction mixture at 1,4,7 and 10 minutes and mixed with 1.0 ml of 10% trichloroacetic acid to stop the reaction. After allowing a few minutes for the protein to precipitate, 2.0 ml of Nash reagent (2 M ammonium acetate, 0.05 M acetic acid, and 0.02 M 2,4-pentanedione) was added to each tube and the mixtures were heated at 60°C for 10 min to develop the color. After cooling and centrifugation for about 10 min, the absorbance of the supernatant was measured at 412 nm against a blank consisting of 1.0 ml 10% trichloroacetic acid, 1.0 ml buffer, and 2.0 ml Nash reagent. An extinction coefficient of 7.08×10^3

$M^{-1}cm^{-1}$ and a dilution factor of 4 were used to calculate the formaldehyde content.

The microsomal cytochrome P448-dependent hydroxylase activity using the substrate benzo[*a*]pyrene was measured by the method of Gielen *et al.* (115) and is also described in detail by Moore (53). Fluorescence readings were taken within 6 minutes after the NaOH-extraction to avoid fluorescence decay of the hydroxylated metabolites of benzo[*a*]-pyrene.

UDP-glucuronyl transferase was measured using *p*-nitrophenol as a substrate according to the method of Grote *et al.* (116) except that KCl was omitted. The reaction rate was obtained by recording the decrease in absorbance at 403 nm due to the conversion of the substrate to a colorless glucuronic acid conjugate. An extinction coefficient of 12.6 $mM^{-1}cm^{-1}$ was used to calculate the conjugation activity.

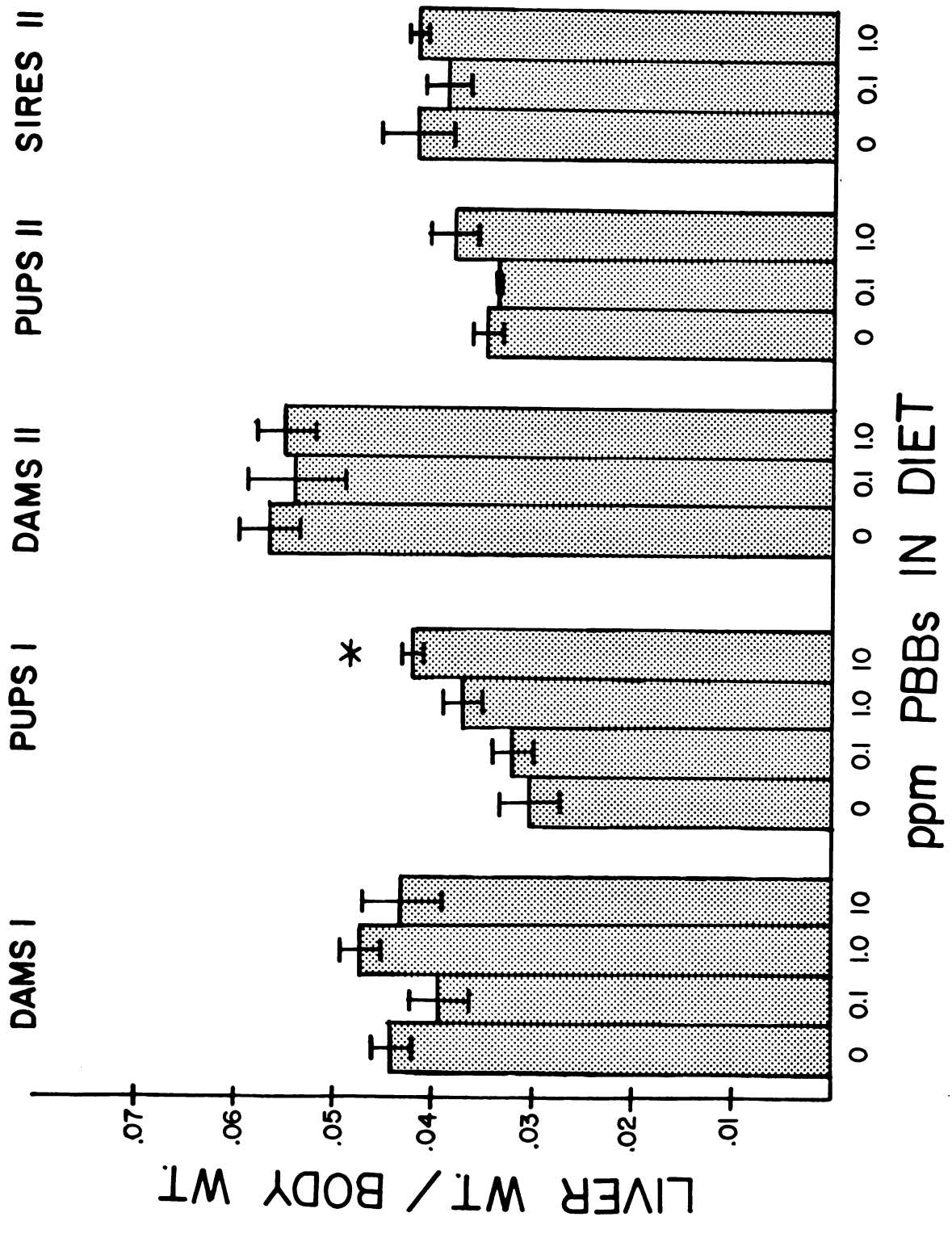
RESULTS

Effects of 0.1, 1.0, or 10 ppm PBBs Feeding During the Nursing Period on Drug Metabolizing Enzymes in Lactating Rats and Their Nursing Pups

The effects of post partum exposure to low levels of PBBs in the diet on the liver drug metabolizing enzymes of lactating rats and their nursing offspring were investigated. PBBs had no effect on the liver weights of the mothers while it caused a significant increase of 39% in the liver weights of pups nursed by mothers fed 10

Figure 2. EFFECTS OF PBBs IN THE MATERNAL DIET ON LIVER WEIGHTS IN DAMS AND PUPS OVER TWO SUCCESSIVE GENERATIONS (I and II).

In the first generation dams were exposed to PBBs for 18 days following parturition. Several pups from 0, 0.1 and 1.0 ppm groups were kept and raised on their mothers diets and allowed to mate. They and their pups were killed 18 days after parturition. Values are means $\pm$ SEM. N = 4 for the first generation study, while N = 3 for the second generation. *Represents values that are significantly different from corresponding controls at $p < 0.05$.


DAMS I
PUPS I
DAMS II
PUPS II
SIRES II
*
LIVER WT / BODY WT
.07
.06
.05
.04
.03
.02
.01
0 0.1 1.0 10
ppm PBBs IN DIET

ppm PBBs in the diet (Fig. 2). Pups nursing mothers fed 1 ppm PBBs had a non-significant 22% increase in their liver weights. Also, there were no significant increases in the microsomal yield (expressed as protein/liver weight) of the mothers or their pups even though exposure at all levels of PBBs resulted in slight increases in this parameter among all rats (Fig. 3).

The content of cytochrome(s) P450 increased 64% in the microsomes of the dams on 10 ppm PBBs diet, but no significant increases were evident in dams on the lower PBBs-containing diets (Fig. 4). Pups nursing from dams fed 1 and 10 ppm PBBS, however, showed significant increases of 36 and 206%, respectively, in their microsomal cytochrome P450 content. Even though the NADPH-dependent cytochrome P450 reductase of all dams and their nursing pups underwent a slight gradual increase that paralleled the increasing PBBs in the diets, all such increases were statistically insignificant (Fig. 5).

Aminopyrine demethylase was used as a measure of PB-like induction of drug metabolizing enzymes (11) and benzo[a] pyrene hydroxylase was the indicator for MC-type induction (49). Mothers fed 10 ppm PBBs showed significant increases in both parameters. Aminopyrine demethylase was increased 58% while benzo[a] pyrene hydroxylase increased 650% (Figs. 6 and 7). No such increases were observed in dams fed diets containing lower levels of PBBs. However, significant increases in both drug metabolizing parameters

Figure 3. EFFECTS OF PBBs IN THE MATERNAL DIET ON MICROSOMAL PROTEIN YIELD IN DAMS AND PUPS OVER TWO SUCCESSIVE GENERATIONS.

Details as in Figure 2.

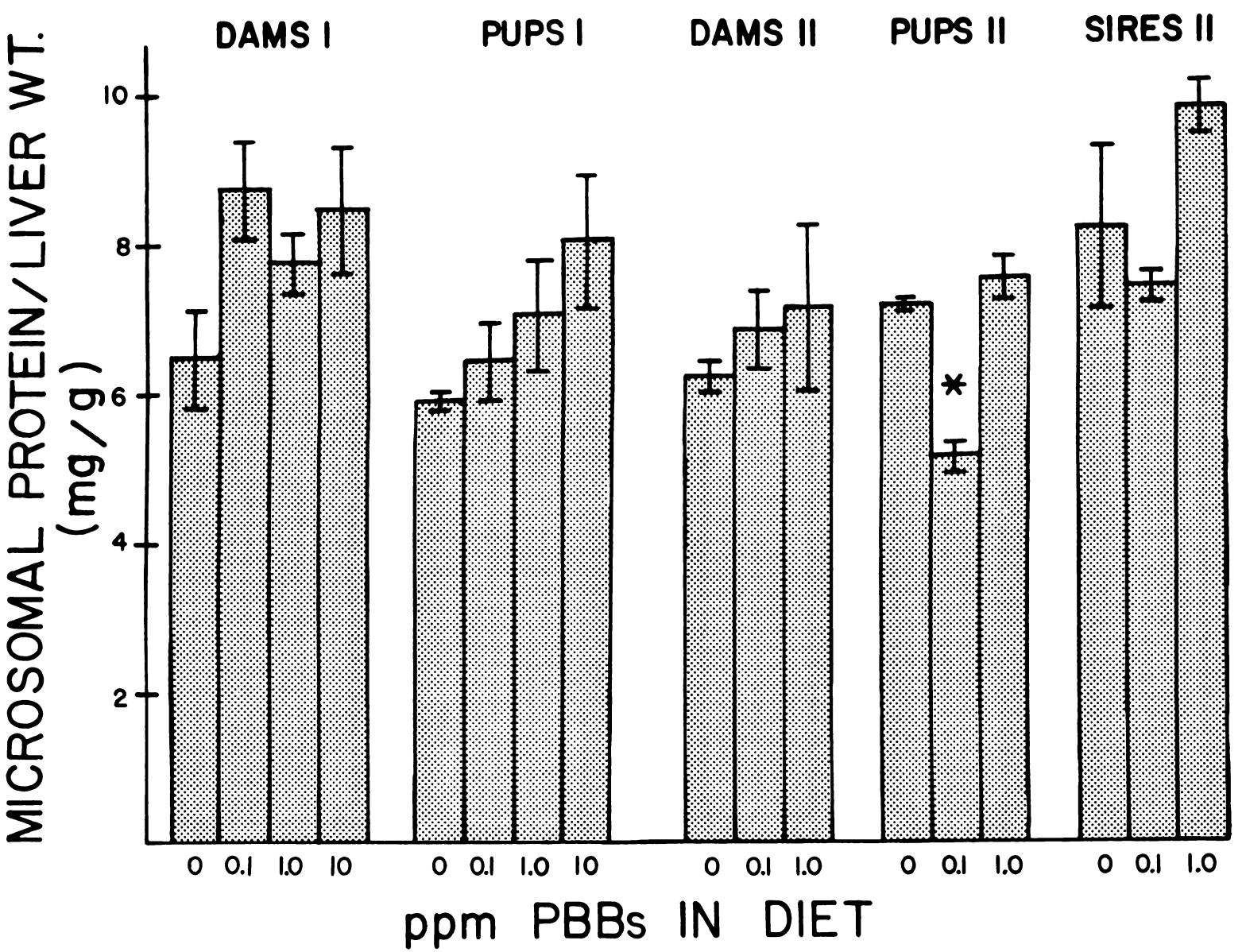
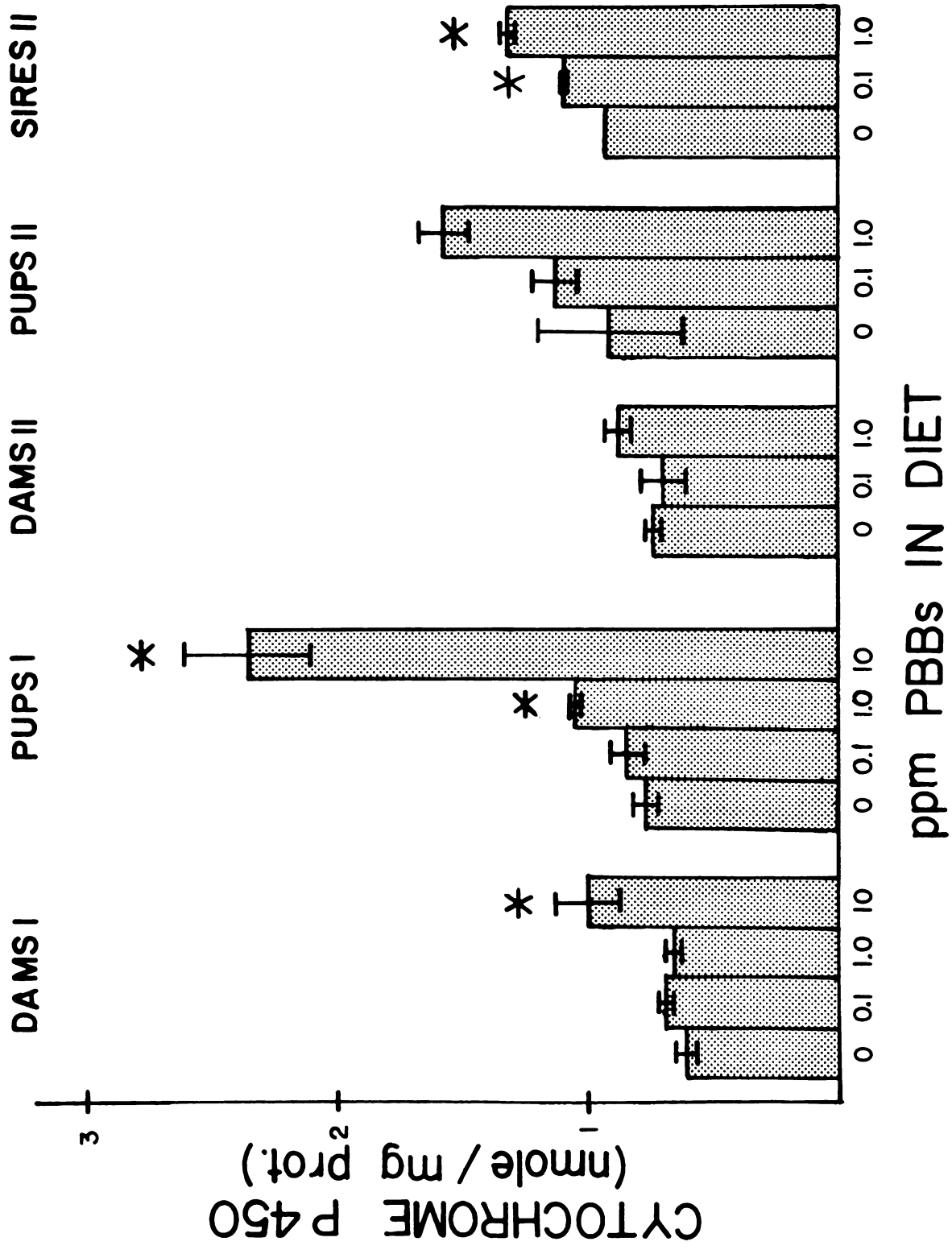

