INDUCTION OF CYTOCHROMES P450 1A, P450 2B, AND P450 3A IN LONG-EVANS RATS BY LOW-LEVEL EXPOSURE TO AROCLOR 1260

by

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ABSTRACT

Polychlorinated biphenyls (PCBs) are highly persistent environmental contaminants that are capable of inducing cytochrome P450 enzymes in exposed animals. In the present study, one set of male Long-Evans rats was treated with various doses of Aroclor 1260 ranging from 0.5 to 50 mg/kg in an attempt to achieve a level of exposure similar to that observed in the environment. Treatment was via oral gavage for seven days followed by sacrifice three days later. A second set of rats was treated at a dose of 10 mg/kg using the same protocol, but were killed from one to 48 days after the last treatment. Total cytochrome P450 content and several cytochrome P450 mediated enzyme activities were measured.

Significant increases in enzyme activities indicative of cytochrome P450 2B were found at doses of 5.0 mg/kg and greater. Immunoquantitation of blots probed with antibody to P450 2B1 confirmed a marked induction of cytochrome P450 2B isozymes at doses of 5 mg/kg and greater. Dose-dependent increases in cytochrome P450 1A1 protein levels and cytochrome P450 1A-mediated enzyme activities were observed. Enzyme activities catalyzed by cytochrome P450 3A and immunoreactive cytochrome P450 3A protein were increased significantly by Aroclor 1260 treatment at doses of 5.0 mg/kg and greater.

Cytochrome P450 1A1 and P450 3A levels correlated with hepatic PCB levels, while there was no more than a weak correlation between hepatic PCB levels and cytochrome P450 2B levels.

Significant increases in cytochrome P450 2B-mediated activities and protein levels persisted for at least 48 days post-treatment. Induction of cytochrome P450 1A1

protein levels and enzyme activities was relatively short-lived, while cytochrome P450 3A protein levels and enzyme activities were increased significantly for up to 24 days post-treatment.

In summary, the study indicates that subchronic low-level exposure to Aroclor 1260 induces cytochromes P450 1A, P450 2B, and P450 3A enzymes, but that cytochrome P450 2B1 is induced to the greatest extent and the induction is more persistent.

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LIST OF ABBREVIATIONS

Ah	aromatic hydrocarbon
AHH	aryl hydrocarbon hydroxylase
AhRE	aromatic hydrocarbon response element
Bis	N,N′-methylene-bis-acrylamide
BROD	benzyloxyresorufin *O*-dealkylase
BSA	bovine serum albumin
C	control
CV	coefficient of variation
DCM	dichloromethane
DDT	dichloro-diphenyl-trichloroethane
EDTA	ethylenediaminetetraacetic acid
EROD	ethoxyresorufin *O*-dealkylase
GC	gas chromatography
HEPES	N-2-hydrozyethylpiperazine-N′-2-ethanesulfonic acid
HEX	hexane
HPLC	high performance liquid chromatography
IgG	immunoglobulin G
i.p.	intra-peritoneal
LOD	limit of detection
LOQ	limit of quantitation
3-MC	3-methylcholanthrene
mRNA	messenger ribonucleic acid
MS	mass spectrometry
n	number of samples in a group
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
p	probability of a Type I error, α
PAGE	polyacrylamide gel electrophoresis
PB	phenobarbital
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo dioxin
PCDF	polychlorinated dibenzo furan
PBS	phosphate buffered saline
PROD	pentoxyresorufin *O*-dealkylase
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEMED	N,N,N′,N′-tetramethyethylenediamine
TRIS	2-amino-2-(hydroxymethyl)-1,3-propanediol
UV	ultraviolet radiation

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1. INTRODUCTION

Contamination of the environment by organochlorines such as polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzo-furans (PCDFs), and pesticides has been known for many years (Cockerman and Shane 1994). These anthropogenic chemicals are extremely persistent and widespread in the environment with the result that small amounts have been detected in fish, wildlife, human tissue including breast milk, marine and estuarine sediments, and even air samples from highly industrialized and urban areas (Safe 1990, Dustman and Stickel 1969). Factors that contribute to the persistence of PCBs, PCDDs, and PCDFs in environmental compartments include relatively strong lipophilic character and slow metabolism *in vivo* (De Jongh *et al.* 1995). These compounds are also stable and resistant to breakdown by acids, bases, heat, and hydrolysis (Safe 1990).

A considerable amount of information exists on the toxic and biochemical responses observed in animals exposed to organochlorines. Both biochemical and toxic effects of PCBs and related organochlorines are dependent upon age, sex, species, and strain of the animal as well as the composition of the organochlorine. Toxic responses include dermal toxicity, immunotoxicity, hepatotoxicity, carcinogenicity, teratogenicity and neurobehavioural effects (Ahlborg *et al.* 1992). Exposure of laboratory animals to PCBs and related organochlorines also results in increased levels (induction) of several hepatic cytochrome P450 enzymes (Yu *et al.* 1996, Safe 1990, McFarland *et al.* 1989, Snyder *et al.* 1979). Because induction of hepatic cytochrome P450 enzymes is a relatively sensitive and common biochemical response to PCB and related organochlorine

exposure, it has been proposed as an indicator or marker of exposure (Lubet *et al.* 1992).

The following introduction will briefly describe the catalytic properties of cytochrome P450 enzymes, various inducers of cytochrome P450, and their proposed mechanisms of induction. In addition, the biochemical and toxic effects of PCBs will be outlined followed by a brief description of the impact of PCB contamination in the environment.

1.1. *CYTOCHROME P450 ENZYMES*

Cytochrome P450 enzymes have been identified in bacteria, plants, insects, fish, and mammals (Ioannides and Parke 1990). In mammals, the hepatic cytochrome P450 enzymes are phase I enzymes that catalyze the biotransformation of both exogenous and endogenous lipophilic compounds such as certain steroids, fatty acids, vitamins, bile acids, pesticides, carcinogens, therapeutic drugs and industrial chemicals. Cytochrome P450 was first discovered by its carbon-monoxide binding pigment which absorbs light maximally at 450 nm (Omura and Sato 1964). Cytochrome P450 is a hemeprotein consisting of an iron protoporphyrin IX heme moiety and a single polypeptide chain or apoprotein of 45,000 to 55,000 Da (Guengerich and Martin 1980). The iron of the heme prosthetic group is located at the center of the protoporphyrin ring, with four coordinate bonds radiating from the heme iron to the porphyrin ring. The fifth ligand of the heme moiety is a cysteinyl residue of the apoprotein and the sixth ligand is normally molecular oxygen (Goeptar *et al.* 1995).

The cytochrome P450-dependent monooxygenase system is localized in the

membrane of the endoplasmic reticulum and includes NADPH-dependent cytochrome P450 reductase (Imai and Sato 1974). NADPH-dependent cytochrome P450 reductase functions by transporting electrons from NADPH to the cytochrome P450 enzymes (Taniguchi *et al.* 1984). The electrons can be transported either directly from NADPH to cytochrome P450 or indirectly from NADH via cytochrome b_5 (Omura and Sato 1964). Cytochrome P450, as the terminal enzyme in the electron transport chain, contains the catalytic and oxygen binding site. Once the electrons are transferred to cytochrome P450 they are used to reduce molecular oxygen to generate an active oxygen species, which is then inserted into the substrate. Cytochrome P450 enzymes catalyze a wide range of monooxygenase reactions such as hydroxylations, epoxidations, *N*-demethylations, sulfoxidations, and oxidative dehalogenations (Goeptar *et al.* 1995). A typical reaction scheme of the cytochrome P450 monooxygenase system is illustrated in Figure 1.1. The hydroxylated product is generally more hydrophilic than the original compound and in turn may undergo further biotransformation via Phase I or Phase II enzymes before being excreted from the body. Although the hydroxylated product usually has less pharmacological activity than the parent molecule, some metabolites are more toxic than the parent molecule and may bind covalently to macromolecules such as DNA (Nebert and Gonzalez 1987).

Since the discovery of cytochrome P450, researchers have identified and characterized many distinct forms of the enzyme and these various forms or isozymes have been grouped into a cytochrome P450 superfamily. With the use of molecular cloning and recombinant DNA technologies, numerous cytochrome P450 genes have been isolated and identified. With a few exceptions, each cytochrome P450 gene almost

always produces a single protein (*Nebert et al.* 1993). The exceptions involve differential processing of the cytochrome P450 transcript in which entire translated exons or portions of exons are exchanged to produce a cytochrome P450 enzyme with a new catalytic activity or tissue-specific expression (Lephart *et al.* 1990, Miles *et al.* 1990, Means *et al.* 1991).

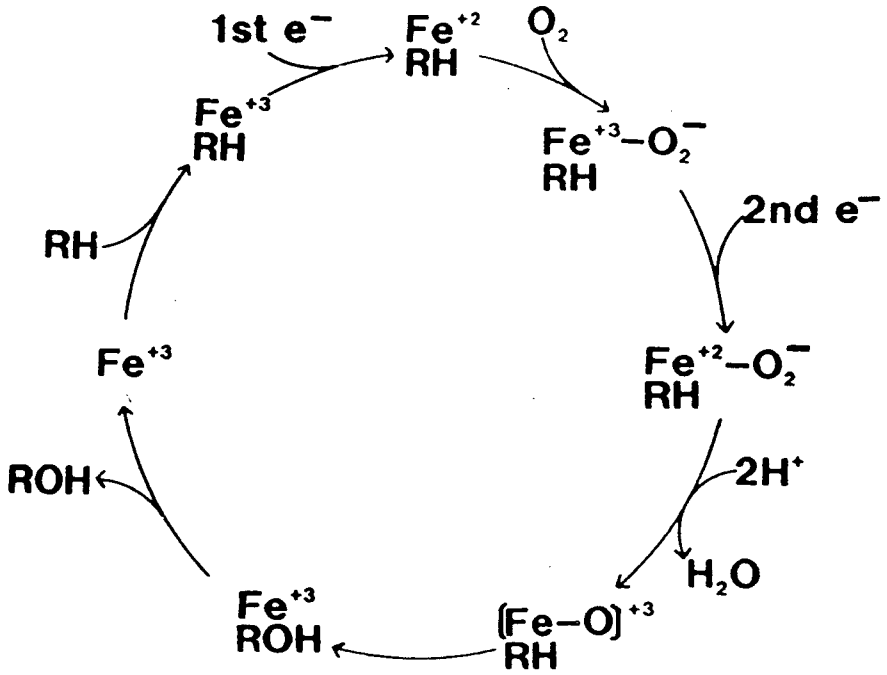

Figure 1.1. Schematic diagram of a cytochrome P450 monooxygenase reaction. RH represents a substrate and ROH the product. Adapted from Omura *et al.* (1993).