DAMS I
PUPS I
DAMS II
PUPS II
SIRES II
MICROSOMAL PROTEIN/LIVER WT. (mg/g)
10
8
6
4
2
0
0.1
1.0
10
*
ppm PBBs IN DIET

Figure 4. EFFECTS OF PBBs IN THE MATERNAL DIET ON CYTOCHROME P450 IN DAMS AND PUPS OVER TWO SUCCESSIVE GENERATIONS.

Details as in Figure 2.


CYTOCHROME P450
(nmole / mg prot.)
3
2
1
DAMS I
PUPS I
DAMS II
PUPS II
SIRES II
0 0.1 1.0 10
ppm PBBs IN DIET
*

were seen in pups nursing from dams on 1 and 10 ppm PBBs diets. Aminopyrine demethylase was increased 76 and 192%, while benzo [*a*]pyrene hydroxylase went up by 280 and 370%, respectively. Pups nursing from 0.1 ppm PBBs fed dams showed a slight insignificant decrease of 20% and 14% of aminopyrine demethylase and benzo[*a*] pyrene hydroxylase, respectively.

UDP-glucuronyltransferase has been shown to exist in more than one form after induction by PB or MC (117). Using *p*-nitrophenol as a substrate it is possible to measure the activity of the enzyme(s) resulting from MC-type induction to conjugate hydroxylated substrates including, possibly, PBBs metabolites. Rats fed the 10 ppm PBBs diet and their nursing pups had 115 and 240% increases in this enzymatic activity (Fig. 8). While the same parameter was increased over 50% in both dams and pups in the 1 ppm group, the increases were not significant.

Effects of Lifetime Exposure to 0.1 and 1.0 ppm PBBs in the Diet on Rat Drug Metabolizing Enzymes

Three pairs of pups from each of 0, 0.1 and 1.0 ppm groups were saved and raised on their mothers' diets and allowed to mate. Eighteen days after delivery, the dams, adult males and their pups were sacrificed and their liver microsomes obtained and the drug metabolizing enzymes assayed. Liver weights and microsomal proteins (Figs.2 and 3) were not significantly changed in adult males or females at either PBBs level, while the pups of 0.1 ppm group

Figure 5. EFFECTS OF PBBs IN THE MATERNAL DIET ON NADPH-CYTOCHROME P450 REDUCTASE IN DAMS AND PUPS OVER TWO SUCCESSIVE GENERATIONS.

Details as in Figure 2.

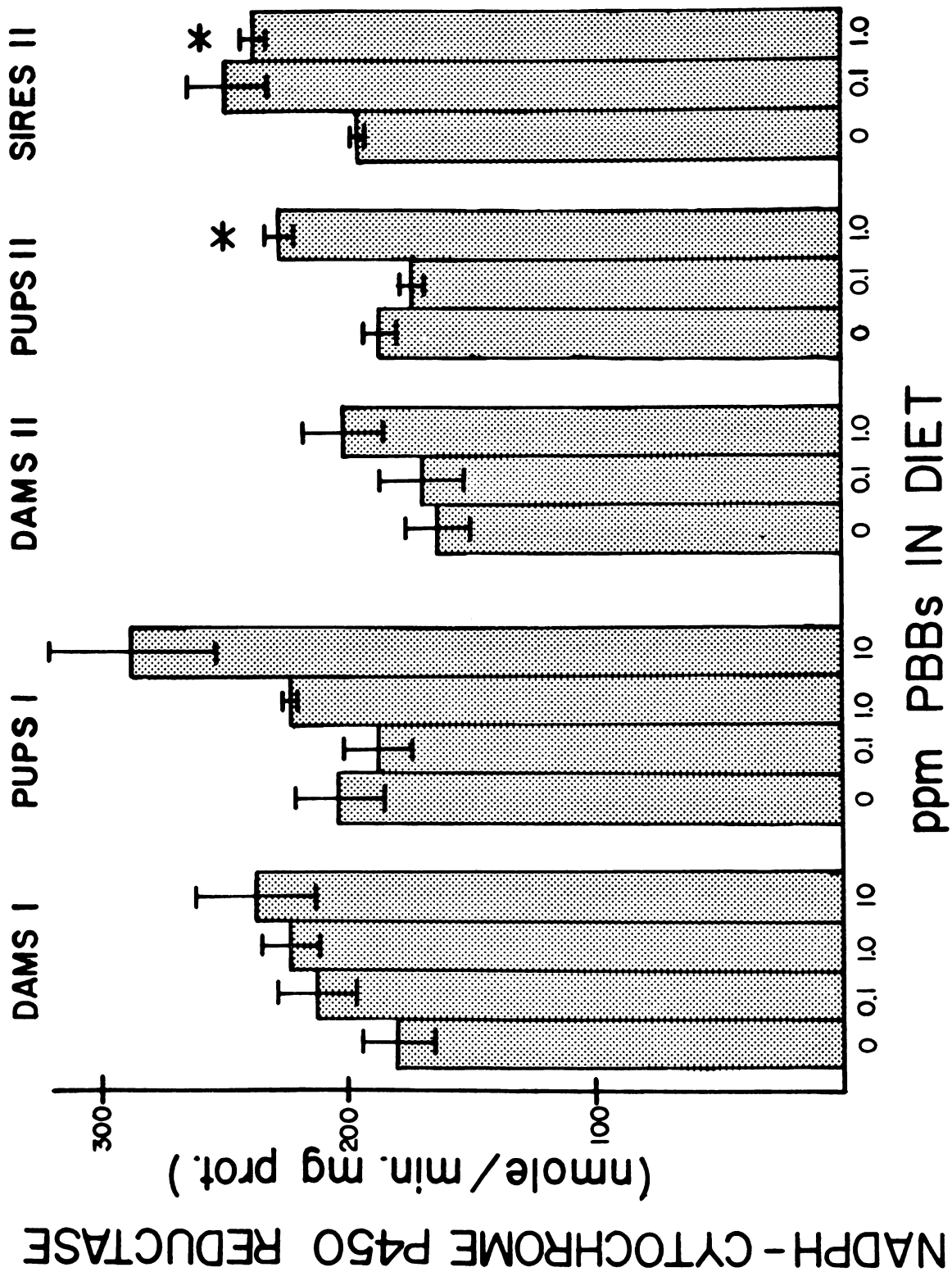
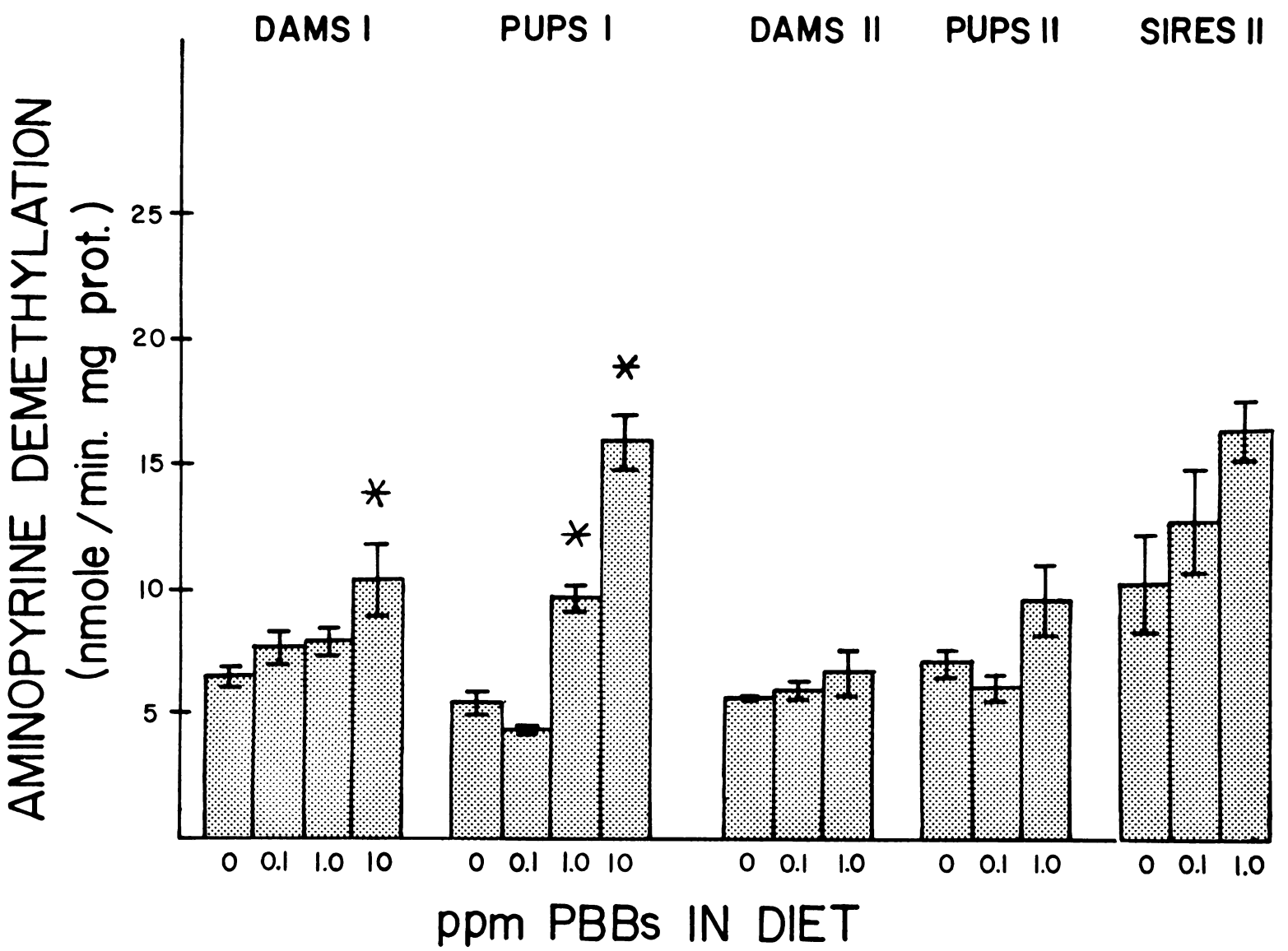

NADPH-CYTOCHROME P450 REDUCTASE
(nmole/min. mg prot.)
300
200
100
DAMS I
PUPS I
DAMS II
PUPS II
SIRES II
0 0.1 1.0 10
ppm PBBs IN DIET
*
*

Figure 6. EFFECTS OF PBBs IN THE MATERNAL DIET ON AMINOPYRINE DEMETHYLATION IN DAMS AND PUPS OVER TWO SUCCESSIVE GENERATIONS.

Details as in Figure 2.


DAMS I
PUPS I
DAMS II
PUPS II
SIRES II
AMINOPYRINE DEMETHYLATION
(nmole/min. mg prot.)
25
20
15
10
5
0 0.1 1.0 10
0 0.1 1.0 10
0 0.1 1.0
0 0.1 1.0
0 0.1 1.0
ppm PBBs IN DIET

had a 30% decrease in their microsomal protein. This unexpected decrease is most likely the result of a technical error, such as incomplete recovery of the microsomal pellet. Cytochrome P450 underwent a significant increase of about 20 and 40% in adult males of 0.1 and 1.0 ppm groups, respectively. No significant changes in the content of this cytochrome was seen in the females or their pups even though it was increased by 75% in the 1.0 ppm pups. NADPH-cytochrome P450 reductase activity was significantly increased by 22% in both adult males and pups in the 1.0 ppm group, but no significant increases were noticeable in the females. Although aminopyrine demethylase tended to increase in all animals in the 1.0 ppm group, the increases were not statistically significant. However, the other drug metabolizing activity, benzo[α]pyrene hydroxylase, was significantly increased by 230, 80 and 340% in adult females, males and their pups, respectively, in the 1.0 ppm group. The UDP-glucuronyltransferase activity was significantly increased only in the 1.0 ppm pups (115% higher than the control).

DISCUSSION

Polybrominated biphenyls have been found to be potent inducers of the drug metabolizing enzymes in different tissues among different species (60-67,118-120). In rats, PBBs cause a mixed-type induction similar to treatment by both PB and MC (60-66). The two major components of

Figure 7. EFFECTS OF PBBs IN THE MATERNAL DIET ON BENZO[α]PYRENE HYDROXYLATION IN DAMS AND PUPS OVER TWO SUCCESSIVE GENERATIONS.

Details as in Figure 2.

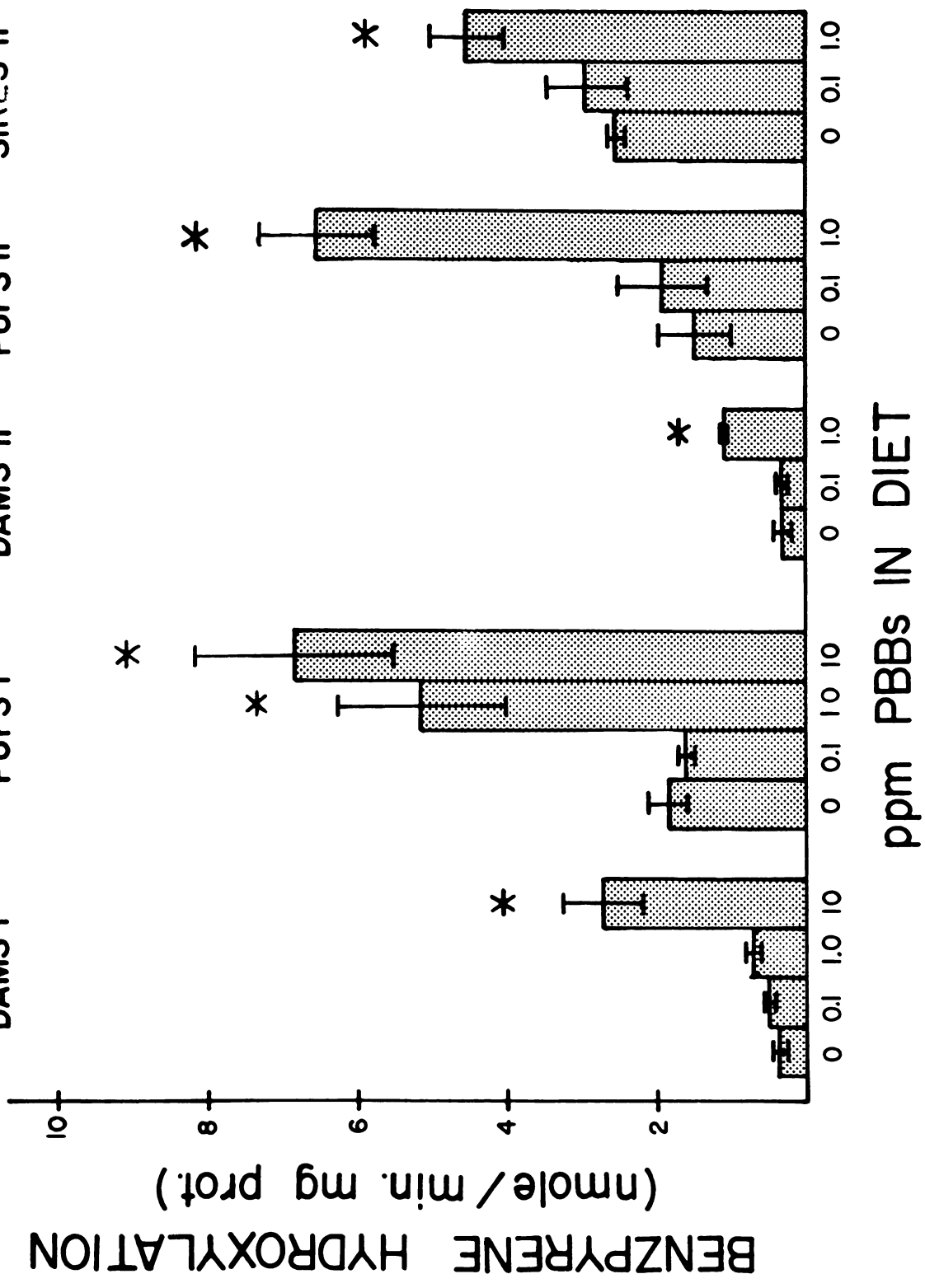
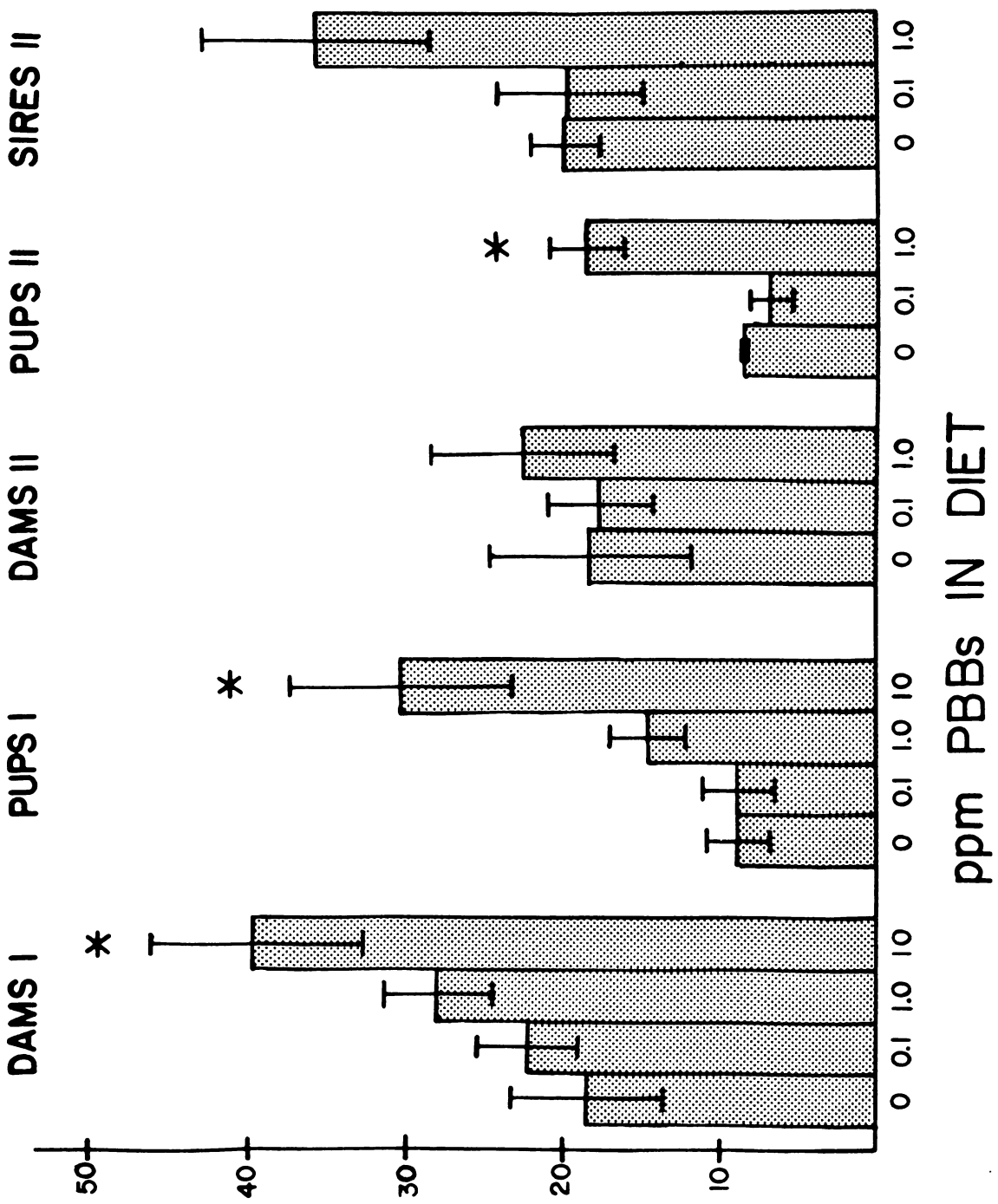

BENZPYRENE HYDROXYLATION
(nmole/min. mg prot.)
2
4
6
8
10
DAMS I
PUPS I
DAMS II
PUPS II
SIRES II
0 0.1 1.0 10
ppm PBBs IN DIET

Figure 8. EFFECTS OF PBBs IN THE MATERNAL DIET ON UDP-GLUCURONYLTRANSFERASE IN DAMS AND PUPS OVER TWO SUCCESSIVE GENERATIONS.

Details as in Figure 2.


UDP - GLUCURONYLTRANSFERASE
(nmole / min. mg prot.)
50
40
30
20
10
DAMS I
PUPS I
DAMS II
PUPS II
SIRES II
*
*
*
0 0.1 1.0 10
0 0.1 1.0
ppm PBBs IN DIET

PBBs, peaks 4 and 8 (2,4,5,2',4',5'-hexa- and 2,3,4,5,2',4',-5'-hepta-bromobiphenyls, respectively), comprising about 83% of the total mixture, have been found to be strict PB-type inducers in the rat (65,66). One or more components of the remaining PBBs must be responsible for the MC-type induction. Although PBB components have previously been shown to be concentrated in rat mammary tissue and cows milk (70,85,86) there was no information on their effect on nursing animals. The present research was therefore undertaken to determine if the PBB components responsible for inducing the full range microsomal drug metabolizing enzymes, could be transmitted from lactating rats to their nursing offspring. It was equally important to determine a limit for PBBs in the diet of lactating rats below which the drug metabolizing enzymes in their nursing pups would be insensitive.

The results presented in this chapter show that the liver drug metabolizing enzymes underwent mixed-type inductions in pups nursing from rats fed PBBs in their diets. Therefore, PBBs belonging to both classes of inducers can be transmitted through rat milk. Similar results were obtained by Alvares and Kappas (121) following treatment of lactating rats with Aroclor 1254, a mixture of polychlorinated biphenyls that are known to have mixed-type induction properties (26). The significant increases in neonates liver weights, cytochrome P450, and aminopyrine demethylase activity can be attributed to transmission of PB-type inducers through

the milk. In rat milk (Fig. 15) both peaks 4 and 8, which have been shown to belong to PB-type inducers (65,66), were present in substantial quantities and could, by themselves, account for the PB-type induction seen in neonates. However, the contribution to PB-like induction by other PBBs present in the milk cannot be ruled out. The large inductions of both benzo[α]pyrene hydroxylase and UDP-glucuronyltransferase in the pups are taken as indications for the transmission through the milk of one or more components with MC-like induction properties. Even though the gas chromatographic analysis of the PBBs in the milk looks different from PBBs Firemaster (Fig. 15), due to preferential metabolism and/or solubility in the milk, most of the twelve major components appear to be present in appreciable quantities. Even though the milk that was analyzed for PBBs was obtained after treatment with 90 mg PBBs/kg i.p. following parturition, rats fed PBBs in their diets are assumed to have the same profile of PBBs in their milk.

The main goal of this research was to determine the dose-response relationship between PBBs and the induction of microsomal drug metabolizing enzymes in lactating rats and their nursing offspring. Therefore, the levels of the microsomal enzymes in the pups should be dependent on the concentrations of PBBs in their mothers' milk, especially since the pups had no access to the PBBs containing feed. Lactating rats fed 10 ppm PBBs and their

litters showed pronounced and significant increases in cytochrome P450, aminopyrine demethylation, benzo[a] pyrene hydroxylation, and UDP-glucuronyltransferase, in addition the pups showed an increase in liver weight. Except for the transferase activity all the other enzymes had higher activities in the pups than their mothers in the 10 ppm group. On the other hand, compared to control animals, the increases in all the four enzymes, except benzo[a] pyrene hydroxylase, were higher in the pups than their mothers in the same group. While mothers fed the 1.0 ppm PBBs diet had no significant increases in any of the investigated parameters, their nursing pups showed significant increases in cytochrome P450 as well as the two drug metabolizing activities, aminopyrine demethylase and benzo[a] pyrene hydroxylase. However, all of these increases were less pronounced than those observed for the pups of the 10 ppm group. On the other hand, none of the microsomal parameters was sensitive to PBBs among the pups or their dams on the 0.1 ppm PBBs diet. Therefore, a dose-response relationship seems to exist between PBBs in the diets of lactating rats and the microsomal enzyme induction in both the lactating rats and their offspring. On the other hand, the nursing pups appeared to be more sensitive to PBBs in the milk than their mothers that were fed the PBBs diets.

Not only the neonatal microsomal enzymes appeared more sensitive than those of the lactating rats, but also the neonatal P450 hemoproteins were modified. Benzidine heme staining of the SDS gels loaded with different microsomes showed mainly a single P450 band in all four types of microsomes from lactating rats. However, microsomes from the pups of the 10 ppm group had three P450 hemoprotein bands (or more), one higher and the other lower in molecular weight than the one in common with the other microsomes from 1.0, 0.1 and 0 ppm pups (data not shown). This was taken as an additional support for the higher sensitivity of the neonatal microsomal drug metabolizing enzymes.