A systematic nomenclature based on the similarity of amino acid sequences is currently in use. In this nomenclature, all cytochrome P450 enzymes are grouped into families and subfamilies on the basis of their gene and protein sequences. A cytochrome P450 protein in one gene family is defined as having ≤ 40% sequence identity with that

from any other family, and cytochrome P450 proteins with genes that share ≥ 55% sequence homology are grouped in the same subfamily (Nelson *et al.* 1993). To date, there are 36 gene families, including 12 mammalian gene families, that are further subdivided into 22 mammalian subfamilies (Nelson *et al.* 1993). Each cytochrome P450 isozyme is identified by a three character code such as cytochrome P450 1A1 in which the family is designated by an Arabic number, followed by the subfamily grouping indicated by a capital alphabetical letter, and ending in an Arabic number designating the individual gene (Nelson *et al.* 1993).

The cytochrome P450 1, cytochrome P450 2, and cytochrome P450 3 gene families are the most important hepatic cytochrome P450 enzymes in the metabolism of xenobiotic compounds, while the cytochrome P450 4 gene family is primarily responsible for the metabolism of endogenous compounds. In the present study, cytochrome P450 enzymes belonging to the cytochrome P450 1A, P450 2B, and P450 3A subfamilies will be investigated. Previous studies have observed increased protein levels corresponding to cytochrome P450 1A, P450 2B, and P450 3A subfamilies in animals that had been exposed to PCBs (Safe *et al.* 1994, Dragnev *et al.* 1994, Harris *et al.* 1993)

1.1.1. *The cytochrome P450 1A subfamily*

The mammalian cytochrome P450 1A subfamily includes two forms or isozymes, cytochromes P450 1A1 and P450 1A2. These two isozymes share 68% amino acid sequence similarity (Hines *et al.* 1985). The constitutive level of cytochrome P450 1A1

in rat liver is very low, but is highly inducible upon exposure to lipophilic planar arenes such as 3-methylcholanthrene (3-MC), β-naphthoflavone, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and certain coplanar PCB isomers. Compounds that induce cytochrome P450 1A are termed 3-MC type or Type I inducers. In this thesis, constitutive levels of cytochrome P450 enzymes are defined as the basal levels found in untreated animals. The constitutive level of cytochrome P450 1A2 in rat liver is higher than that of cytochrome P450 1A1, and expression of cytochrome P450 1A2 is largely restricted to the liver (Gonzalez 1990). Cytochrome P450 1A2 is also induced by the 3-MC like inducers. The cytochrome P450 1A subfamily is thought to play an important role in the activation of various carcinogens.

The regulation of cytochrome P450 1A induction is mediated by the TCDD or Ah receptor (Okey 1990). The Ah receptor has been identified in several mammalian and nonmammalian tissues (Okey *et al.* 1994). In rodents, the Ah receptor has been found in extrahepatic tissues such as lung, kidney, spleen, and intestine (Okey *et al.* 1994). In humans, the Ah receptor has been detected in lymphocytes, placenta, and lung (Okey *et al.* 1994).

Figures 1.2 and 1.3 illustrate Ah receptor mediated enzyme induction and the sequence of events in the transformation of cytosolic Ah receptor to the DNA-binding state, respectively. The Ah receptor, which is located in the cytosol, is complexed with a heat shock protein 90 (Okey *et al.* 1994). When an inducer such as TCDD enters the cell by diffusion and is recognized by the cytosolic Ah receptor, an inducer-receptor complex is formed. This complex formation undergoes an energy-requiring transformation process, during which the Ah receptor releases heat shock protein 90 and converts into a

form that can associate with the 5′ flanking region of DNA sequences, referred to as Ah-responsive elements (AhRE). This nuclear form of the Ah receptor is composed of a ligand binding subunit and an Ah receptor nuclear translocator protein (Arnt protein), however it is unknown whether the Arnt protein is a component of the unliganded cytosolic Ah receptor or if the Arnt protein associates with the Ah receptor once the heat shock protein 90 is released. The binding of the nuclear Ah receptor and the AhRE results in the transcription of cytochrome P450 1A1 and P450 1A2 genes. This in turn, results in an increase in cytoplasmic mRNA with a corresponding increase in the synthesis of cytochrome P450 1A1 and P450 1A2 proteins. In addition to cytochrome P450 enzymes, binding of the Ah receptor also induces Phase II conjugating proteins such as uridine-diphosphate-glucuronosyltransferases, glutathione *S*-transferases and aldehyde dehydrogenases (Okey *et al.* 1994).

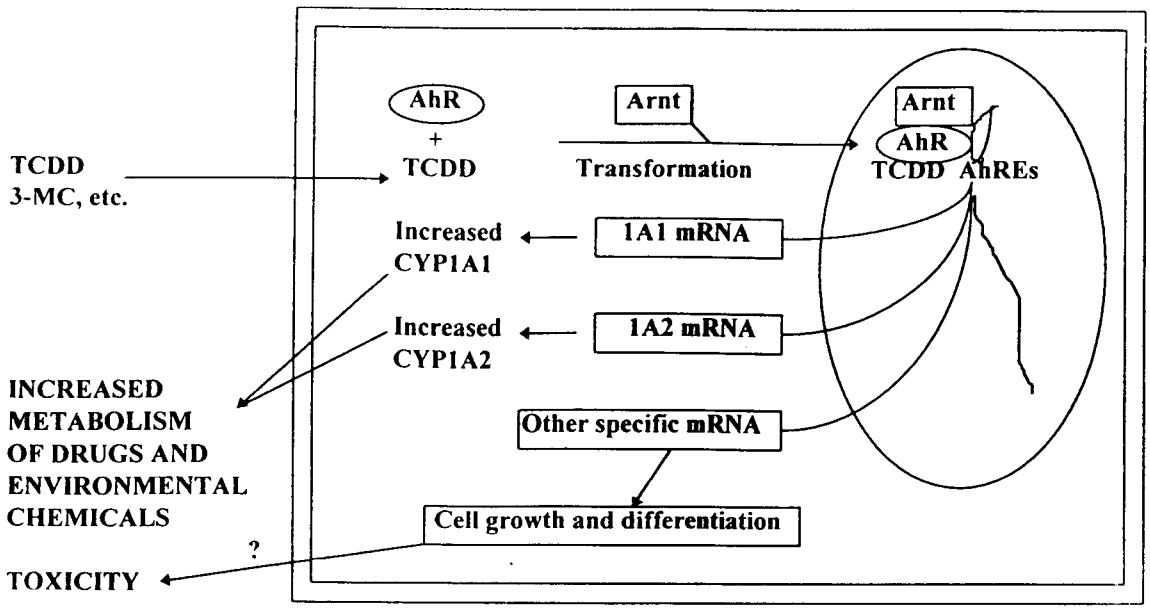

Figure 1.2. A model illustrating the Ah-receptor-mediated enzyme induction. Adapted from Okey *et al.* (1990).

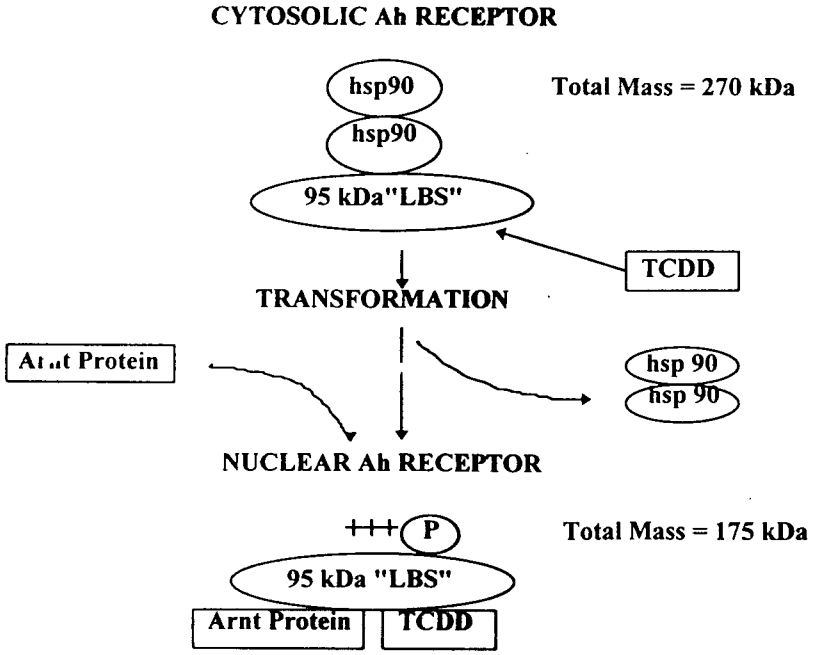

Figure 1.3. Postulated oligomeric structures of cytosolic and nuclear forms of the Ah receptor and the sequence of events in transformation of cytosolic Ah receptor to the DNA-binding state. The receptor may require phosphorylation in order to bind AhRE, which is indicated by "P". Adapted from Okey *et al.* (1990).

1.1.2. *The cytochrome P450 2B subfamily*

The cytochrome P450 2 family includes five subfamilies in rat liver, cytochrome P450 2A, P450 2B, P450 2C, P450 2D and P450 2E (Nelson *et al.* 1993). The cytochrome P450 2A subfamily shows sexually differentiated expression that is thought to be partly controlled by the pattern of pituitary growth hormone secretion (Waxman *et al.* 1985). The cytochrome P450 2C subfamily, which includes seven different genes, is a class of constitutively expressed enzymes that are under developmental and sexual regulation in the rat (Gonzalez *et al.* 1986). The cytochrome P450 2D subfamily consists of five different genes in the rat (Matsunaga *et al.* 1989). Cytochrome P450 2D-mediated

catalytic activity has been correlated to various types of cancer (Ladero *et al.* 1991). A single form of the cytochrome P450 2E subfamily enzyme exists in rats (Song *et al.* 1986) and has been reported to be under developmental regulation (Thomas *et al.* 1987).

To date, sixteen genes have been identified in the cytochrome P450 2B subfamily (Nebert *et al.* 1993). In rats, four cytochrome P450 2B isozymes have been characterized, namely cytochromes P450 2B1, P450 2B2, P450 2B3, and P450 2B8. Of the four, cytochromes P450 2B1 and P450 2B2 have been studied extensively due to their marked induction by phenobarbital (PB). Although cytochromes P450 2B1 and P450 2B2 share 97% amino acid sequence homology and are immunochemically cross-reactive, they can be distinguished by polyacrylamide gels due to slight differences in their mass to charge ratio (Fujii-Kuriyama *et al.* 1982, Kumar *et al.* 1983).

In untreated rats, both cytochromes P450 2B1 and P450 2B2 are minor constituents of total cytochrome P450 content, and the constitutive levels of cytochrome P450 2B1 are 5- to 10-fold lower than those of cytochrome P450 2B2 (Waxman and Azaroff 1992). In untreated rats, cytochrome P450 2B1 content is highest in lung relative to the liver and other extrahepatic tissue, while cytochrome P450 2B2 content is highest in the liver (Christou *et al.* 1987). Cytochromes P450 2B1 and P450 2B2 are also expressed differentially in kidney, adrenal gland, and small intestine (Christou *et al.* 1987).

Cytochrome P450 2B3 is expressed constitutively in the liver of male and female rats (Labbé *et al.* 1988). Cytochrome P450 2B3 has been found to be 77% similar in amino acid sequence to cytochromes P450 2B1 and P450 2B2, and is immunochemically cross-reactive with these two isozymes (Labbé *et al.* 1988). However, the biological role

of cytochrome P450 2B3 is unknown.