One or more of the following reasons could be responsible for the apparent differences in sensitivity to PBBs between the lactating rats and their nursing offspring. Since, as shown in Fig. 15, the profile of PBBs in the milk looks markedly changed from Firemaster PBBs it is possible that PBBs passed over to the nursing neonates have a higher proportion of the more potent inducing congeners. Some PCBs are known to be more potent inducers than others depending not only on their chlorine content but also on the distribution of the chlorines on the biphenyl nucleus (80). It is worth noting that the congeners with the higher potency are probably the ones that are resistent to metabolism in the mother rat since lack of metabolism is thought to be at least one reason for the increased effectiveness of certain PCBs (80) and barbiturates (49)

in inducing the drug metabolizing enzymes. Peaks 1 and 3 of PBBs were susceptible to metabolism *in vitro* (Chapter Two) and they seem to be decreased in the milk (Fig. 15). Some of the other congeners that were resistent to metabolism might be enriched in the milk and therefore might relieve the microsomal enzymes of the lactating rats while at the same time subject the neonatal enzymes to their effects. It should be mentioned that this reason must be coupled to the relatively very small size of the pups and their livers, especially during their early days, to make the neonates become more sensitive to PBBs than their mothers. Another possible reason is the excretion in the milk of some PBBs metabolites that have greater efficacy than the parent compound(s). However, this does not seem likely because on one hand no metabolites were observed in the milk of cows contaminated with PBBs (88), although a faulty procedure for metabolite analysis could have been the reason. Even if some hydroxylated metabolites were in the milk, they still might be less potent inducers than the parent compound(s) as have been found with certain hydroxylated PCBs (80). And conjugated metabolites of PBBs, if present in the milk, might be inactive since conjugation in general is believed to result in loss of biological activity (1). A third possible reason is that milk secretion relieves the dams of some PBBs which brings down their level in the liver to below the threshold that is necessary for significant inductions to occur in non-

lactating female rats of the same weight. However, this possibility is not supported by the observations of Dent et al. (61) where the drug metabolizing enzymes of female non-lactating rats were insensitive to PBBs at 4.69 ppm in the diet over two weeks. At the next higher PBBs level of 18.75 ppm in the diet there was significant mixed-type inductions in almost all investigated parameters (61). Therefore, these results on the significant enzymatic inductions, of both PB- and MC-types, in the dams of the 10 ppm group, but not the 1.0 ppm group, seem to agree with those of Dent et al. (61) on the female rats.

The insensitivity of the female rat to 1 ppm PBBs or lower in the diet seems to be independent of the secretion of PBBs in the milk, and similar results might have been expected had female non-lactating rats been fed similar diets. One last possibility, that seems to be the most plausible, is the inherently higher refractiveness of neonatal rat as compared to the adult. The drug metabolizing enzymes seem to be largely latent or absent in the newly born rat, but they rapidly start to increase right after parturition (47,49). On the other hand, they seem to respond to induction soon after birth (47). However, the basal levels of these enzymes as well as their responsiveness to inducers seem to decline with age (122). Therefore, PBBs seem to be at least ten times as effective inducers of the neonatal microsomal enzymes as compared to those of the adult female rat, most probably because

of the inherently higher sensitivity of the neonate as compared to the mother.

Some liver specimens from the present studies were given to Dr. Sleight, of the Department of Pathology at MSU, for microscopic examination to see if the biochemical data on the induction of the microsomal enzymes coincides with the pathological state of the livers. The results were published (120) as part of a larger study on the pathological toxicosis of PBBs in the rat and guinea pig. There were several obvious changes in the livers of pups in the 10 ppm group including increased cytoplasmic vacuolation and myelin bodies in addition to some changes in the endoplasmic reticulum and degenerative changes of some mitochondria. Both the mothers fed 10 ppm PBBs and the pups which nursed dams fed the 1.0 ppm PBBs diet had swollen mitochondria. However, in agreement with the results on the lack of microsomal inductions, there were no obvious hepatic lesions in the mothers fed the 1.0 or 0.1 ppm PBBs diets, nor in the pups which nursed the dams of the 0.1 ppm group.

A comparative study on the effects of pre- and postnatal exposure of rat pups to PBBs was done by Dent *et al*. (123). Liver weights, benzo[*a*]pyrene hydroxylase (an index of MC-like induction), and epoxide hydratase (an index of PB-like induction) were significantly increased in pups nursing mothers fed 50 ppm PBBs for the two weeks following parturition. Comparable results were seen in

other pups due to prenatal exposure through their mothers which had the same level of PBBs in their diets for the last two weeks of pregnancy. A combination of pre- and post-natal exposure resulted in the highest increases. However, analysis of tissue samples from pups exposed pre- or post-natally showed that the transfer of PBBs through the milk is much more important for their appearance in the newborns than their placental transfer (70). There were higher concentrations of PBBs in the livers of neonates exposed through the milk than in the livers of pups exposed transplacentally (22.3 vs. 1.1 µg PBBs/g wet liver, respectively). More PBBs were also present in the mammary tissue of pregnant rat in comparison to the same tissue of lactating animal (317.5 vs. 65.2 g/g wet weight, respectively). All of these findings seem to indicate that the mammary tissue in the female rat is a major concentrating organ for PBBs, which could then be transferred through the milk to the nursing pup. It is also interesting to note that there were higher concentrations of PBBs in the livers of nursing neonates than in the livers of their lactating mothers (22.3 vs. 2.8 µg PBBs/g wet liver, respectively). Whether there was a similar trend of concentrating PBBs in the neonatal liver as opposed to the dams' in the studies presented in this thesis is not known. However, it does not seem possible to directly correlate the levels of PBBs in the livers with the extent of induction of the microsomal enzymes.

The neonates that had the highest inductions through both pre- and post-natal exposure to PBBs had significantly much lower levels of PBBs in their livers than the pups exposed to PBBs through the milk only (70,123).

Since it was interesting to know the long term effects of PBBs at the low levels of 1.0, 0.1 and 0 ppm in the feed, some pups were saved and raised on the same diets their mothers had, and were allowed to mate and raise pups until they were 18 days old. A different trend in the activities of the drug metabolizing enzymes was observed between the adult males and females. As rats grew up from weanlings to adults the enzyme activities in females were depressed while they were increased in males (Figs. 2-7, compare the parameters of Pups I with the corresponding ones of Dams II and Sires II). This phenomenon happened regardless of the presence of PBBs or their level in the diet, and most probably was due to the onset of sexual maturation (38,39,49). However, the levels of the different parameters tended to closely match between the pups of the first (Pups I) and second (Pups II) studies, and also between their dams in both studies (Dams I and Dams II). Therefore, lifetime exposure to PBBs in the diet, at 0.1, or 1.0 ppm, had no apparent accumulative effect on the induction of drug metabolizing enzymes. In addition, there was no interference with the expected increases in these enzymes that usually occur in males as opposed to female rats upon sexual maturation. However,

while the lifetime exposure to PBBs at 1.0 ppm in the diet significantly enhanced some of the enzymatic activities (see Figs. 4, 5 and 7) in the adult males (Sires II), the adult females (Dams II), underwent no significant inductions of their enzymes (with the exception of benzo[a]-pyrene hydroxylase). Therefore, it seems likely that the female rat is inherently less sensitive to PBBs than the male, regardless of milk secretion.

It is also worth noting that the continued exposure to PBBs resulted in induction that appeared to be more characteristic of MC- than PB-type. For example, in the 1.0 ppm group aminopyrine-demethylase was not significantly increased in the second generation pups, or their fathers, even though this activity was induced in the first generation pups. However, benzo[a] pyrene hydroxylase was increased in both. UDP-glucuronyltransferase was induced only in the second generation pups. The females of the 1.0 ppm group showed a significant increase in only one parameter, namely benzo[a] pyrene hydroxylase (compare Dams I and Dams II of Fig. 7). These observations seem to agree with the observation that the pattern of induction changes from PB-like initially, following a single PBBs injection, to MC-like at later times (124). Such a process might be totally dependent on the dose of PBBs since MC-like inducers in the mixture must be present in small quantities as compared to PB-type PBB congeners [the two major components of Firemaster, peaks 4 and 8 which are

83% of the total mixture, are PB-like inducers (65,66)]. However, it should be cautioned that the results may not be conclusive in this regard, although they can be suggestive, mainly because the number of animals in each group was not large enough (N = 4 in the first part of the study, while N = 3 for the continued second part). More definitive conclusions, in this regard, require the use of larger numbers of animals, as well as applying certain other experimental methods, such as the ethylisocyanide difference spectroscopy as well as comparative gel electrophoresis.

Finally, it is tempting to theoretically estimate the cumulative minimum amount of injested PBBs over the 18 days period before significant inductions are triggered in both the lactating females and their pups. However, the following basic information is required: 1), Amount of feed ingested per day by each dam; 2), The concentration of PBBs in secreted milk; and 3), The amount of milk that each rat produces per day. Since feed consumption was not monitored in my studies, I had to resort to other similar values reported in the literature. Male rats of 250-300 g weight were reported to have consumed 28 g feed (of the same type used in my studies) per day per rat (60). On the other hand, female rats of less than 200 g weight consumed an average of 15 g of a powder diet per day per rat (61). Since the rats in my studies weighed between 300-350 g and were breast feeding, an estimated 40 g feed is assumed to have been consumed per rat per

day. Therefore, a total of 720 g of feed is the amount estimated to be ingested by each dam over the entire 18 days nursing period. This much feed of the 10, 1.0 and 0.1 ppm diets must have contained 7200, 720 and 72 g PBBs, respectively. Therefore, each of the last three figures represents the amount of PBBs ingested by each dam in each of the three groups over 18 days. To estimate the amount of PBBs passed to each of the pups during the nursing period, the concentration of PBBs in their mother's milk must be known. PBBs were found at a concentration of 10-15 ppm in the milk sample collected from a lactating rat between days two and nine following a single injection (i.p.) of 90 mg PBBs/kg body wt. (roughly 30 mg/rat), see Fig. 15. Assuming that a lactating rat produces 20 ml milk per day, it follows that 12-18% of the 30 mg PBBs dose is secreted in the milk over 18 days of nursing. On the other hand, over a month period, cows have been reported (86) to have secreted 2.22% of the total injested PBBs in their 50 ppm diet for two weeks. This value is much lower than what has been reported (87) for PCBs (Aroclor 1254) secretion in cows milk (21% of their daily intake of 12.5 ppm diet). Based on this last rate for PCBs secretion in cows milk, and the estimated 12-18% secretion of PBBs over 18 days after a single 90 mg PBBs/kg in the rat, it was assumed that rats on different regimens have secreted 15% of all the ingested PBBs over the 18 days nursing period. It is realized that such an assumption

overlooks the possibility that rats on the different diets might secrete different concentrations of PBBs in their milk. It follows that each rat passed 1080, 108, and 10.8 µg PBBs to its litter in the 10, 1.0, and 0.1 ppm groups, respectively over eighteen days. On per pup basis, these values amounted to 188, 9.2, and 1.2 µg PBBs in the same order just mentioned. These values correspond to: 20, 2.4, and 0.2 µg PBBs/g body weight for the dams on the 10, 1.0, and 0.1 ppm diets, respectively. Each of their corresponding pups injested 9.4, 0.46, and 0.06 µg/g body weight, respectively (20 g is considered the mean weight of each pup between 1-18 days old, where 5-6 g is its weight at birth and 33 g is its weight after 18 days). While the mother rat needed 20 µg PBBs/g body weight to have its microsomal enzymes significantly induced, 0.46 µg/g body weight was enough to elicit similar inductions in the pup. The obvious conclusion is that the rat pup is roughly forty times more sensitive to PBBs than its mother.

CHAPTER TWO

STUDIES ON THE *IN VITRO* MICROSOMAL METABOLISM OF POLYBROMINATED BIPHENYLS (PBBs)

ABSTRACT

Polybrominated biphenyls (PBBs), a mixture of twelve major components, are thought to be quite persistent in the body, even though nothing is known about their susceptibility to biotransformation. The metabolism of PBBs was therefore investigated by incubating Firemaster PBBs with rat liver microsomes in presence of NADPH and atmospheric O_2. Quantitative recoveries of all PBBs were obtained after incubations with control or 3-methylcholanthrene (MC) induced microsomes. Of the twelve major components, losses of only peaks 1 (2,4,5,2',5'-pentabromobiphenyl) and 3 (a hexabromobiphenyl) were observed following incubations with microsomes from phenobarbital (PB)- or PBBs-pretreated rats. Of seven structurally identified PBB components, only peak 1 has a bromine-free para position. Peaks 1, 2 and 5 all have two adjacent unsubstituted carbons, yet only peak 1 is metabolized. Of two dibromobiphenyl model compounds studied, the 2,2'-congener was very rapidly metabolized by PB-induced microsomes, whereas its 4,4'isomer was not. These results suggest that the presence of a free para position is required for the metabolism of brominated biphenyls. Of lesser importance appears to be the number of bromines or the availability of two adjacent unsubstituted carbons. In vivo evidence for the metabolism of peaks 1 and 3 was also provided by their drastically diminished levels in liver and milk extracts.

Also notable, in these extracts, was the elevated concentrations of peaks 2, 5 and 6. These variations may reflect differences in both metabolism and distribution among the PBB congeners.

INTRODUCTION

Induction of hepatic drug metabolizing mixed-function oxidases is caused by a wide variety of xenobiotics including drugs, pesticides as well as carcinogens (9,11,12) most of which are susceptible to metabolic conversion by the same inducible enzymes. In chapter one of this thesis, it was shown that PBBs cause significant enzymatic inductions at dietary levels of 10 ppm in adult female rats as well as in neonates nursing rats fed 10 or 1.0 ppm PBBs in the diet. My studies on adult female rats and their neonates (see chapter one) as well as studies by others (60-66) on adult rats have clearly shown that PBBs are mixed-type inducers similar to both PB and MC in their induction properties. However, no information is available, yet, on the metabolic fate of PBBs which are believed to be highly persistent in the body (58,70,86). Since the closely related PCBs also cause a mixed-type induction in the rat and since certain PCBs are metabolized by the hepatic mixed-function oxidases *in vivo* (89-91) as well as *in vitro* (100-103) it was necessary to look for evidence of the metabolism of PBBs by the same enzymes. Because the individual separate

components of PBBs (nonlabelled or ^{14}C-labelled) were not available, it was not possible to search for an evidence on the *in vivo* metabolism of PBBs by isolating their metabolites from animals excreta in a manner similar to what has been done on PCBs (89-91). For the same reason, it was not possible to isolate metabolites from *in vitro* studies utilizing liver microsomes supplemented with NADPH as has been done with certain PCBs (100-102). On the other hand, even if the individual components were available, the efforts of isolating their metabolites from *in vivo* or *in vitro* systems might have been tedious or nonproductive since it was expected that some PBB components, like certain PCBs (72,98,99,103) might be resistent to metabolism. An easier and faster way to identify the components that could be metabolized is to incubate Firemaster PBBs mixture in an *in vitro* microsomal system containing NADPH followed by extraction and analysis of the remaining PBB-components by gas chromatography. In the absence of metabolism all components should be quantitatively recovered under ideal extraction conditions with no change in the pattern of PBBs in comparison to a standard of PBBs. However, if certain components were metabolized, then the recovered PBBs would have a different pattern from that of a standard due to the reduced chromatographic responses resulting from metabolism.

The *in vitro* metabolism of PBBs was initially tested exclusively by using microsomes from PB-pretreated rats in addition to an NADPH-generating system identical to the one used for the aminopyrine metabolism assay as described in the first chapter. Incubations and extractions of PBBs were done as described under Materials and Methods except that PBBs were delivered in petroleum ether instead of PEG, and at the end of the incubations PBBs were extracted by petroleum ether also, instead of ethylacetate. However, several problems were encountered including large variations in recoveries and failure to duplicate the results. However, at a relatively early stage in this initial work, it was noticed that out of the recovered PBBs, peaks 1 and 3 seemed to be diminished in comparison to other components. Failure to reproduce these results as well as to achieve complete recoveries of PBBs made it difficult to reach any significant conclusions. A breakthrough was made, however, when it was decided to replace petroleum ether by PEG as a carrier for delivering PBBs to the microsomal incubations as well as using ethyl acetate for extracting PBBs at the end of the incubations. Due to the high viscosity and boiling point of PEG it has become possible to deliver exact amounts of PBBs (10 µg) in as little as 5 µl volumes of PEG, a requirement that was harder to meet when petroleum ether was used. On the other hand, the switch to ethyl acetate extraction was made after, in one experiment, it was decided to

further extract the microsomal system by ethyl acetate following the repeated (3 times) petroleum ether extraction. While petroleum ether extracted 60-70% of the added PBBs, futher extraction by ethyl acetate recovered most of the remaining PBBs to make the total recovery add up to greater than 95%. In the following experiments, in which petroleum ether was completely replaced by PEG as the PBBs carrier and by ethyl acetate as the extracting solvent, almost complete recoveries of all PBBs were achieved except for two components, namely peaks 1 (2,4,5,2',5'-pentabromobiphenyl) and 3 (a hexabromobiphenyl). After 1 hr incubation with PB- microsomes peaks 1 and 3 were significantly diminished in comparison to the same peaks at 0 time. That the loss of both components was dependent on the NADPH-dependent microsomal monooxygenases was shown by the next experiment in which $NADP^+$ was omitted from the incubation mixture. As shown in Table 1, NADPH was absolutely required for the metabolism of both peaks, a result that clearly implicates the involvement of the microsomal monooxygenase system containing cytochrome(s) P450.

Therefore, using an aerobic NADPH-generating system which contains rat liver microsomes, it has become possible to study the metabolism of brominated biphenyls by measuring their recoveries, by the GLC, before (at 0 time) and after (at t time) incubation where a loss in the recovery of a certain component after its incubation for a certain time is considered to be due to its metabolism.

TABLE 1

RECOVERIES OF PBBs FOLLOWING INCUBATIONS WITH PB-INDUCED MICROSOMES

Ten µg of Firemaster PBBs were aerobically incubated with 5 mg protein of PB-induced rat liver microsomes for one hour at 37°C in the presence and absence of NADPH. PBBs extractions and assays were performed as described in Materials and Methods. The GC profile of PBBs of Zero time incubation was identical to that of Firemaster PBBs, and the recoveries of all Zero time peaks were >85%. Each value is an average of two determinations and represents the per cent recovery after one hour incubation as compared to the corresponding value at Zero time. The average SEM in per cent recovery for the control incubations (-NADPH) were 6.6 and 3.4 for the Zero time and one hour incubations, respectively. For the incubations with NADPH, they were 1.6 and 3.0 for Zero time and one hour, respectively.

PEAK	+NADPH	-NADPH
1	41*	88
2	101	114
3	41*	94
4	111	95
5	104	95
6	104	106
7	104	105
8	113*	96
9	124	90
10	96	100
11	98	109
12	125	103

*Significantly different from those at Zero time ($p<0.05$).

MATERIALS AND METHODS

Chemicals

All materials used in this study were described under Chemicals of Chapter one.

Animals and Treatments

Male rats of the Sprague-Dawley strain were used and were obtained from Spartan Research Animals, Inc., Haslett, Mich. Control rats, weighing between 250-275 g, were not given any treatment before sacrifice. PBBs-pretreated rats were between 150-175 g when injected, i.p., with 90 mg PBBs/kg (in 2 ml PEG/kg) one week before sacrifice. Rats treated with PB weighed 225-250 g and were given four daily i.p. injections at 50 mg/kg (in 1 ml H_2O/kg) and killed on the fifth day. Rats given MC ranged between 75-100 g and were injected, i.p., with 20 mg MC/kg in 2 ml PEG/kg 36 and 24 hours before sacrifice. Pelleted feed and water were available ad libitum except for the night before sacrifice when only water was accessible.

Isolation of Microsomes

Microsomes were isolated and stored under the identical conditions used in Chapter one.

Collection of Rat's Milk and Its Extraction for PBBs

One day after parturition, lactating rats weighing about 300 g were injected, i.p., either with 90 mg PBBs/kg (in 1.5 ml corn oil/kg) or with the equivalent amount of corn oil. Milk was obtained on the afternoons of days

2,4,6,7 and 9 after the injections. The samples from each dam were pooled together for later extraction. The dams were separated from their litters 2-3 hours before the milking process. Milk ejection was stimulated with a 0.15 ml i.p. injection of oxytocin (pitocin), 10 U/ml. Five minutes later the rat was lightly anesthesized by injecting 0.1-0.15 ml of 60 mg/ml sodium pentobarbital. Milk was collected by gentle suction using a simple hand-made suction apparatus employing a water aspirator. Solvent extractions and acetonitrile partitioning of PBBs in the milk were done according to the Pesticide Analytical Manual procedure for extraction of organochlorine residues from milk (125) which was scaled down for samples of 2-3 ml. The extracts were run over a small Florisil column and analyzed for PBBs by a Hewlett-Packard 402 gas chromatograph. The gas chromatograph was equipped with a pulsed-^{63}Ni electron capture detector, fitted with a 6 ft. glass column, and operated at 270°C. 95% Argon-5% methane was used both as a carrier and purge gas.

In Vitro Microsomal Metabolism of PBBs

The NADPH-dependent metabolism of PBBs was studied by assaying for their time-dependent disappearance when incubated with liver microsomes. Microsomal incubations were carried out aerobically at 37°C in 50 mM Tris-HCl buffer, pH 7.5, in the presence of an NADPH generating system consisting of 5 µM $MnCl_2$, 5 mM $MgCL_2$, 0.5 mM $NADP^+$, 4 mM D,L-isocitrate, and 0.5 units of isocitrate dehydrogenase.

The microsomes were added at 1 mg protein/ml except for the metabolism of peaks 8 where 2 mg protein/ml was used. The following amounts of substrates were delivered in 5 µl PEG: 10 µg PBBs, and roughly 0.27 µg peak 1, 0.37 µg peak 3, 0.5 µg peak 4, 1 µg peak 8 and 50 µg of either 2,2'- or 4,4'-dibromobiphenyls. Incubations were carried out in a Dubnoff shaker, and three 10 ml ethyl acetate extractions were performed at the end of each incubation period, as well as for the 0 time, non-incubated samples. The extracts were water washed and the organic solvent dried over sodium sulfate before evaporation to about 1 ml. The concentrated extract was then run on a small Florisil column to remove undesirable polar material and concentrated to a suitable volume before GLC analysis. For the analysis of the dibromobiphenyls the column temperature was reduced to 200°C. The recoveries of the different bromobiphenyls were determined by cutting and weighing their corresponding gas chromatographic peaks. Since the gas chromatographic detector responses for peaks 1 and 3 are not known, the response to pure peak 4 was used to quantitate these congeners, assuming all three responses to be identical. All experiments were run in duplicate and the difference between the means of 0-time and t-time recoveries for each peak was statistically analyzed by the student's t-test, $p < 0.05$. The PBBs present in the liver microsomes from PBBs pretreated rats were extracted and analyzed by the same procedure.

RESULTS

In Vitro Metabolism Of PBBs by Different Types of Microsomes

The NADPH-dependent metabolism of PBBs was studied aerobically using different types of induced microsomes. Microsomes from PB- or PBBs-pretreated rats were found to cause preferential losses of the same two components, namely peaks 1 and 3 (penta- and hexa-bromobiphenyls, respectively) from the PBBs mixture in a time-dependent manner. These two components are estimated to be present in approximately 2 and 1%, respectively, of the total PBBs mixture. In the PBBs mixture, the PB-induced microsomes metabolized peak 1 at a maximal rate of 1.6 pmoles/min. mg protein during the period between 15 and 30 minutes, while peak 3 was maximally metabolized during the first 15 minutes at a rate of 0.9 pmoles/min.mg protein (Fig. 9). Microsomes from PBBs-pretreated rats metabolized the same two PBB components at activities of 2.4 and 0.9 pmoles/min.mg protein, respectively, during the same time periods (Fig. 10). Microsomes from control or MC-pretreated rats were unable to significantly affect the recovery of any component of the PBBs mixture (Figs. 11&12).

To determine whether or not metabolism is affected by the presence of the other PBB components the metabolism of several pure congeners was investigated (Fig. 13). Peak 1 at a purity close to 80% (the rest being peak 4) had a maximal rate of metabolism of 1.7 pmoles/min.mg protein achieved at the first 15 minutes. Peak 3 with

Figure 9. TIME COURSE FOR THE METABOLISM OF PBBs BY LIVER MICROSOMES FROM PB-PRETREATED RATS.

Aerobic incubations for different times were carried out separately in 5 ml total volume. Each incubation mixture contained the NADPH-generating components described under Materials and Methods, 5 mg liver microsomal protein and 10 μg PBBs delivered in 5 μl PEG. PBBs extractions and assays were performed as described in Materials and Methods. Each point represents an average of two determinations. Peaks 1 and 3 at all time points were statistically different from those at 0 time ($p < 0.05$). All other peaks at all time points were not statistically different.

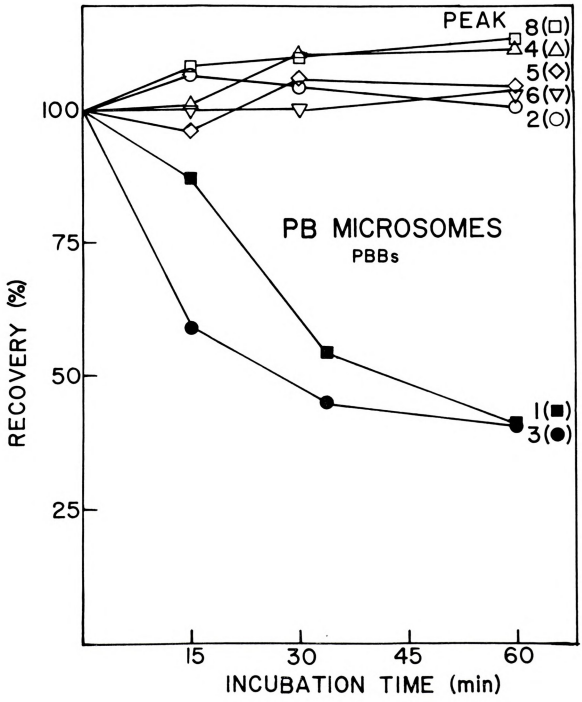

PEAK
8(□)
4(△)
5(◇)
6(▽)
2(○)
1(■)
3(●)
PB MICROSOMES
PBBs
RECOVERY (%)
100
75
50
25
15
30
45
60
INCUBATION TIME (min)

Figure 10. TIME COURSE FOR THE METABOLISM OF PBBs BY LIVER MICROSOMES FROM PBBs-PRETREATED RATS.