The cytochrome P450 2B subfamily has been identified in other mammals such as rabbits, mice, and humans. The cytochrome P450 2B isozymes found in these mammals share from 75 to 95% sequence similarity with the rat cytochrome P450 2B isozymes (Komori *et al.* 1988, Noshiro *et al.* 1988, Miles *et al.* 1988).

Upon exposure of animals to PB or PB-like inducers, both cytochrome P450 2B1 and P450 2B2 levels are elevated significantly above constitutive levels (20- to > 100-fold increase), with the expression of hepatic cytochrome P450 2B1 induced to a greater extent (approximately 2-fold) than that of cytochrome P450 2B2 (Christou *et al.* 1987). In extrahepatic tissues, cytochrome P450 2B1 is induced only modestly while cytochrome P450 2B2 is induced by PB only in the adrenal gland (Christou *et al.* 1987).

There are several PB-like or Type II inducers of the cytochrome P450 2B subfamily. Halogenated hydrocarbons that do not possess coplanar aromatic conformations, such as DDT, chlordanes, and some *ortho*-chlorinated PCBs induce cytochrome P450 2B (Figure 1.4) (Waxman and Azaroff 1992).

Induction of cytochrome P450 2B involves transcriptional activation of cytochrome P450 2B genes leading to accumulation of mRNA and increased cytochrome P450 2B-mediated enzymatic activities. The mechanism of how the cell recognizes the inducer and conveys the information to the transcriptional machinery has not been determined, however many models and working hypotheses have been proposed (Waxman and Azaroff 1992). Waxman and Azaroff (1992) suggested that PB binds to an extracellular receptor that, in turn, leads to the formation of intracellular secondary

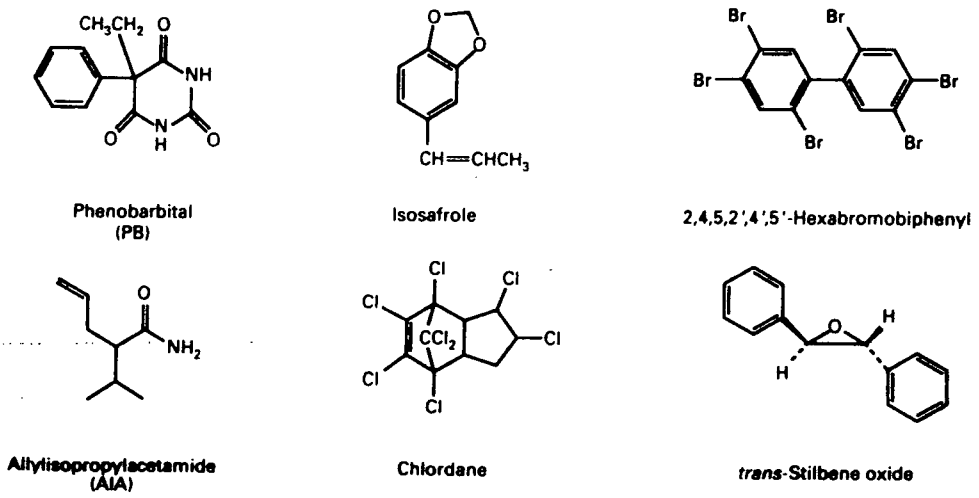

Figure 1.4. Chemical structures of PB-like or Type II inducers of the cytochrome P450 2B subfamily

messengers leading to the activation of cytochrome P450 2B gene transcription. In another model, Waxman and Azaroff (1992) suggested a model similar to the Ah receptor. In this model, PB, due to its lipophilic properties, enters the cell and binds to an intracellular receptor resulting in an inducer-receptor complex. This complex would then undergo a conformational change and bind to a PB-responsive element leading to transcriptional activation. However, a "PB receptor" has not yet been identified. Furthermore, due to the structurally diverse nature of PB-like inducers, the existence of a "PB receptor" is not likely (Okey 1990).

With this in mind, other models were developed that do not include the use of a "PB receptor". One model, suggested by Waxman and Azaroff (1992) involved the metabolism of an endogenous substrate by cytochrome P450 2B. Normal basal levels of free cytochrome P450 2B would keep the substrate and metabolite levels very low,

however, once PB was bound to cytochrome P450 2B, production of the endogenous substrate would outweigh the metabolism of the substrate resulting in higher substrate levels. At some threshold level of substrate transcriptional activation of cytochrome P450 2B genes would occur.

Another model outlined by Waxman and Azaroff (1992) involved the maintenance of a "repressor precursor". Repression of cytochrome P450 2B transcription would be accomplished by metabolism of the "repressor precursor" to an active form by basal levels of cytochrome P450 2B. Binding of PB to cytochrome P450 2B enzymes would prevent formation of the active repressor, leading to increased cytochrome P450 2B transcription.

Many models have been proposed to account for the induction of cytochrome P450 2B enzymes, however none of the models are supported by sufficient experimental evidence and the mechanism of cytochrome P450 2B induction remains to be solved.

1.1.3. *The cytochrome P450 3A subfamily*

The cytochrome P450 3A subfamily in the rat consists of five isoforms, namely cytochromes P450 3A1, P450 3A2, P450 3A9, P450 3A18, and P450 3A23 (Mahnke *et al.* 1997). Both cytochrome P450 3A1 and P450 3A2 isozymes are immunochemically cross-reactive.

Cytochrome P450 3A1 is undetectable in untreated male or female rats, while cytochrome P450 3A2 is expressed constitutively in immature and mature male rats and

in immature female rats (Cooper *et al.* 1992). Expression of both cytochromes P450 3A1 and P450 3A2 can be induced by treatment with compounds such as pregnenolone-16α-carbonitrile, dexamethasone, and PB (Cooper *et al.* 1992). Few studies have investigated induction of cytochrome P450 3A by PCBs.

In humans, the cytochrome P450 3A subfamily represents the most abundantly expressed set of cytochrome P450 isozymes in the liver. There are four closely related proteins of the cytochrome P450 3A subfamily in humans that account for about 60% of the total cytochrome P450 content (Guengerich 1990). One of the isozymes, cytochrome P450 3A7, is expressed in fetal liver only, while another isozyme, cytochrome P450 3A5 is found in adults but only in 25% of liver specimens tested (Spatzenegger and Jaeger 1995). Cytochrome P450 3A4 is the most important isozyme of the cytochrome P450 3A subfamily as it is responsible for the metabolism of many clinically important drugs such as corticosteroids, antifungal agents, macrolide antibiotics, and antineoplastic agents (Spatznegger and Jaeger 1995). In light of the role and abundance of the cytochrome P450 3A subfamily in humans, it is relevant to investigate the effects of PCBs on cytochrome P450 3A induction.

There is a diverse group of cytochrome P450 3A inducers, including steroid antagonists, endogenous and synthetic glucocorticoids, macrolide antibiotics, various antifungal agents, PB and PB-like compounds (Okey 1990). The extent of induction of cytochromes P450 3A1 and P450 3A2 differs among the various inducers and the inducing compounds can be divided into two classes on the basis of potency. The first class includes compounds that have an inductive effect on both cytochromes P450 3A1 and P450 3A2, such as dexamethasone, triacetyloleandomycin, and pregnenolone-16α-

carbonitrile (Cooper *et al.* 1992). The second class includes compounds that preferentially induce one cytochrome P450 3A isozyme over the other. For example, rifampicin and PB increase cytochrome P450 3A1 and P450 3A2 levels but have a lesser effect on cytochrome P450 3A2, whereas 3-MC induces cytochrome P450 3A2 but has no effect on cytochrome P450 3A1 (Cooper *et al.* 1992). Schuetz *et al.* (1986) reported that PCBs substituted at more than two *ortho*-positions were found to be the most efficacious inducers of cytochrome P450 3A in rat hepatocyte cultures and *in vivo*. This differs from cytochrome P450 2B induction by PCBs in which isomers substituted in two *ortho*-positions induced cytochrome P450 2B enzymes more effectively (Parkinson *et al.* 1983).

The mechanism responsible for cytochrome P450 3A induction varies among the different inducing compounds. Transcriptional activation is responsible for cytochrome P450 3A induction by pregnenolone-16α-carbonitrile and dexamethasone. An increase in transcription of cytochrome P450 3A genes of 3- to 6-fold has been reported for pregnenolone-16α-carbonitrile and dexamethasone, respectively (Simmons *et al.* 1987). Induction by triacetyloleandomycin, PB, and PB-like compounds involves mRNA and protein stabilization of cytochrome P450 3A (Schuetz *et al.* 1986). Other factors such as the age and sex of the animal are important determinants of the effects of various inducers on cytochrome P450 3A expression (Cooper *et al.* 1992).

1.2. *POLYCHLORINATED BIPHENYLS (PCBS)*

There are 209 possible PCB isomers arising from substitution of one to ten chlorine atoms on the biphenyl nucleus (Figure 1.5). Each isomer has been given an identification number as developed by the International Union of Pure and Applied Chemistry (Table 1.1) (Ballschmitter and Zell 1980). Commercial preparations of PCBs are mixtures containing approximately 100 different isomers. The PCB mixtures are usually identified by the percentage of chlorine by weight in the mixture. For the commercial preparations of Aroclor such as 1254 and 1260, manufactured by the Monsanto Co., the type of molecule present is identified by the first two digits (12 = biphenyl) and the amount of chlorine is specified by the last two digits. For example, Aroclor 1242, 1248, 1254, and 1260 contain 42%, 48%, 54%, and 60% chlorine by weight, respectively (Tanabe *et al.* 1981, Albro *et al.* 1981). Aroclor mixtures with a lower chlorine content have a larger ratio of lower chlorinated isomers to higher chlorinated isomers, and the opposite is observed as the chlorine content increases (Table 1.2). Aroclor 1242, 1254, and 1260 were produced in the largest amount and are generally considered the most prevalent in the environment (Steichen *et al.* 1982).

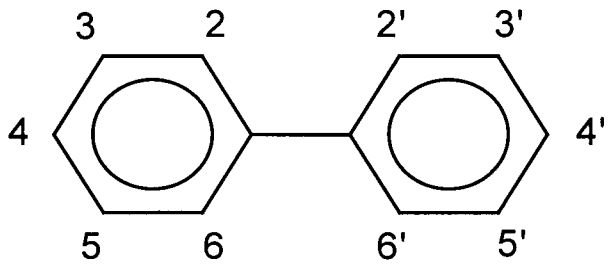

Figure 1.5. Structure of a Biphenyl

Table 1.1. The International Union of Pure and Applied Chemistry numbers for all PCB isomers (Ballschmiter & Zell 1980).