Details as in Figure 9. Peaks 1 and 3 at all time points were statistically different from those at 0 time ($p<0.05$). All other peaks at all time points were not statistically different from their corresponding values at 0 time ($p<0.05$).

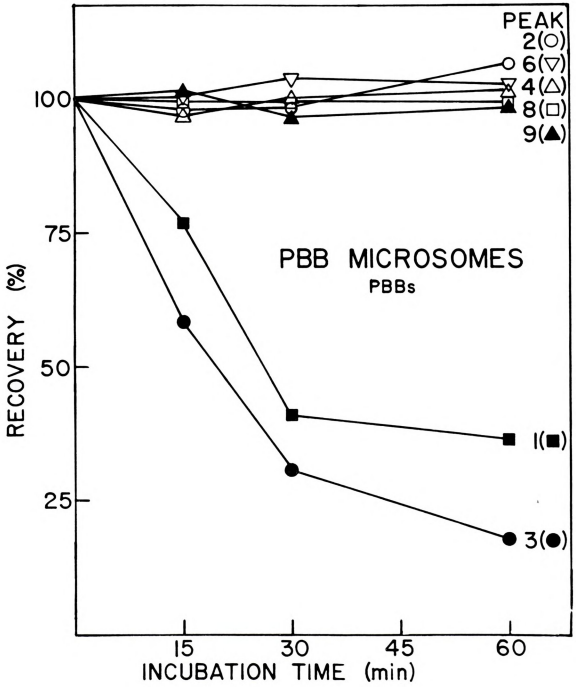

PEAK
2(○)
6(▽)
4(△)
8(□)
9(▲)
100
75
50
25
RECOVERY (%)
PBB MICROSOMES
PBBs
1(■)
3(●)
15
30
45
60
INCUBATION TIME (min)

Figure 11. TIME COURSE FOR THE METABOLISM OF PBBs BY LIVER MICROSOMES FROM CONTROL RATS.

Details as in Figure 9. All peaks at all time points were not statistically different from their corresponding values at 0 time ($p<0.05$).

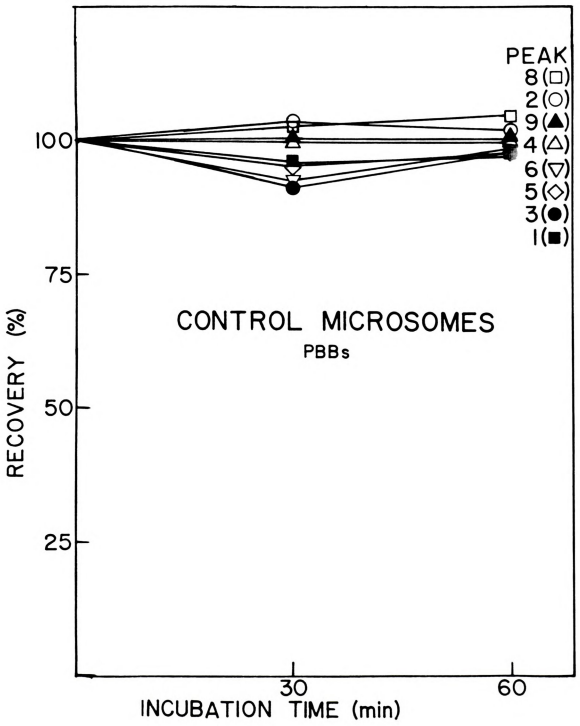
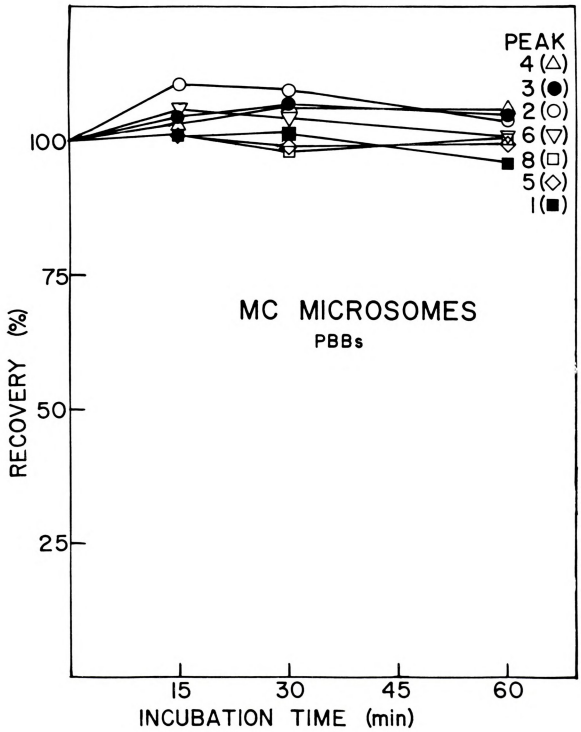

PEAK
8(□)
2(○)
9(▲)
4(△)
6(▽)
5(◇)
3(●)
1(■)
100
75
50
25
RECOVERY (%)
CONTROL MICROSOMES
PBBs
30
60
INCUBATION TIME (min)

Figure 12. TIME COURSE FOR THE METABOLISM OF PBBs BY LIVER MICROSOMES FROM MC-PRETREATED RATS.

Details as in Figure 9. All peaks at all time points were not statistically different from their corresponding values at 0 time ($p<0.05$).


PEAK
4 (△)
3 (●)
2 (○)
6 (▽)
8 (□)
5 (◇)
1 (■)
100
75
50
25
RECOVERY (%)
MC MICROSOMES
PBBs
15
30
45
60
INCUBATION TIME (min)

an over 85% purity (slightly contaminated by each of peaks 1, 4, 5, 6, 7 and 8) was metabolized at a maximal rate of 3 pmoles/min.mg protein also during the first 15 minutes. No significant losses of pure peaks 4 or 8 were observed. When an incomplete NADPH-generating system (-$NADP^+$) was used all peaks were recovered almost quantitatively after one hour incubation with PB-induced microsomes (Table 1).

In Vitro Metabolism of Two Dibromobiphenyls by Microsomes From PB-Pretreated Rats

Incubations of two dibromobiphenyl model congeners with PB-induced microsomes resulted in quantitative recovery of 4,4'-dibromobiphenyl, whereas its 2,2'-dibromo isomer was very rapidly metabolized (Fig. 14). In fact, about 160 nmoles had disappeared after the first time point was taken (15 minutes). Studies by others indicated that the biphenyl is metabolized at 3 nmoles/min.mg protein, while 2,2'-dichlorobiphenyl is metabolized at a rate 2/3 that of the biphenyl (103).

DISCUSSION

The studies presented in Chapter one, as well as studies of others (60-66), have shown that PBBs are potent inducers of the liver drug metabolizing enzymes, and that they can cause a mixed-type induction similar to that caused by treatment with both PB and MC together, or to treatment by certain PCB mixtures (26,75-78). However, the metabolic fate of the brominated biphenyls is not

Figure 13. TIME COURSE FOR THE METABOLISM OF PURIFIED PBB PEAKS 1, 3, 4, and 8 BY LIVER MICROSOMES FROM PB-PRETREATED RATS.

Details as in Figure 9 except that 10 mg microsomal protein was used for peak 8 metabolism. The following rough amounts were used of peaks 1, 3, 4 and 8: 0.27, 0.37, 0.5 and 1 μg, respectively. All time points for peaks 1 and 3 were statistically different from those at 0 time ($p<0.05$). All time points for peaks 4 and 8 were not statistically different from their corresponding values at 0 time ($p<0.05$).

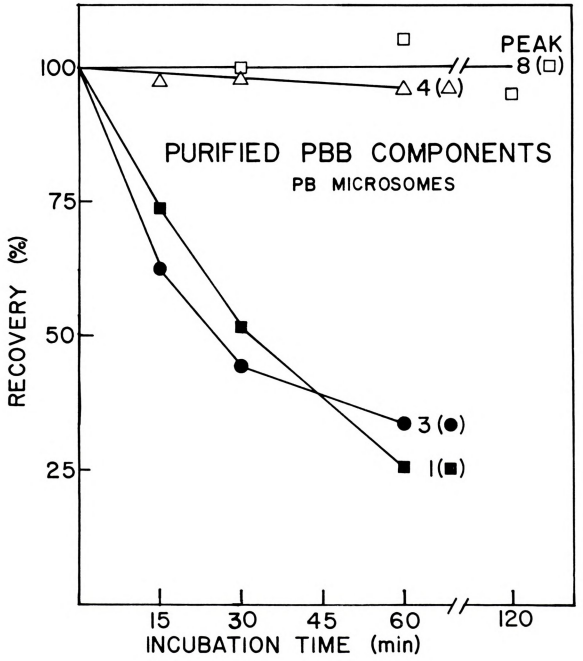
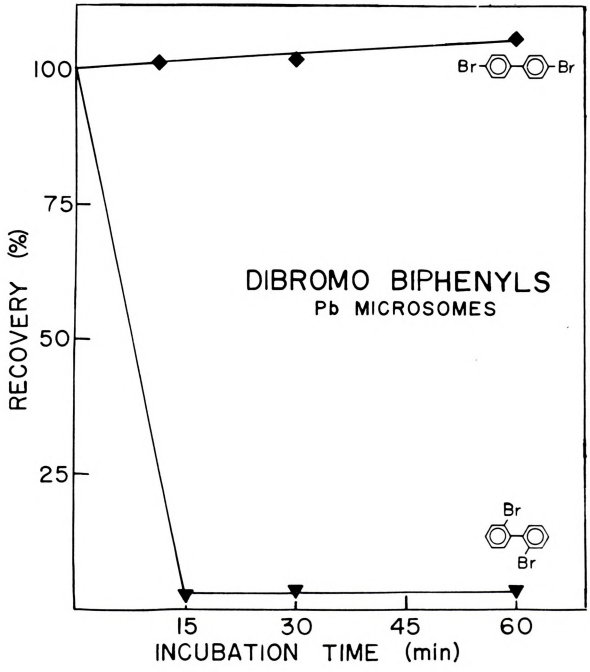

PEAK
8 (□)
4 (△)
PURIFIED PBB COMPONENTS
PB MICROSOMES
100
75
50
25
RECOVERY (%)
3 (●)
1 (■)
15
30
45
60
120
INCUBATION TIME (min)

Figure 14. TIME COURSE FOR THE METABOLISM OF TWO DIBROMOBIPHENYL MODEL COMPOUNDS BY LIVER MICROSOMES FROM PB-PRETREATED RATS.

Experimental conditions were similar to those described for Figure 9 except that each of the two dibromobiphenyls was added to a final concentration of 34 mM.


100
75
50
25
RECOVERY (%)
DIBROMO BIPHENYLS
Pb MICROSOMES
Br
Br
Br
Br
15
30
45
60
INCUBATION TIME (min)

known but at least certain PBBs are suspected to be metabolized into hydroxy derivatives by the microsomal mixed-function oxidases in a manner similar to PCBs (89-91,100-103). The metabolic hydroxylation of PCBs was found to be dependent not only on the number of chlorines on the biphenyl nucleus (89,100,103) but also on their distribution (72,98,99,101). A model PBB compound, 4,4'-dibromobiphenyl, (not known to be present in the PBBs mixture) was found to be metabolized in vivo into hydroxylated derivatives in a manner suggestive of an arene-oxide intermediate (91). Certain PCBs (90,91,93) as well as other aromatic compounds (92) are known to be metabolized via arene-oxides which can covalently bind to macromolecules such as protein, DNA and RNA (92,126,127). The covalent binding of the arene-oxides to the cellular constituents, especially DNA, is thought to be one way of causing cell mutation which may ultimately lead to cancer. Therefore, the metabolism of the aromatic compounds, may not be absolutely harmless.

Even though the experiments reported in this chapter on the in vitro metabolism of PBBs are not directly concerned with the identity of the PBB metabolites they still clearly show that two out of the twelve major PBB-components can readily be metabolized by the liver microsomal enzymes. The gas chromatographic profile of the PBBs mixture used for the in vitro metabolism studies is shown in Fig. 1. Also the numbering system for the different components

is shown along with the bromine content and structures of the identified PBBs. The microsomal enzymes from control rats were incapable of metabolizing any of the PBBs in the presence of NADPH (Fig. 11). This finding agrees with the inactivity of control rat microsomes to metabolize those PCBs that PB-induced microsomes could (100), including the chlorinated homolog of PBBs peak 1 [2,4,5,2',5'-pentabromobiphenyl(53)]. However, microsomes from PB- or PBBs-pretreated rats were able to cause time-dependent losses of two peaks out of the twelve major PBB components. These two components, peaks 1 (2,4,5,2',5'-pentabromobiphenyl) and 3 (a hexabromobiphenyl), are estimated to be present in approximately 2 and 1%, respectively of the total PBBs mixture, assuming they have a similar GLC response to peak 4 (2,4,5,2',4',5'-hexabromobiphenyl). Of the mixture, peak 3 was metabolized at the same maximum rate of 0.9 pmoles/min.mg protein by both PB- or PBBs-induced microsomes during the first 15 minutes. However, the metabolism of peak 1 did not reach its maximum rate of 1.6 and 2.4 pmoles/min.mg protein by PB- and PBBs-induced microsomes, respectively, until the second 15 minutes period. Therefore, it appears that the first and third components of PBBs are metabolized by the same hepatic monooxygenase system namely that of the PB-type, and that the third component (a hexabromobiphenyl) is the preferred substrate. In the mixture, as the concentration of peak 3 goes down the rate of peak 1 metabolism goes up to reach its maximum

value during the second 15 minutes, after most of peak 3 is already gone. However, the maximum rate of metabolism of peak 3 (0.9 pmoles/min.mg protein) is lower than that of peak 1 (1.6 pmoles/min.mg protein) a result that could be due to the presence of at least twice of peak 1 as compared to peak 3 in the mixture. Support of this has been provided by the results on the metabolism of partially purified peaks 1 and 3 by PB-induced microsomes. In the absence of peak 3, partially purified peak 1 (80% pure) at a concentration similar to that of the same peak used in the mixture, was metabolized maximally during the first 15 minutes. However, its metabolic rate was almost identical to that of peak 1 in the mixture (1.7 vs. 1.6 pmoles/min.mg protein, respectively), a finding that may exclude the possibility of competition for the same monooxygenase(s) by the other PBBs. On the other hand, purified peak 3 (85% pure) underwent metabolism at a rate of 3 pmoles/min.mg protein, a value more than three times that of the same component in the PBBs mixture. However, the fact that it was used at a concentration more than three times of that present in the mixture argues against the possibility of competition by the other PBBS.