No.	Structure	No.	Structure	No.	Structure	No.	Structure
	Monochlorobiphenyls		Tetrachlorobiphenyls		Pentachlorobiphenyls		Hexachlorobiphenyls
1	2	52	2,2',5,5'	105	2,3,3',4,4'	161	2,3,3',4,5',6
2	3	53	2,2',5,6'	106	2,3,3',4,5	162	2,3,3',4',5,5'
3	4	54	2,2',6,6'	107	2,3,3',4',5	163	2,3,3',4',5,6
	Dichlorobiphenyls	55	2,3,3',4	108	2,3,3',4,5'	164	2,3,3',4',5',6
		56	2,3,3',4'	109	2,3,3',4,6	165	2,3,3',5,5',6
4	2,2'	57	2,3,3',5	110	2,3,3',4',6	166	2,3,4,4',5,6
5	2,3	58	2,3,3',5'	111	2,3,3',5,5'	167	2,3',4,4',5,5'
6	2,3'	59	2,3,3',6	112	2,3,3',5,6	168	2,3',4,4',5',6
7	2,4	60	2,3,4,4'	113	2,3,3',5',6	169	3,3',4,4',5,5'
8	2,4'	61	2,3,4,5	114	2,3,4,4',5		Heptachlorobiphenyls
9	2,5	62	2,3,4,6	115	2,3,4,4',6		
10	2,6	63	2,3,4',5	116	2,3,4,5,6	170	2,2',3,3',4,4',5
11	3,3'	64	2,3,4',6	117	2,3,4',5,6	171	2,2',3,3',4,4',6
12	3,4	65	2,3,5,6	118	2,3',4,4',5	172	2,2',3,3',4,5,5'
13	3,4'	66	2,3',4,4'	119	2,3',4,4',6	173	2,2',3,3',4,5,6
14	3,5	67	2,3',4,5	120	2,3',4,5,5'	174	2,2',3,3',4,5,6'
15	4,4'	68	2,3',4,5'	121	2,3',4,5',6	175	2,2',3,3',4,5',6
	Trichlorobiphenyls	69	2,3',4,6	122	2',3,3',4,5	176	2,2',3,3',4,6,6'
		70	2,3',4',5	123	2',3,4,4',5	177	2,2',3,3',4',5,6
16	2,2',3	71	2,3',4',6	124	2',3,4,5,5'	178	2,2',3,3',5,5',6
17	2,2',4	72	2,3',5,5'	125	2',3,4,5,6'	179	2,2',3,3',5,6,6'
18	2,2',5	73	2,3',5',6	126	3,3',4,4',5	180	2,2',3,4,4',5,5'
19	2,2',6	74	2,4,4',5	127	3,3',4,5,5'	181	2,2',3,4,4',5,6
20	2,3,3'	75	2,4,4',6		Hexachlorobiphenyls	182	2,2',3,4,4',5,6'
21	2,3,4	76	2',3,4,5			183	2,2',3,4,4',5',6
22	2,3,4'	77	3,3',4,4'	128	2,2',3,3',4,4'	184	2,2',3,4,4',6,6'
23	2,3,5	78	3,3',4,5	129	2,2',3,3',4,5	185	2,2',3,4,5,5',6
24	2,3,6	79	3,3',4,5'	130	2,2',3,3',4,5'	186	2,2',3,4,5,6,6'
25	2,3',4	80	3,3',5,5'	131	2,2',3,3',4,6	187	2,2',3,4',5,5',6
26	2,3',5	81	3,4,4',5	132	2,2',3,3',4,6'	188	2,2',3,4',5,6,6'
27	2,3',6		Pentachlorobiphenyls	133	2,2',3,3',5,5'	189	2,3,3',4,4',5,5'
28	2,4,4'			134	2,2',3,3',5,6	190	2,3,3',4,4',5,6
29	2,4,5	82	2,2',3,3',4	135	2,2',3,3',5,6'	191	2,3,3',4,4',5',6
30	2,4,6	83	2,2',3,3',5	136	2,2',3,3',6,6'	192	2,3,3',4,5,5',6
31	2,4',5	84	2,2',3,3',6	137	2,2',3,4,4',5	193	2,3,3',4',5,5',6
32	2,4',6	85	2,2',3,4,4'	138	2,2',3,4,4',5'		Octachlorobiphenyls
33	2',3,4	86	2,2',3,4,5	139	2,2',3,4,4',6		
34	2',3,5	87	2,2',3,4,5'	140	2,2',3,4,4',6'	194	2,2',3,3',4,4',5,5'
35	3,3',4	88	2,2',3,4,6	141	2,2',3,4,5,5'	195	2,2',3,3',4,4',5,6
36	3,3',5	89	2,2',3,4,6'	142	2,2',3,4,5,6	196	2,2',3,3',4,4',5',6
37	3,4,4'	90	2,2',3,4',5	143	2,2',3,4,5,6'	197	2,2',3,3',4,4',6,6'
38	3,4,5	91	2,2',3,4',6	144	2,2',3,4,5',6	198	2,2',3,3',4,5,5',6
39	3,4',5	92	2,2',3,5,5'	145	2,2',3,4,6,6'	199	2,2',3,3',4,5,6,6'
	Tetrachlorobiphenyls	93	2,2',3,5,6	146	2,2',3,4',5,5'	200	2,2',3,3',4,5',6,6'
		94	2,2',3,5,6'	147	2,2',3,4',5,6	201	2,2',3,3',4',5,5',6
40	2,2',3,3'	95	2,2',3,5',6	148	2,2',3,4',5,6'	202	2,2',3,3',5,5',6,6'
41	2,2',3,4	96	2,2',3,6,6'	149	2,2',3,4',5',6	203	2,2',3,4,4',5,5',6
42	2,2',3,4'	97	2,2',3',4,5	150	2,2',3,4',6,6'	204	2,2',3,4,4',5,6,6'
43	2,2',3,5	98	2,2',3',4,6	151	2,2',3,5,5',6	205	2,3,3',4,4',5,5',6
44	2,2',3,5'	99	2,2',4,4',5	152	2,2',3,5,6,6'		Nonachlorobiphenyls
45	2,2',3,6	100	2,2',4,4',6	153	2,2',4,4',5,5'		
46	2,2',3,6'	101	2,2',4,5,5'	154	2,2',4,4',5,6'	206	2,2',3,3',4,4',5,5',6
47	2,2',4,4'	102	2,2',4,5,6'	155	2,2',4,4',6,6'	207	2,2',3,3',4,4',5,6,6'
48	2,2',4,5	103	2,2',4,5',6	156	2,3,3',4,4',5	208	2,2',3,3',4,5,5',6,6'
49	2,2',4,5'	104	2,2',4,6,6'	157	2,3,3',4,4',5'		Decachlorobiphenyl
50	2,2',4,6			158	2,3,3',4,4',6		
51	2,2',4,6'			159	2,3,3',4,5,5'	209	2,2',3,3',4,4',5,5',6,6'
				160	2,3,3',4,5,6		

Table 1.2. Approximate weight percentages of chlorobiphenyls in some commercial Aroclor mixtures (Table 8.7. Cockerman and Shane 1994)

Isomer	Aroclor 1242	Aroclor 1254	Aroclor 1260
MonoCl	1.0	<0.1	ND
DiCl	16.0	0.5	ND
TriCl	49.0	1.0	<0.5
TetraCl	25.0	21.0	1.5
PentaCl	8.0	48.0	12.0
HexaCl	1.0	23.0	38.0
HeptaCl	<0.1	6.0	41.0
OctaCl	ND	ND	8.0
DecaCl	ND	ND	<0.1

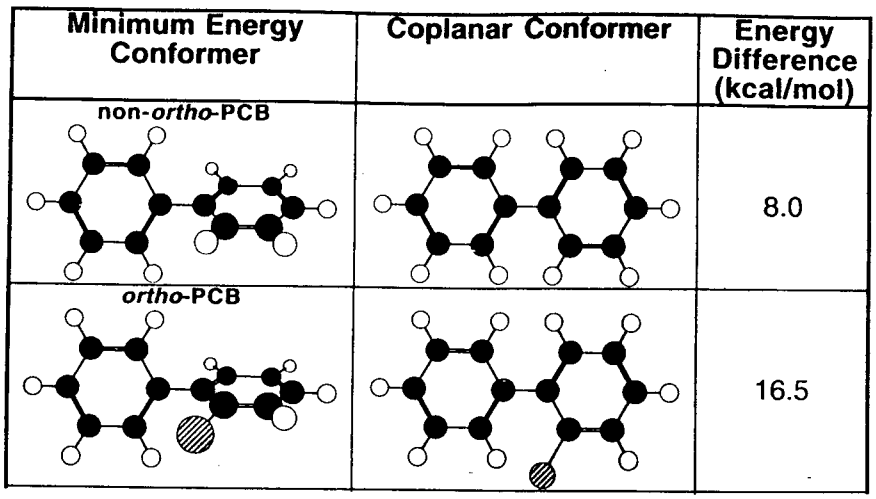

Figure 1.6. Conformations of PCB isomers (adapted from Kodavanti *et al.* 1996)

The biphenyl structure of the PCB molecule can adopt different conformations depending on the chlorine substitution pattern. The two phenyl rings of the PCB molecule can either be in line with one another (coplanar) or the two phenyl rings can rotate relative to one another and adopt a twisted rather than a flat conformation (non-coplanar). The existence of the coplanar conformation in PCB isomers and congeners has been observed using X-ray crystallography (McKinney and Singh, 1981). A more recent study used molecular mechanical studies to determine the energy required to obtain coplanarity of the two phenyl rings in PCB molecules (Kodavanti *et al.* 1996). These studies indicated that the minimal energy required for an unsubstituted biphenyl molecule to achieve a coplanar conformation was 8.0 kcal/mol. However, if the biphenyl is substituted at one *ortho* position, the minimal energy needed to achieve coplanarity increased to 16.5 kcal/mol (Figure 1.6) (Kodavanti *et al.* 1996). A di*ortho*-chlorinated biphenyl would require more energy to achieve a planar conformation. Thus, the coplanar conformation in *ortho*-substituted PCB isomers is disfavored. The minimum energy state of *ortho*-chlorinated PCBs would result in a twist angle of approximately 65° (Kodavanti *et al.* 1996).

1.2.1. *Biochemical effects of PCBs*

It has been found that the induction of specific cytochrome P450 isozymes is dependent upon the structure of the PCB to which the animal is exposed (Ikegwuonu *et al.* 1996). PCB isomers can be divided into Type I, Type II, and Mixed type inducers

depending on their ability to induce cytochromes P450 in a similar fashion to 3-methylcholanthrene (Type I), phenobarbital (Type II), or both (mixed type). Table 1.3 is a list of PCB isomers known to be classified as Type I, Type II, or Mixed type inducers.

Table 1.3. Type I, Type II, and Mixed Type PCB Isomers (Adapted from Letcher *et al.* 1996, Parkinson *et al.* 1981, Parkinson *et al.* 1980)

Type I (3-MC like inducer)	Type II (PB like inducer)		Mixed Type (3-MC and PB like inducers)	
PCB-77	PCB-47	PCB-180	PCB-37	PCB-156
PCB-81	PCB-49	PCB-183	PCB-75	PCB-157
PCB-126	PCB-52	PCB-194	PCB-105	PCB-158
PCB-169	PCB-99		PCB-118	PCB-166
	PCB-101		PCB-119	PCB-168
	PCB-153		PCB-128	PCB-170
	PCB-154		PCB-137	PCB-189
	PCB-155		PCB-138	

The PCBs that are the most efficacious inducers of cytochrome P450 1A1 and P450 1A2 are substituted in two *para-* and at least one *meta-* position of both phenyl rings and do not possess *ortho*-chlorinated substitutions (Safe *et al.* 1990). These PCBs are relatively flat or coplanar molecules and are collectively referred to as 3-MC like or Type I inducers.

Ortho-substituted isomers, which assume non-coplanar conformations, were found to induce cytochromes P450 2B, P450 3A, P450 2C6, and P450 2A1 (Ikegwuonu *et al.* 1996). These PCB isomers are collectively referred to as PB-like inducers or as Type II inducers.

PCB isomers classified as Mixed type iducers do not follow a common substitution pattern, and have both 3-MC- and PB-like inductive effects. However, some PCB isomers, such as those which contain only one or two chlorines, do not induce cytochrome P450 and may produce little or no toxicity (Safe 1990).

1.2.2. *Toxic effects of PCBs*

Among dioxins and related compounds, TCDD is considered the most toxic compound and is used as a reference to which the toxicity of all other PCDD isomers and related compounds, such as PCDFs and PCBs are compared. This reference system uses units known as toxic equivalency factors and 2,3,7,8-TCDD has a maximum value of one (Safe 1990).

The toxic effects of PCBs may involve at least three mechanisms of action, (1) reversible interaction (binding) of the PCB with specific molecular sites such as receptors and enzymes, (2) irreversible covalent interaction (binding) between the PCB (most likely a reactive metabolite) and target molecules (most likely macromolecules such as DNA and proteins), and (3) accumulation of highly lipid-soluble, metabolically stable PCBs in lipid-rich tissues or tissue compartments (McKinney and Waller 1994).

The toxic effects of the PCB isomers are also dependent upon the chlorination pattern and the conformation, either coplanar or non-coplanar, of the molecule. Coplanar isomers have a high affinity for the Ah receptor and thus have TCDD-like activity in biological systems (Tithof *et al.* 1996, Tithof *et al.* 1995, Brown and Ganey 1995). Safe

(1990, 1994) outlined the differences in relative toxic potency of the different PCB isomers by assigning the Type I inducers larger TEF values than the other PCBs. Most non-coplanar isomers have a low affinity for the Ah receptor and may possibly act through several different mechanisms (Moore *et al.* 1997). In the past, these non-coplanar isomers were thought to produce relatively few toxic effects. Current studies show that *ortho*-chlorinated isomers that cause induction of cytochrome P450 2B enzymes cause greater decreases in neurotransmitter levels (Shain *et al.* 1991), perturbations of Ca^{+2} homeostasis in neural cells (Kodavanti *et al.* 1995, Kodavanti *et al.* 1994), and the degranulation and production of the superoxide anion (O^{2-}) by neutrophils (Tithof *et al.* 1996, Tithof *et al.* 1995, Brown and Ganey 1995), when compared to coplanar isomers.

1.2.3. *Biotransformation of PCBs*

Once PCBs have been taken up into the body, they are distributed to different organs. Matthews and Dedrick (1984) reported that once PCBs are absorbed they are initially distributed to highly perfused organs such as liver and muscle. Following this initial distribution, most PCB isomers are redistributed, depending on their lipophilicity, to poorly perfused organs such as adipose tissue and skin which sequester PCBs. It has been reported that by the end of the redistribution phase about three quarters of the PCB body-burden is present in adipose tissue which may make up only 7% of total body weight (Lutz *et al.* 1977). Table 1.4 shows the tissue distribution of PCBs in rats fed various Aroclor mixtures.

Table 1.4. Tissue distribution of PCBs (mg/kg wet weight) in rats fed Aroclor 1254 (Grant *et al.* 1974), Aroclor 1242, or Aroclor 1016 (Burse *et al.* 1974) at 100 mg/kg for about six months

	PCB levels in tissue (mg/kg)		
Tissue	Aroclor 1254	Aroclor 1242	Aroclor 1016
Blood	0.40	0.53 (plasma)	0.38 (plasma)
Liver	16.0	4.21	7.86
Brain	3.4	1.69	2.98
Kidneys	-	1.89	3.21
Heart	7.3	-	-
Fat	32.0	110.0	236.0
Urine	-	0.03	0.28

PCBs are eliminated from the body primarily via oxidative pathways. PCBs are metabolized to more water soluble, hydroxylated metabolites, which may then be conjugated to glucuronides and excreted in the bile (Matthews and Dedrick 1984). The metabolism of PCBs may result in the formation of various metabolites including hydroxylated PCBs, dihydrodiols and catechols, phenolic conjugates, glutathione

conjugates, and methylsulfonyl metabolites (Figure 1.7) (Safe 1994, Moore *et al.* 1997). The toxicity of hydroxylated PCB metabolites has been reported to be lower than that of the parent compound (Yoshimura *et al.* 1987). Yoshimura *et al.* (1987) found that the two major metabolites of 3,3′,4,4′-tetrachlorobiphenyl were consiberably less toxic than the parent compound and did not induce Ah-receptor-mediated responses.

However, hydroxylated PCB metabolites are biologically active. Such activities include acting as uncouplers and inhibitors of mitochondrial oxidative phosphorylation, (Narasimhan *et al.* 1991, Nishihara 1988, Nishihara *et al.* 1987), binding competitively to the estrogen receptor, and increasing mouse uterine wet weight *in vivo* (Korach *et al.* 1988), inhibiting various cytochrome P450-dependent enzyme activities (Schmoldt *et al.* 1977), and binding to prealbumin, a major serum thyroxine-binding protein (Rickenbacher *et al.* 1986).

Another route of PCB metabolism involves the formation of glutathione conjugates that are excreted in the bile as a mercapturic acid. The glutathione conjugate is degraded to a cysteine conjugate that is further cleaved by intestinal mucosal β-lyase to form methylthiols. The methylthiols are reabsorbed from the intestinal tract, transferred to the liver and *S*-oxidized by cytochrome P450 enzymes to form methylsulfonyl PCB metabolites, which have been identified in human and animal serum and several organs and tissues (Safe 94, Haraguchi *et al.* 1992, Goldstein and Safe 1989). Methylsulfonyl metabolites are also biologically active and bind to endogenous proteins such as uteroglobulin and fatty acid-binding proteins (Gillner *et al.* 1988, Larsen *et al.* 1992). Methylsulfonyl metabolites have also been found to inhibit aryl hydrocarbon hydroxylase (AHH) activity (Kiyohara *et al.* 1990, Kiyohara *et al.* 1992). However the toxicological

significance of methylsulfonyl PCB metabolites has not been determined (Safe 1994).

The rate of elimination of PCBs is dependent upon the chlorination pattern of the isomer. The primary oxidative pathway is hydroxylation at the *meta-* and *para-* postitions, most likely via an arene oxide intermediate, which is believed to be catalyzed by cytochromes P450 2B1 and P450 2B2 (Clevenger *et al.* 1989). Hydroxylation at the *ortho-* position is most likely catalyzed by cytochrome P450 1A1. Clevenger *et al.* (1989) reported that PCBs substituted in the *meta-* and *para*-positions are associated with a slow rate of elimination as well as a tendency to accumulate in tissues. Not only are these PCB isomers eliminated more slowly but they are among the most toxic PCB isomers and have been positively correlated with binding to the cytosolic Ah receptor (Safe *et al.* 1985).

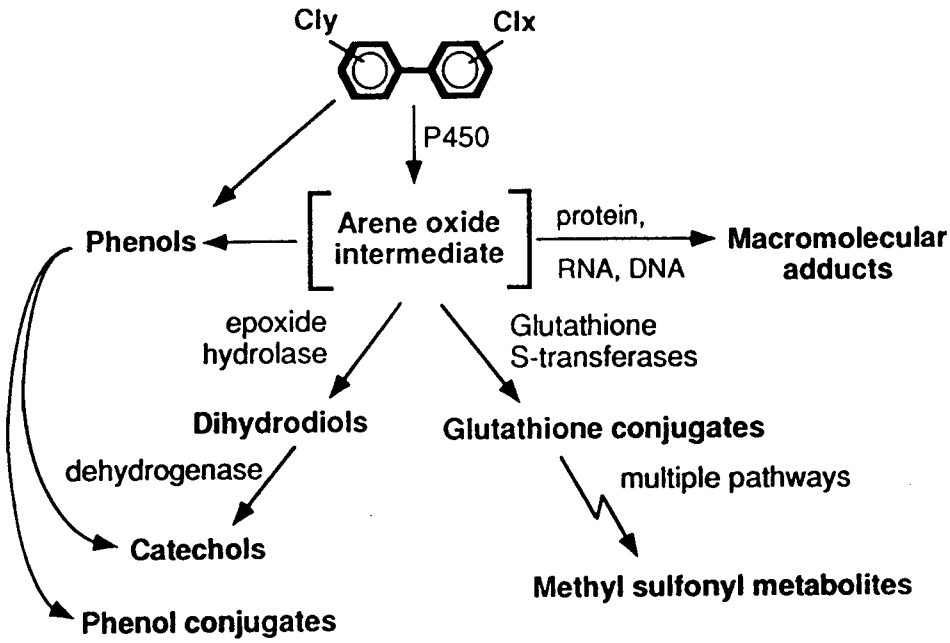

Figure 1.7. Scheme for the metabolism of PCBs (Figure 5 Safe 1994)

1.3. *PCBs IN THE ENVIRONMENT*

The persistent nature of PCBs have made them desirable as heat transfer fluids, organic diluents, plasticizers, fire retardents, and dielectric fluids for capacitors and transformers. The high lipophilicity and chemical stability of these compounds contributes to their bioaccumulation and biomagnification in higher trophic levels of the food chain.

The initial release of PCBs into the environment occurred primarily by leakage and disposal of electrical transformers and hydraulic equipment. The continuing use of existing PCB-containing stock has been limited to closed-systems, however, leakage from these systems continues to be a source of environmental contamination. The major sources of PCBs in the global atmosphere are considered to be: (1) evaporation from landfills, (2) incineration of hazardous wastes, and (3) re-volatization from environmental compartments (Standley and Hites 1991). Of the estimated total global production of 1.2 to 1.5 million tonnes of PCBs, 31% (370,000 tonnes) was estimated to have been released into the environment as of 1988 (Tanabe 1988).

Once the commercial PCB mixtures enter the environment, the composition and relative concentration of PCB isomers in the mixture are altered by several factors. These factors include the different rates of breakdown of congeners by microorganisms in sediment deposits, and the varying lipophilicity of different congeners which affects their transport in water or their uptake by biological organisms, with the result that the PCB composition varies constantly among different environmental niches or compartments. Environmental changes in the composition of the PCB mixture and possible synergistic

or antagonistic effects resulting from combinations of PCBs makes it difficult to track and examine the specific effects of each congener.