The absolute requirement for NADPH lends direct support for the involvement of the microsomal monooxygenase system containing cytochrome P450 (15). It also appears that the enzymes involved in the metabolism of these two PBB components are of the type induced by PB. Even though

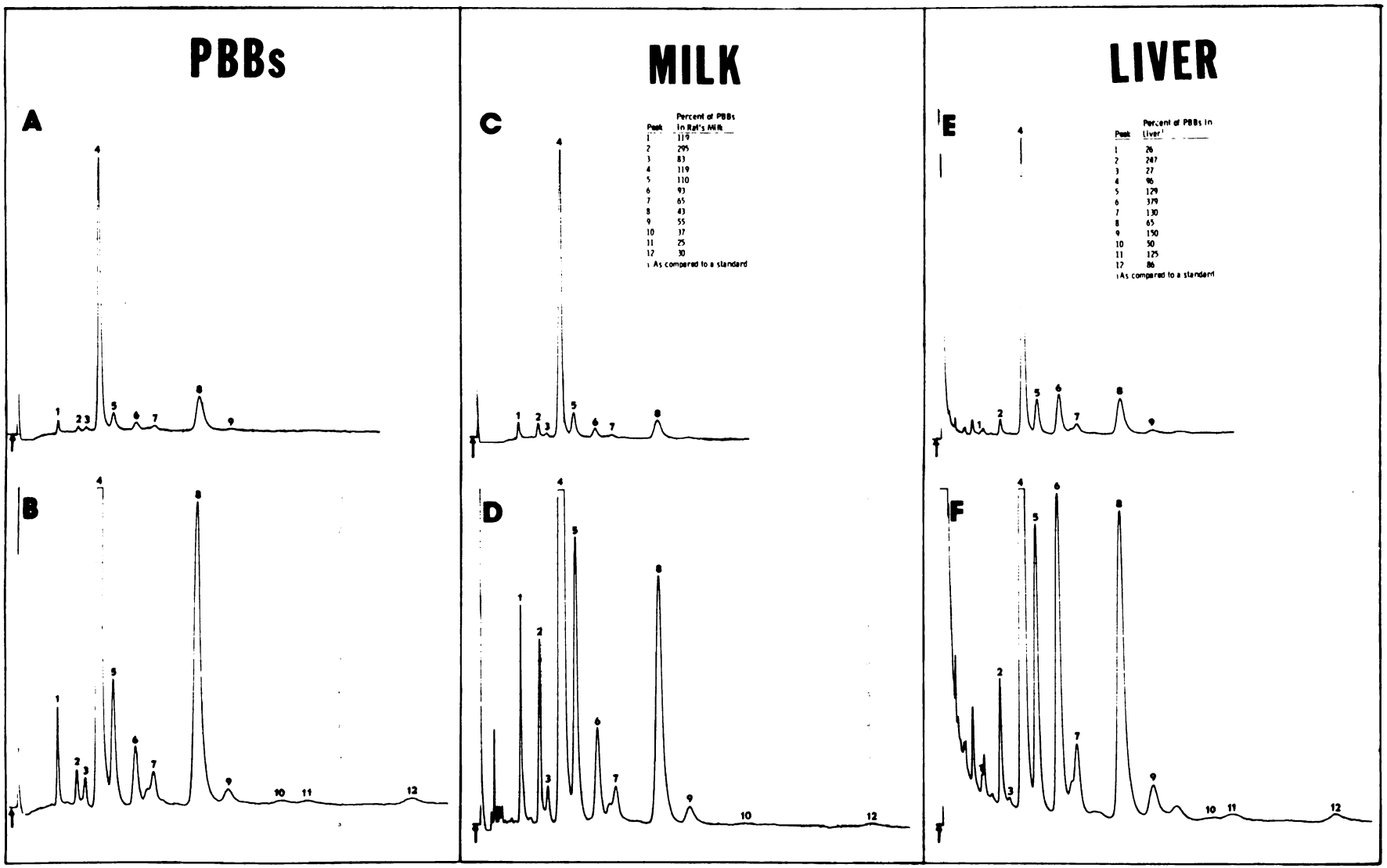
PBBs are known to cause a mixed-type induction (60-66), microsomes from MC-treated rats seemed incapable of metabolizing any of the major components of PBBs. It is clear, therefore, that the PB-type induced enzymes in microsomes from PBBs-treated rats are responsible for metabolizing those two PBB components. These findings appear to be in rough agreement with the results from studies on the covalent binding of a ^{14}C-PBB mixture, comprised almost exclusively of peaks 4 and 8, to microsomes following in vitro incubations under conditions very similar to those used in these studies (53). Even though the ^{14}C-PBB mixture used was lacking in peaks 1 and 3, microsomes from PB- or PBBs-pretreated rats had traces of associated radioactivity (0.05% of added substrate) that was significantly higher than the radioactivity associated with MC-induced microsomes and especially control microsomes (53). On the other hand, there was no binding to exogenously added DNA following similar in vitro incubations in presence of different types of microsomes. This seems to agree with the results on the lack of metabolism of peaks 4 and 8, almost the only constituents of the ^{14}C-PBB mixture, where binding to DNA usually requires the metabolic activation of aromatic compounds (92,126,127).

The results on the in vitro metabolism of PBBs seem to indicate that the bromine content of the molecule is not as important as is the distribution of bromines on the biphenyl nucleus. The metabolism of peak 1 (2,4,5,2',5'-pentabromobiphenyl) seems to agree with the metabolism

of the same pentachlorinated molecule into hydroxylated derivatives in vitro (100) as well as in vivo (128). Peak 2, the second pentabrominated component of PBBs, with bromines at the 2,4,5,3', and 4' positions was found to be resistent to metabolism. On the other hand, the same pentachlorinated biphenyl homologe was noticed to be persistent in tissues from humans (72) as well as chickens (98). Peaks 3 through 6 are all hexa brominated biphenyls, but only peak 3 is metabolized. Unfortunately, at the time this thesis was written, the structure of peak 3 was not known, but all the seven PBBs whose structures are known so far have a bromine at each of the para positions except peak 1 which has one bromine-free para position. On the other hand, each of peaks 1, 2 and 5 has two adjacent unsubstituted carbons yet only peak 1 is metabolized. These results seem to agree with the persistence in human tissue of 2,4,5,2',4',5'- and 2,3,4,2',4',5'-hexachlorobiphenyls (the two equivalents of peaks 4 and 5 in PBBs, respectively) and the apparent resistence to in vivo metabolism of all those PCBs with a 4,4'- substitution in avians (72,98,99). Therefore, it appears that the presence of two adjacent unsubstituted carbons is not sufficient by itself to render the brominated biphenyl molecule vulnerable to microsomal oxidation. Bromination at both para positions seems to render the PBB molecule resistant to microsomal metabolism regardless of the number of bromines or their distribution on the biphenyl nucleus, or the presence of two adjacent unsubstituted carbons.

Figure 15. THE DISTRIBUTION OF PBBs IN FIREMASTER (A,B), MILK (C,D), AND LIVER MICROSOMES (E,F).

A and B represent the GC responses to 15 and 100 ng PBBs injections, respectively.



Peak	Percent of PBBs in Rat's Milk
1	119
2	295
3	83
4	119
5	110
6	93
7	65
8	43
9	55
10	37
11	25
12	30

1 As compared to a standard

Peak	Percent of PBBs in Liver[1]
1	26
2	247
3	27
4	96
5	129
6	379
7	130
8	65
9	150
10	50
11	125
12	86

1 As compared to a standard

Of the two model dibromobiphenyls studied, the 2,2'-dibromobiphenyl was rapidly metabolized while its 4,4'-isomer was not metabolized. This may lend further support for the necessity of a free *para* position to render the halogenated biphenyl susceptible to metabolism. The rapid metabolism of 2,2'-dibromobiphenyl agrees with the results on the rapid metabolism of 2,2'-dichlorobiphenyl by microsomes from both PB-pretreated female rats (101) and male rabbits (103). In the first study, there was a reduction in the metabolism and diversity of the hydroxylated metabolites when 2,4'-dichlorobiphenyl was used instead of the 2,2'-dichlorobiphenyl isomer.

Data was obtained to correlate *in vitro* metabolism with *in vivo* tissue levels of the various PBBs. Even though the distribution of PBBs in rat milk and male rat liver microsomes are not the same, they both are significantly changed from standard PBBs (Fig. 15). Most noticeable is the preferential reduction in peaks 1 and 3 in rat liver microsomes and, to a lesser extent, in rat milk especially when both components are compared to peak 2. It is also worth noting that the hepta- (peaks 8-10) and octa- (peaks 11 and 12) brominated biphenyls are reduced in the milk as well as peaks 8 and 10 in the liver. Furthermore, peaks 2, 5 and 6 seem to be preferentially enriched in both milk and liver. It should be pointed out that all of these observations could be due to differences in the distribution of the PBB components in different

animal tissues rather than to their metabolic conversion per se.

Although the present studies on the *in vitro* metabolism of PBBs do not reveal the identity of the metabolites, they still provide enough evidence on the ability of PB- or PBBs-induced microsomes to preferentially metabolize peaks 1 and 3. Even though the structure of peak 3 is not known yet, the data in general seem to indicate that the presence of a bromine-free *para* position on a brominated biphenyl molecule is the primary requirement for its metabolism. Future studies should concentrate on isolating and identifying the metabolites resulting from peaks 1 and 3 in order to gain more insight into the toxicity and metabolism of PBBs.

REFERENCES

REFERENCES

1. Kappas, A. and Alvares, A.P. (1975) Sci. Am., 232, No. 6, 22.

2. Gillett, J.R. (1966) Adv. in Pharmacol., 4, 219.

3. Sperber, I. (1963) Proc. 1st Int. Pharmacol. Meet., 4, Drugs and Membranes 137 (Hogben, C.A.M., ed.), Pergamon Press Ltd., London.

4. Millburn, P., Smith, R.L. and Williams, R.T. (1967) Biochem. J., 105, 1275.

5. Wada, F., Slibata, H., Goto, M. and Sakamoto, Y. (1968) Biochim. Biophys. Acta, 162, 518.

6. Tephly, T.R. and Mannering, G.J. (1968) Mol. Pharmacol., 4, 10.

7. Wakabayashi, K. and Shimazono, N. (1963) Biochim. Biophys. Acta, 70, 132.

8. Abou-El-Makarem, M.M., Millburn, P., Smith, R.L. and Williams, R.T. (1967) Biochem. J., 105, 1269.

9. Conney, A.H. and Burns, J.J. (1972) Science, 178, 576.

10. Kuntzman, R. (1969) An. Rev. Pharmacol., 9, 21.

11. Conney, A.H. (1967) Pharmacol. Rev., 19, 317.

12. Sher, S.P. (1971) Toxicol. Appl. Pharmacol., 18, 780.

13. Brown, R., Miller, J.A. and Miller, E.C. (1954) J. Biol. Chem., 209, 211.

14. Mueller, G.C. and Miller, J.A. (1953) J. Biol. Chem., 202, 579.

15. Mason, H.S. (1957) Science, 125, 1185.

16. Posner, H.S., Mitoma, C., Rothberg, S. and Udenfriend, S. (1961) Arch. Biochem. Biophys., 94, 280.

17. Omura, T. and Sato, R. (1964) J. Biol. Chem., 239, 2370.

18. Klingenberg, M. (1958) Arch. Biochem. Biophys., 75, 376.

19. Garfinkel, D. (1958) Arch. Biochem. Biophys., 77, 493.

20. Imai, Y. and Sato, R.J. (1967) J. Biochem., 62, 239.

21. Lu, A.Y.H. and Coon, M.J. (1968) J. Biol. Chem., 243, 1331.

22. Lu, A.Y.H., Strobel, H.W. and Coon, M.J. (1969) Biochem. Biophys. Res. Comm., 36, 545.

23. Strobel, H.W., Lu, A.Y.H., Heidema, J. and Coon, M.J. (1970) J. Biol. Chem., 245, 4851.

24. Lu, A.Y.H., Levin, W. and Kuntzman, R. (1974) Biochem. Biophys. Res. Comm., 60, 266.

25. Lu, A.Y.H., Junk, K.W. and Coon, M.J. (1969) J. Biol. Chem., 244, 3714.

26. Alvares, A.P., Bickers, D. and Kappas, A. (1973) Proc. Nat. Acad. Sci., 70, 1321.

27. Lu, A.Y.H., Kuntzman, R., West, S., Jacobson, M. and Conney, A.H. (1972) J. Biol. Chem., 247, 1727.

28. Pederson, T. and Aust, S.D. (1970) Biochem. Pharmacol., 19, 2221.

29. Aust, S.D. and Stevens, J.B. (1971) Biochem. Pharmacol., 20, 1061.

30. Creaven, P.J. and Parke, D.V. (1966) Biochem. Pharmacol., 15, 7.

31. Welton, A.F. and Aust, S.D. (1974) Biochem. Biophys. Res. Comm., 56, 898.

32. Lu, A.Y.H., Levin, W., West, S.B., Jacobson, M., Ryan, D., Kuntzman, R. and Conney, A.H. (1973) J. Biol. Chem., 248, 456.