Organochlorines can move from the atmosphere into the aquatic environment from either the vapor or particle phase. The atmospheric distribution of organochlorines in the two phases is dependent on size (i.e. chlorination), surface area, and organic carbon content of the particle phase as well as the organochlorines' vapor pressure. In general, the lower the volatility of the compound the greater the tendency to be associated with the particle phase. Aerial movement of organic contaminants occurs predominantly in the vapor phase (90%) and deposition into aquatic environments relies primarily on particle deposition (Cockerman and Shane 1994). The vapor pressure of organochlorines is dependent upon temperature (Bidleman *et al.* 1987). A "cold condensation" effect is believed to occur for organochlorines which results in their migration from warmer to colder regions (Ottar 1981, Mackay and Wania 1995). A negative gradient with increasing latitude exists for less volatile organochlorines such as highly chlorinated PCBs, indicating more efficient removal of organochlorines close to emission sources at mid-latitudes.

Due to the movement of PCBs in the environment, PCBs and other organochlorines have been found in circumpolar Arctic regions. They have been identified in snow, ice, water, and air, and have contaminated every compartment of the food web. The food web of the Arctic is diverse including phytoplankton, zooplankton, fish, seal, birds, whales, and polar bears. Lipophilic contaminants are passed from prey to predator, eventually resulting in their bioaccumulation and biomagnification in the higher trophic levels of the Arctic food web. For example, mean total PCB levels in

pooled adipose tissue of polar bears (*Ursus maritimus*) from the Canadian arctic were 6- to 15-fold greater than those measured in the blubber of ringed seals (*Phoca hispida*), the primary prey of polar bears (Muir *et al.* 1988). The difference in PCB levels is especially apparent in this food chain as polar bears feed primarily on ringed seal skin and blubber, which serve as storage depots for lipophilic xenobiotics.

Studies that investigated the PCB isomers present in the polar bear liver found a distinct pattern that is unlike patterns found in other marine mammals (Figure 1.8). Over 95% of the total residual PCBs present in polar bear liver is represented by five congeners: CB-153 (2,2′,4,4′,5,5′ hexachlorobiphenyl) which makes up 40% of this total, CB-99 (2,2′,4,4′,5 pentachlorobiphenyl), CB-138 (2,2′,3,4,4′,5 hexachlorobiphenyl), CB-170 (2,2′,3,3′,4,4′,5 heptachlorobiphenyl), and CB-180 (2,2′,3,4,4′,5,5′ heptachlorobiphenyl) (Letcher *et al.* 1995). In the polar bear, the non-*ortho* coplanar PCB isomers (Type I inducers) that induce primarily cytochrome P450 1A accounted for less than 1% of the total PCBs in liver. In comparison, PCBs that are Mixed Type inducers and induce both cytochromes P450 1A and P450 2B comprised 25%. Type II inducers that induce cytochrome P450 2B accounted for about 71%. This is different from the PCB congeners found to be concentrated in ringed seal, the primary food source of the polar bear. The PCBs found in the highest concentration in ringed seal liver includes non-*ortho* substituted isomers such as CB-77 (3,3′,4,4′ tetrachlorobiphenyl), CB-126 (3,3′,4,4′,5 pentachlorobiphenyl), and CB-169 (3,3′,4,4′,5,5′ hexachlorobiphenyl), and mono-*ortho* isomers such as CB-105 (2,3,3′,4,4′ pentachlorobiphenyl), CB-118 (2,3′,4,4′,5 pentachlorobiphenyl), CB-156 (2,3,3′,4,4′,5 hexachlorobiphenyl), and CB-157 (2,3,3′,4,4′,5′ hexachlorobiphenyl). Both the non- and

mono-*ortho* substituted PCBs found in ringed seal are present at concentrations that are 10-100 fold lower in the polar bear, indicating that the polar bear readily metabolizes these isomers.

PCB contamination studies have been conducted in Inuit that hunt and eat traditional foods such as ringed seal (Ayotte *et al.* 1995, Dewailly *et al.* 1995, Kuhnlein 1995). It was found that the total PCB body burden in the Inuit of Greenland was the highest level ever reported in humans in the world. For example, the total PCB concentration was 15.7 mg/kg in omental fat and 17.0 mg/kg in adipose tissue (Dewailly *et al.* 1995). What is common to both the Inuit people and polar bears is the close similarity of the PCB isomer composition found in both species. This suggests that the distribution and metabolism of PCBs may be similar in polar bears and humans. Furthermore, this finding is not unexpected as both the Inuit people and polar bears feed primarily on seals (Kuhlein 1995, Norstrom *et al.* 1988, Muir *et al.* 1988).

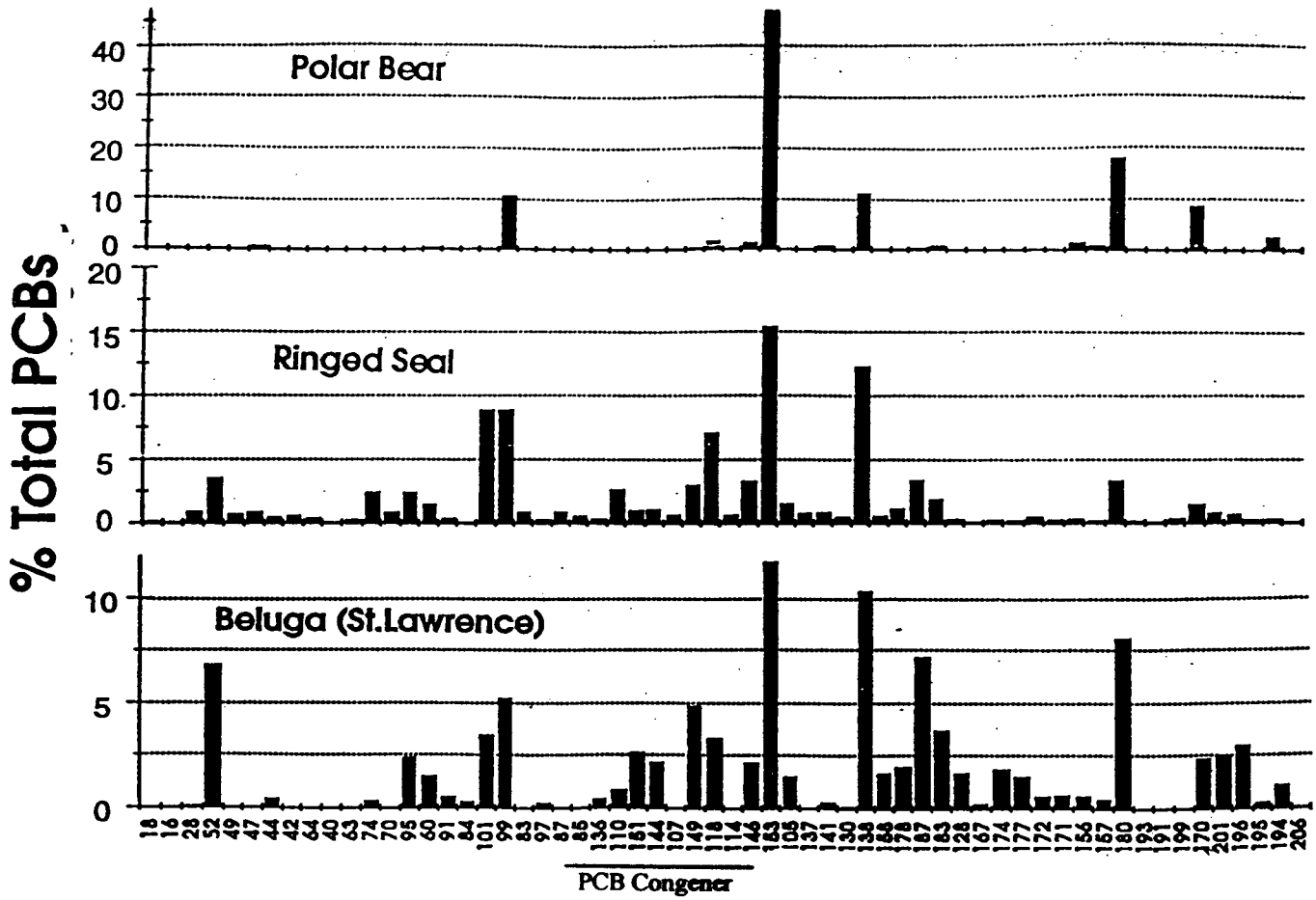

Figure 1.8. PCB isomers in adipose tissue of marine mammals from the Eastern Arctic (Figure 2 Norstrom & Muir 1994)

1.4. *RESEARCH OBJECTIVES*

The aim of the present study was to try to develop a laboratory model of exposure to PCBs at levels similar to those found in the environment. I intended to examine the correlation between residual hepatic PCB concentrations and induction of cytochromes P450 after low level PCB exposure in a laboratory animal model. Many previous studies investigated the biologic effects of exposure to PCBs at high levels that are not found in the environment (Dragnev *et al.* 1995, Dragnev *et al.* 1994, Harris *et al.* 1993). Moreover, several of the studies used pure PCB isomers or commercial mixtures that are not representative of PCBs in the environment. In the present study, male Long-Evans rats were exposed to various doses of Aroclor 1260 in an attempt to mimic levels of exposure similar to those found in the arctic marine environment.

The specific objectives of the project were:

(1) To examine the induction of cytochromes P450 after exposure to various doses of Aroclor 1260 for seven days.

(2) To measure residual hepatic PCB levels in a laboratory rat model after exposure to various doses of Aroclor 1260 for seven days.

(3) To examine the relationship between residual hepatic PCB concentrations and induction of cytochromes P450 after exposure to Aroclor 1260 in an animal model.

(4) To examine the induction of cytochromes P450 at various times after, low level PCB exposure.

1.5. *RESEARCH PLAN AND RATIONALE*

To study the effects of sub-chronic, low level PCB exposure, I proposed to treat Long-Evans rats with varying doses (0.5, 5.0, 10, 20, and 50 mg/kg) of Aroclor 1260 by oral gavage for seven consecutive days. Hepatic microsomes were prepared from these animals and the levels of cytochromes P450 1A, P450 2B, and P450 3A were quantitated. A second part of the study involved measuring induction of cytochromes P450 1A, P450 2B, and P450 3A at various times after treatment (1, 3, 6, 12, 24, and 48 days) with Aroclor 1260 for seven consecutive days at a dose of 10 mg/kg. Cytochrome P450-mediated activities including alkoxyresorufin *O*-dealkylase, aryl hydrocarbon hydroxylase, and testosterone hydroxylase were measured. In addition, cytochrome P450 isozyme levels were measured using immunoblots and immunoquantitation. A GC-MS method was used to determine residual hepatic PCB levels in rats.

In the present study, Aroclor 1260 was used to treat male Long-Evans rats because of its large percentage of the more highly chlorinated biphenyls, in particular isomers PCB-138, -153, -170, and -180, all of which have been found to be highly concentrated in marine mammals (Letcher *et al.* 1995). For example, three hexachlorobiphenyl isomers, namely PCB-138, -153, and -180 account for 5.01%, 8.22%, and 7.20%, (by weight) respectively, of the total PCB composition of Aroclor 1260 (Albro *et al.* 1981), and account for more than 15%, 30%, and 15%, respectively, of the total PCBs found in polar bear liver (Bandiera *et al.* 1997). However, the molar percentages for PCB-99 and -170 (0.82 and 0.62, respectively) are quite low in Aroclor 1260 compared to levels found in polar bears (Albro *et al.* 1981). We initially considered

preparing a synthetic mixture using purified PCB congeners but the cost of the purified congeners in the amounts needed was excessive.

All dosages will be administered daily by oral gavage for seven consecutive days. The doses chosen for the present study were selected after examination of previous studies (Dragnev *et al.* 1994, Harris *et al.* 1993, Nims *et al.* 1992, Lubet *et al.* 1991). Both Lubet *et al.* (1991) and Nims *et al.* (1992) found that a significant increase of cytochrome P450 1A-mediated enzyme activity occurred with Aroclor 1254 at concentration of 1.0 mg/kg in the diet for seven days. However, Lubet *et al.* (1991) found that an Aroclor 1254 concentration of at least 10 mg/kg in the diet was necessary to produce a significant increase in cytochrome P450 2B-mediated enzyme activity. In contrast, Dragnev *et al.* (1994) found that an Aroclor 1254 concentration of 3.3 mg/kg in the diet significantly elevated cytochrome P450 2B-mediated enzyme activity, but the rats were administered the PCB enhanced diet for 84 days. For rats administered a single intraperitoneal injection of Aroclor 1254, cytochrome P450 1A- and P450 2B-mediated enzyme activities were increased significantly at doses of 10 and 40 mg/kg, respectively (Harris *et al.* 1993).

The concentration of Aroclor 1254 that a rat would be exposed to during dietary intake is dependent on the amount of food the rat eats in a particular day, and would most likely result in a lower concentration than the concentration in the food. This factor is important when interpreting results from previous studies (Dragnev *et al.* 1994, Nims *et al.* 1992, Lubet *et al.* 1991). The actual amount of Aroclor 1254 ingested by the rats can be calculated by assuming that the daily intake for a single rat is 15g of rat chow and the average weight of the rats in that study (Lubet *et al.* 1991) used was 163g. For example a

concentration of 100 ppm in the food translates into a dose of 9 mg/kg for a rat weighing 163 g.

$$(15\ g)(100\ ppm)/1{,}000{,}000 = 0.0015\ g\ of\ Aroclor\ 1254/163\ g\ rat$$

$$\Rightarrow (0.0015\ g)(1000kg/163g) = 0.009g/kg\ or\ 9mg/kg$$

Table 1.5. Intake of dietary Aroclor 1254 in F344/CrlBr rats

Aroclor 1254 Concentration in Feed ppm (mg/kg)	Daily Calculated Aroclor 1254 Concentration Ingested by Rats	Calculated Aroclor 1254 Concentration in Rats After Seven Days Exposure
1 ppm	0.09 mg/kg	0.64 mg/kg
3.3 ppm	0.3 mg/kg	2.13 mg/kg
10 ppm	0.9 mg/kg	6.3 mg/kg
33 ppm	3 mg/kg	21 mg/kg
100 ppm	9 mg/kg	63 mg/kg

Data adapted from Dragnev *et al.* (1994).

From existing studies, it is apparent that the elevation of cytochrome P450-mediated enzyme activities is dependent upon the PCB dose, the route of delivery, and duration of treatment. It should also be noted that Aroclor 1260 is less potent than Aroclor 1254 (Harris *et al.* 1993). Taking all of these considerations into account, it was decided that Aroclor 1260 doses ranging from 0.5 to 50 mg/kg would be used for the

present study. Oral gavage was selected as the route of administration to ensure that each rat received the entire daily Aroclor 1260 dose. Unlike the study by Garthoff *et al.* (1981), whereby dose calculations were based on animal weights at the start of the experiment and not adjusted for changing body weights, dose calculations in the present study were based upon individual body weights at each time of dosing.

Few studies examined the effect of time after PCB exposure on cytochrome P450-mediated enzyme activities and hepatic cytochrome P450 levels. Dragnev *et al.* (1994) observed that cytochrome P450 1A-mediated enzyme activity decreased slightly by 21 days after dietary exposure to Aroclor 1254. In the present study, I have chosen to kill the rats exposed to Aroclor 1260 at a mid-range dose from one to 48 days after treatment.

2. EXPERIMENTAL

2.1. *CHEMICALS*

Chemicals and reagents used in this study were obtained from the following sources:

Aldrich Chemical Company Inc. (Milwaukee, Wisconsin, U.S.A.):

Resorufin

Anachemia (Mississauga, Ontario, Canada):

Hydrochloric acid

BDH Chemicals (Toronto, Ontario, Canada):

Ethanol (95%); ethylenediaminetetraacetic acid, disodium salt (EDTA); Folin and Ciocalteu phenol reagent; magnesium chloride; potassium chloride; potassium ferricyanide; sodium carbonate, anhydrous; sodium hydroxide, sucrose.

Bio-Rad Laboratories (Mississauga, Ontario, Canada):

Acrylamide 99.9%; N,N′-methylene-bis-acrylamide (BIS); 2-mercaptoethanol; N,N,N′,N′-tetramethylethylenediamine (TEMED); Bio-Beads S-X3 (200-400 mesh).

Boehringer Mannheim Canada Ltd. (Laval, Quebec, Canada):

Nicotinamide adenine dinucleotide phosphate, tetrasodium salt (NADPH).

Caledon Chemicals, Inc. (Georgetown, Ontario, Canada)

Acetone, ACS reagent grade

Carnation Inc. (Toronto, Ontario, Canada):

Skim milk powder.

Fisher Scientific Ltd. (Vancouver, British Columbia, Canada):

Acetonitrile (HPLC-grade); ascorbic acid; methylene choride (HPLC-grade); glycerin; glycine; methanol (ACS grade); methanol (HPLC-grade); hexane (Optima-grade); methylene chloride (Optima-grade); toluene (Optima-grade); perchloric acid; potassium phosphate monobasic; sodium chloride; sodium dodecyl sulphate (SDS);

sodium phosphate; sodium sulfate, anhydrous.

ICN Biomedicals Canada Ltd. (St-Laurent, Quebec, Canada):

Bovine serum albumin (globulin and fatty acid free, fraction V); tris(hydroxymethyl)aminomethane (Trizma base); silica (100-200); alumina N-super 1.

Immunocorp (Montreal, Quebec, Canada):

Affinity-isolated, alkaline phosphatase conjugated, goat $F(ab')_2$ anti-rabbit IgG (gamma and light chain specific, human IgG adsorbed) from TAGO Immunologicals; affinity-isolated, alkaline phosphatase conjugated, goat $F(ab')_2$ anti-mouse IgG (gamma and light chain specific, human IgG adsorbed) from TAGO Immunologicals.

J.T. Baker Chemical Co. (Phillipsburg, New Jersey, U.S.A.):

Sodium dithionite; concentrated sulfuric acid.

Mandel Scientific Company Ltd. (Edmonton, Alberta, Canada):

Blotting paper; nitrocellulose membrane (Schleicher & Schnell); glass wool (pesticide grade).

Molecular Probes, Inc. (Eugene, Oregon, U.S.A.):

7-benzyloxyresorufin; 7-ethoxyresorufin; 7-pentoxyresorufin.

NCI Chemical Carcinogen Repository Midwest Research Institute

3-OH benzo[a]pyrene.

Pierce (Rockford, Illinois, U.S.A.):

4-Nitro-blue tetrazolium chloride.

Praxair (Vancouver, British Columbia, Canada):

Carbon monoxide gas (99.5% purity); Nitrogen gas.

Schwarz/Mann Biotech (Cleveland, Ohio, U.S.A.):

Ammonium persulfate (electrophoresis grade).

Sigma Chemical Co. (St. Louis, Missouri, U.S.A.):

Benzo[a]pyrene (98%); bromphenol blue; corn oil; cupric sulfate pentahydrate;

dimethyl sulfoxide; N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES); pyronin Y (certified); sodium potassium tartrate tetrahydrate; Tris(hydroxymethyl)aminomethane hydrochloride (Trizma HCl) and polyoxyethylene sorbitan monolaurate (Tween 20).

Steraloids Inc. (Wilton, New Hampshire, U.S.A.):

Testosterone; 2α-hydroxytestosterone; 2β-hydroxytestosterone; 6β-hydroxytestosterone; 7α-hydroxytestosterone; 11β-hydroxytestosterone; 16α-hydroxytestosterone and 16β-hydroxytestosterone.

Xymotech Biosystems (Mt. Royal, Quebec, Canada)

5-bromo-4-chloro-3-indolyl phosphate disodium salt.

Dr. S. M. Bandiera (Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, British Columbia, Canada):

Purified rat cytochrome P450 1A1; Purified rat cytochrome P450 2B1; purified cytochrome P450 3A1; polyclonal rabbit anti-rat cytochrome P450 2B1 IgG (backabsorbed and non-backabsorbed); polyclonal rabbit anti-rat cytochrome P450 3A1 IgG (backabsorbed); pooled liver microsomes prepared from phenobarbital-treated adult male Long Evans rats.

Dr. M. Ikonomou (Institute of Ocean Sciences, Sidney, British Columbia, Canada)

Internal and external standards for the PCB extraction method. The internal standard consisted of seven $^{13}C_{12}$-labeled pure PCB isomers (PCB-52, PCB-128, PCB-180, PCB-194, PCB-208, PCB-101, PCB-209) diluted in toluene. The external standard consisted of one ^{13}C-labeled pure PCB isomer (PCB-111) diluted in toluene.

Dr. Stephen Safe (Texas A & M University, College Station, Texas, USA)

Aroclor 1260.

Dr. Paul Thomas (Rutgers University, Piscataway, New Jersey, USA)

Monoclonal mouse anti-rat cytochrome P450 1A1 IgG

2.2. *ANIMALS AND TREATMENT*

Animal treatment for this study was divided into two sets:

Set 1.

Thirty male Long-Evans rats, weighing 250-300 g (7-9 weeks old) were purchased from Charles River Canada, Inc. (Montreal, Quebec). They were housed in polycarbonate cages on corncob bedding in groups of two or three per cage. The animal

room was maintained at a controlled temperature (23°C) and on 12-h light and 12-h dark cycles. The rats were given Purina rat chow (Lab Diet, Purina Mills, Inc.) and water *ad libitum*.

After one week of acclimatization, the rats were divided into six treatment groups consisting of five animals per group. One group of rats was treated with corn oil by oral gavage at a dose of 2.5 mL/kg, and the five other groups were given Aroclor 1260 in corn oil by oral gavage once per day for seven consecutive days at a dose of 0.5, 5.0, 10, 20, or 50 mg/kg in a volume of 2.5 mL/kg. Rats were decapitated three days after the last treatment. A 0.5-1.5 g sample of adipose tissue was removed from the inner thigh of each rat, weighed, and stored at -20°C for later analysis of PCB content.

Set 2.

The second set of rats consisted of five groups of three animals each. The rats were maintained under the same conditions as described above. Rats were treated with Aroclor 1260 given by oral gavage once per day for seven consecutive days at a dose of 10 mg/kg and then killed at different times (1, 6, 12, 24, and 48 days) after the last treatment. A 0.5-1.5 g sample of adipose tissue was removed from the inner thigh of each rat and stored at -20°C for later analysis of PCB content.