33. Thomas, P.E., Lu, A.Y.H., Ryan, D., West, S.B., Kawalek, J. and Levin, W. (1976) Mol. Pharmacol., 12, 746.

34. Warner, M., Vella La Marca, M. and Neims, A.H. (1977) Fed. Proc., 36, Abst. 3804.

35. Guengerich, F.P. (1977) J. Biol. Chem., 252, 3970.

36. Rubin, A., Tephly, T.R. and Mannering, G.J. (1964) Biochem. Pharmacol., 13, 1007.

37. Netter, K.J. and Seidel, G. (1964) J. Pharmacol. Exp. Therap., 146, 61.

38. Quinn, G.P., Axelrod, J. and Brodie, B.B. (1958) Biochem. Pharmacol., 1, 152.

39. Kato, R. and Gillette, J.R. (1965) J. Pharmacol., 150, 279.

40. Jondorf, W.R., Maickel, R.P. and Brodie, B.B. (1958) Biochem. Pharmacol., 1, 352.

41. Fouts, J.R. and Adamson, R.H. (1959) Science, 129, 897.

42. Kats, R., Vassanelli, P., Frontino, G. and Chiesara, E. (1964) Biochem. Pharmacol., 13, 1037.

43. Soyka, L.F. (1969) Biochem. Pharmacol., 18, 1029.

44. Basu, T.K., Dickerson, J.W.T. and Parke, D.V.W. (1971) Biochem. J., 124, 19.

45. Gram, T.E., Guarino, A.M., Schroeder, D.H. and Gillette, J.R. (1969) Biochem. J., 113, 681.

46. Fouts, J.R. and Devereux, T.R. (1972) J. Pharmacol., 183, 458.

47. Guenthner, T.M. and Mannering, G.J. (1977) Biochem. Pharmacol., 26, 567.

48. Guenthner, T.M. and Mannering, G.J. (1977) Biochem. Pharmacol., 26, 577.

49. Parke, D.V. (1975) In Enzyme Induction (Parke, D.V., ed.), 207-271, Plenum Press, N.Y.

50. Breckenridge, A. (1975) In Enzyme Induction (Parke, D.V., ed.), 273-301, Plenum Press, N.Y.

51. Symposium on PCBs (1972) Environ. Health Perspect., Exp. Issue 1 (April), NIEHS, Research Triangle Park, N.C.

52. Carter, L.J. (1976) Science, 192, 240.

53. Moore, R.W. (1978) Ph.D. Thesis, Michigan State University, East Lansing, Michigan.

54. Dunckel, A.E. (1975) J. Am. Vet. Med. Assoc., 167, 838.

55. Mercer, H.D., Teske, R.H., Condon , R.J., Furr, A., Meerdink, G., Buck, W. and Fries, G. (1976) J. Toxicol. Environ. Health, 2, 335.

56. Kay, K. (1977) Environ. Res., 13, 74.

57. Jackson, T.F. and Halbert, F.L. (1974) J. Am. Vet. Med. Assoc., 165, 437.

58. Aftosmis, J.G., Culik, R., Lee, K.P., Sherman, H., and Waritz, R.S. (1972) Toxicol. Appl. Pharmacol., 22, 316 (Abst. 110).

59. Lee, K,P., Hebert, R.R., Sherman, H., Aftosmis, J.G. and Waritz, R.S. (1975) Arch. Environ. Health, 30, 465.

60. Troisi, C.L. (1975) M.S. Thesis, Michigan State University, East Lansing, Michigan.

61. Dent, J.G., Netter, K.J. and Gibson, J.E. (1976) Res. Comm. Chem. Path. Pharmacol., 13, 75.

62. Dent, J.G., Netter, K.J. and Gibson, J.E. (1976) Toxicol. Appl. Pharmacol. 38, 237.

63. Dent, J.G., Cagen, S.Z., McCormack, K.M., Rickert, D.E. and Gibson, J.E. (1977) Life Sciences, 20, 2075.

64. Dent, J.G., McCormack, K.M., Cagen, S.Z. and Gibson, J.E. (1977) Toxicol. Abstracts, Toronto, Abst. 40.

65. Moore, R.W., Sleight, S.D. and Aust, S.D. (1978) Toxicol. Appl. Pharmacol., in press.

66. Moore, R.W., Sleight, S.D. and Aust, S.D. To be submitted to Toxicol. Appl. Pharmacol.

67. Corbett, T.H., Beaudoin, A.R., Cornell, R.G., Anver, M.R., Schumacher, R., Endres, J. and Szwabowska, M. (1975) Environ. Res., 10, 390.

68. Beaudoin, A.R. (1978) Environ. Health Perspect., in press.

69. Cecil, H.C., Harris, S.J. and Bitman, J. (1978) Environ. Health Perspect., in press.

70. Rickert, D.E., Dent, J.G., Cagen, S.Z., McCormack, K.M., Melrose, P. and Gibson, J.E. (1978) Environ. Health Perspect., in press.

71. Risebrough, R.W., Rieche, P., Peakall, D.B., Herman, S.G. and Kirven, M.N. (1968) Nature, 220, 1098.

72. Jensen, S. and Sundstrom, G. (1974) Ambio, 3, 70.

73. Acker, L. and Schulte, E. (1970) Naturwissenschaften, 57, 497.

74. Curley, A., Burse, V.W., Grim, M.E., Jennings, R.W. and Linder, R.E. (1971) Environ. Res., 4, 481.

75. Chen, T.S. and DuBios, K,P. (1973) Toxicol. Appl. Pharmacol., 26, 504.

76. Bickers, D.R., Harber, L.H., Kappas, A. and Alvares, A.P. (1972) Res. Comm. Chem. Path. Pharmacol., 3, 505.

77. Goldstein, J.A., Hickman, P., Burse, V.W. and Bergman, H. (1975) Toxicol. Appl. Pharmacol., 32, 461.

78. Ryan, D., Thomas, P.E. and Levin, W. (1977) Fed. Proc., 36, Abst. 3806.

79. Johnstone, C.J., Echobicon, D.J. and Hutzinger, O. (1974) Toxicol. Appl. Pharmacol., 28, 66.

80. Goldstein, J.A., Hickman, P., Bergman, H., McKinney, J.D. and Walker, M.P. (1977) Chem.-Biol. Interact., 17 69.

81. Turner, J.C. and Green, R.S. (1974) Bull. Environ. Contamin. Toxicol., 12, 669.

82. Villeneuve, D.C. and Grant, D.L. (1971) Bull. Environ. Contamin. Toxicol., 6, 120.

83. Kimbrough, R.D. (1974) Critical Rev. in Toxicol., 2, 445.

84. Catz, C.S. and Giacoia, G.P. (1972) Ped. Clin. N. Amer., 19, 151.

85. Fries, G.F. and Marrow, G.S. (1975) J. Dairy Sci., 58, 947.

86. Gutenmann, W.H. and Lisk, D.L. (1975) J. Agric. Food Chem., 23, 1005.

87. Fries, G.F., Marrow, G.S. and Gordon, C.H. (1973) J. Agric. Food Chem., 21, 117.

88. Gardner, A.M., Righter, H.F. and Roach, J.A.G. (1976) J. Assoc. Offic. Analyt. Chemists, 59, 273.

89. Hutzinger, O., Nash, D.M., Safe, S., DeFreitas, A.S.W., Norstrom, R.J., Wildish, D.J. and Zitko, V. (1972) Science, 178, 312.

90. Gardner, A.M., Chen, J.T., Roach, J.A.G. and Ragelis, E.P. (1973) Biochem. Biophys. Res. Comm., 55, 1377.

91. Safe, S., Jones, D. and Hutzinger, O. (1976) J. Amer. Chem. Soc. (Perkin Transactions I) 357.

92. Jerina, D.M. and Daly, J.W. (1974) Science, 185, 573.

93. Safe, S., Hutzinger, O. and Jones, D. (1975) J. Agr. Food Chem., 23, 851.

94. Block, W.D. and Cornish, H.H. (1959) J. Biol. Chem., 234, 3301.

95. Yoshimura, H. and Yamamoto, H. (1973) Chem. Pharm. Bull., 21, 1168.

96. Yoshimura, H., Yamamoto, H. and Saeki, S. (1973) Chem. Pharm. Bull., 21, 2231.

97. Jondorf, W.R., Parke, D.V. and Williams, R.T. (1955) Biochem. J., 61, 512.

98. Bush, B., Tumasonis, C.F. and Baker, F.D. (1974) Arch. Environ. Contamin. Toxicol. 2, 195.

99. DeFreitas, A.S. and Norstrom, R.J. (1974) Can. J. Physiol. Pharmacol., 52, 1080.

100. Ghiasuddin, S.M., Menzer, R.E. and Nelson, J.O. (1976) Toxicol. Appl. Pharmacol., 36, 187.

101. Greb, W., Klein, W., Coulston, F., Golberg, L. and Korte, F. (1975) Bull. Environ. Contamin. Toxicol. 13, 424.

102. Holm, S. (1977) First Internat. Cong. on Toxicol., Abstracts, Toronto, p. 24.

103. Hesse, S. and Wolff, T. (1977) Biochem. Pharmacol., 26, 2043.

104. Stonard, M.D. (1975) Biochem. Pharmacol., 24, 1959.

105. Stonard, M.D. and Grieg, J.B. (1976) Chem.-Biol. Interact., 15, 365.

106. Sundstrom, G., Hutzinger, O. and Safe, S. (1976) Chemosphere, 11.

107. Jacobs, L.W., Chou, S.F. and Tiedje, J.M. (1976) J. Agric. Food Chem., 24, 1198.

108. Welton, A.F. and Aust, S.D. (1974) Biochim. Biophys. Acta, 373, 197.

109. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem., 193, 265.

110. Rutter, W.J. (1967) In Methods in Developmental Biology (Wilt, F.H. and Wessells, N.K., eds.) p. 671, Thomas Y. Crowell Co., N.Y.

111. Moore, R.W., Welton, A.F. and Aust, S.D. (1977) In Methods in Enzymology, Vol. 51 Biomembranes C - Biological Oxidations (Fleischer, S. and Packer, L., eds.), Academic Press, N.Y.

112. Fairbanks, G., Steck, T.L. and Wallach, D.H. (1971) Biochem., 10, 2606.

113. Massey, W. (1959) Biochim. Biophys. Acta, 34, 255.

114. Nash, T. (1953) Biochem. J., 55, 416.

115. Gielen, J.A., Coujon, F.M. and Nebert, D.W. (1972) J. Biol. Chem., 239, 2370.

116. Grote, W., Schmoldt, A. and Dammann, H.G. (1975) Biochem. Pharmacol., 24, 1121.

117. Bock, K.W., Frohling, W., Remmer, H. and Rexner, B. (1973) Biochim. Biophys. Acta, 327, 46.

118. Dent, J.G., Roes, U., Netter, K.J. and Gibson, J.E. (1977) J. Toxicol. Environ. Health, 6, 637.

119. Farber, T.M., Balazs, T., Marks, E. and Cerra, F. (1976) Fed. Proc. 35, 376.

120. Sleight, S.D. and Sanger, V.L. (1976) J. Am. Vet. Med. Assoc., 169, 1231.

121. Alvares, A.P. and Kappas, A. (1975) F.E.B.S. Lett., 50, 172.

122. Kato, R. and Takanaka, A. (1968) J. Biochem., 63, 406.

123. Dent, J.G., et al. (1977) Submitted Toxicol. Appl. Pharmacol.

124. Dent, J.G. (1978) Environ. Health Perspect., in press.

125. Pesticide Analytical Manual Vol. 1, Extraction and Clean-Up, U.S. Dept. of Health, Education and Welfare, Food and Drug Administration.

126. Wyndham, C., Devenish, J. and Safe, S. (1976) Res. Comm. Chem. Path. Pharmacol., 15, 563.

127. Shimada, T. (1976) Bull. Environ. Contamin. Toxicol., 16, 25.

128. Sundstrom, G. and Wachtmeister (1975) Chemosphere, No. 1, 7.

APPENDIX

APPENDIX

LIST OF PUBLICATIONS

In Press

Robert W. Moore, Ghazi A. Dannan, and Steven D. Aust. Induction of Drug Metabolizing Enzymes in Polybrominated Biphenyl-Fed Lactating Rats and Their Pups. Environ. Health Perspect.

Ghazi A. Dannan, Robert W. Moore, and Steven D. Aust. Studies on the Microsomal Metabolism and Binding of Polybrominated Biphenyls (PBBs). Environ. Health Perspect.

Abstracts

Robert W. Moore, Ghazi Dannan, and Steven D. Aust. Induction of Drug Metabolizing Enzymes in Rats Nursing From Mothers Fed Polybrominated Biphenyls. Fed. Proc. 35, 708 (1976).