2.3. *HEPATIC MICROSOME PREPARATION*

Following decapitation, the livers were removed and weighed. Each liver was homogenized in 20 mL of ice-cold 0.05M Tris buffer, pH 7.5, containing 1.15% KCl, (4 mL/g wet liver weight) using a Potter-Elvehjem glass mortar and motor driven pestle. Homogenization was achieved using five slow-speed passes with a loose fitting pestle followed by five passes with a high speed tight fitting pestle. A 5 mL sample of the homogenate was taken and stored at -20°C for PCB analysis.

Liver microsomes were prepared as described by Thomas *et al.* (1983). The homogenates were transferred to polycarbonate centrifuge tubes and spun at 9,000 x g for 20 min. at 5°C in a Beckman Model J2-21 centrifuge. The resulting supernatants were strained through cheesecloth into ultra-centrifuge bottles and spun at 105,000 x g for 60 min at 5°C using either a Beckman Model L5-50 or Beckman L8-60 ultracentrifuge. The lipid and glycogen were removed and discarded, and the resulting microsomal pellets were resuspended in ice-cold 10 mM EDTA, 1.15 % KCl, pH 7.4, using the loose fitting pestle for three passes. This was then centrifuged at 105,000 x g for 60 min. at 5°C and the resulting pellets were resuspended in a small volume of (approximately 3 to 6 pellet volumes) of ice-cold 0.25 M sucrose by gentle vortexing followed by three or four passes with the tight-fitting pestle. The microsomal preparations were aliquoted into a series of five 1.8 mL vials and stored at -75°C.

2.4. *DETERMINATION OF TOTAL CYTOCHROME P450 CONTENT*

Total microsomal cytochrome P450 content was determined from the sodium dithionite reduced carbon monoxide difference spectrum according to the method of Omura and Sato (1964a). Hepatic microsomes were diluted in 0.1 M sodium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA, and then reduced with sodium dithionite. The diluted microsomal suspension in the sample cuvette was saturated with carbon monoxide. Spectral measurements were performed using a SLM-Aminco DW-2 spectrophotometer. Total hepatic cytochrome P450 content was calculated using a molar extinction coefficient of 91 cm^2/mmol (Omura and Sato 1964b).

2.5. *DETERMINATION OF TOTAL PROTEIN CONCENTRATION*

Protein concentrations were measured using the Lowry protein assay (1951). Bovine serum albumin (BSA) was used as the standard. All samples were measured in duplicate at an absorbance of 650 nm using a Shimadzu UV-160 UV-Visible recording spectrophotometer.

2.6. *DETERMINATION OF CYTOCHROME P450-MEDIATED ENZYME ACTIVITIES*

All enzyme assays were carried out with individual microsomal samples. For

each enzyme assay, samples were run in duplicate and on at least two different occasions.

2.6.1. *Benzyloxyresorufin O-dealkylase assay (BROD)*

Microsomal BROD activity was measured according to the fluorimetric method of Burke *et al.* (1974). The following reaction illustrates the cytochrome P450 catalyzed dealkylation of benzyloxyresorufin (Figure 2.1)

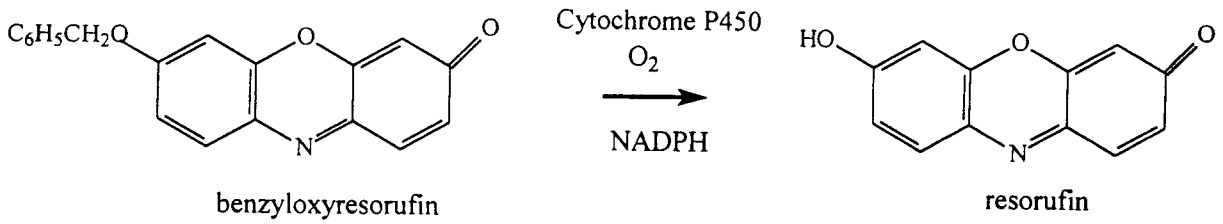

Figure 2.1. The cytochrome P450 catalyzed dealkylation of benzyloxyresorufin

Reaction mixtures contained 50 μL of hepatic microsomal protein at a concentration of 2 mg/mL, 1.93 mL of 0.1 M HEPES buffer, pH 7.4, with 5 mM $MgCl_2$, and 10 μL of 1 mM benzyloxyresorufin in a total of 1.99 mL. After the mixture was preincubated at 37°C for 5 min, the fluorescence intensity was recorded and 10 μL of 50 mM NADPH was then added to initiate the reaction. The reaction was allowed to proceed for 5 min at 37°C and the fluorescence activity was recorded again. All measurements were made using a Shimadzu RF-540 spectrofluorophotometer interfaced with a Shimadzu DR-3 data recorder. The excitation and emission wavelengths were set at 530 nm and 584 nm, respectively, with slit widths of 2 nm. The amount of resorufin

formed was determined from a standard curve of fluorescence intensity versus resorufin concentration.

2.6.2. *Ethoxyresorufin O-deethylase assay (EROD)*

Microsomal EROD activity was determined using the method of Burke *et al.* (1974). The following reaction illustrates the cytochrome P450 catalyzed deethylation of ethoxyresorufin (Figure 2.2).

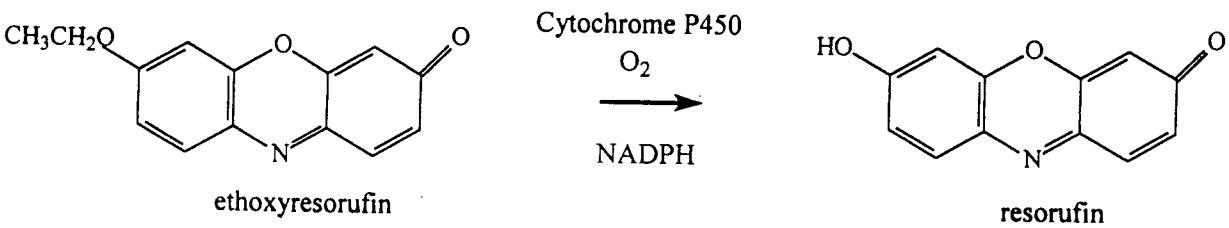

Figure 2.2. The cytochrome P450 catalyzed deethylation of ethoxyresorufin

The method is the same as described above for the BROD assay, except benzyloxyresorufin was replaced by ethoxyresorufin as the substrate.

2.6.3. *Pentoxyresorufin O-dependylase assay (PROD)*

Microsomal PROD activity was determined as described by Lubet *et al.* (1985). The following reaction illustrates the cytochrome P450 catalyzed dependylation of

pentoxyresorufin (Figure 2.3).

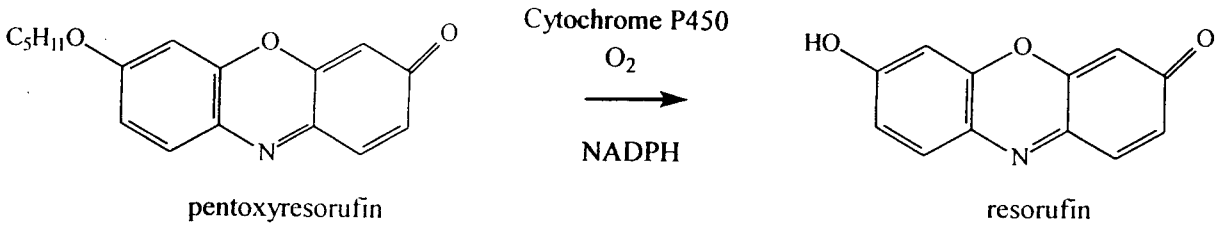

Figure 2.3. The cytochrome P450 catalyzed depentylation of pentoxyresorufin

The method is the same as described above for the BROD assay, except benzyloxyresorufin was replaced by pentoxyresorufin as the substrate.

2.6.4 *Benzo[a]pyrene hydroxylase assay*

Benzo[a]pyrene hydroxylase activity was measured according to the fluorimetric method of Nebert *et al.* (1968). The cytochrome P450 catalyzed hydroxylation of benzo[a]pyrene is illustrated in Figure 2.4.

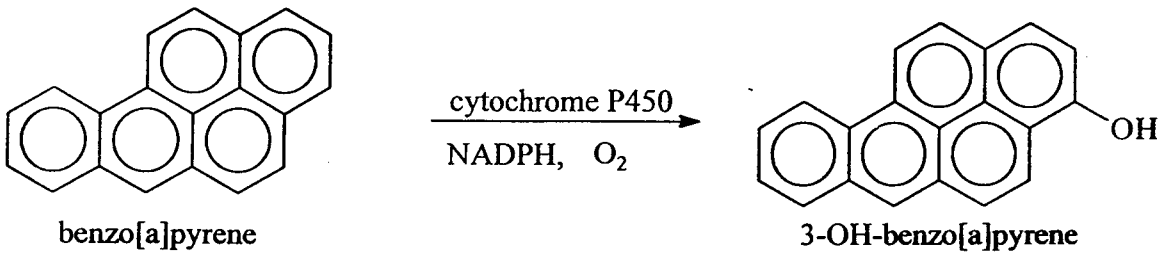

Figure 2.4. The cytochrome P450 catalyzed hydroxylation of benzo[a]pyrene

Because the primary product of this reaction, 3-hydroxy-benzo[a]pyrene, is light sensitive the assay was carried out in dim light. Reaction mixtures contained 50 μL of hepatic microsomal protein at a concentration of 2 mg/mL, 500 μL of 100 mM KPO_4 buffer, pH 7.4, 430 μL distilled water, and 10 μL of 10.0 mM benzo[a]pyrene for a total volume of 990 μL. Reactions were initiated by the addition of 10 μL of 50 mM NADPH after a preincubation period of 5 min. at 37°C. Reaction mixtures were incubated in a shaking water bath at 37°C for 4 min. One mL cold acetone was added to each reaction mixture to stop the reaction. Hexane (3.25 mL) was then added to each tube and the tubes were capped and mixed vigorously for at least one min. These tubes were spun at 3,000 x g for 5 min using a Jouan model CR3000 centrifuge. Two mL of the organic fraction were removed and transferred to a new test tube and 4.0 mL of 1M NaOH was added and mixed. These tubes were spun at 3,000 x g for 5 min using a Jouan model CR3000 centrifuge. The upper organic phase was aspirated and discarded. Fluorescence of the remaining aqueous extract was measured using an excitation wavelength of 396 nm and an emission wavelength of 522 nm. All measurements were made using a

Shimadzu RF-540 spectrofluorophotometer interfaced with a Shimadzu DR-3 data recorder. The amount of 3-hydroxy-benzo[a]pyrene formed was determined from a standard curve of fluorescence intensity versus 3-hydroxy-benzo[a]pyrene concentration.

2.6.5. *Testosterone hydroxylase assay*

The method of Sonderfan *et al.* (1987) was used to measure hepatic microsomal testosterone hydroxylase activities. The cytochrome P450 mediated hydroxylation of testosterone at various positions is illustrated in Figure 2.5.

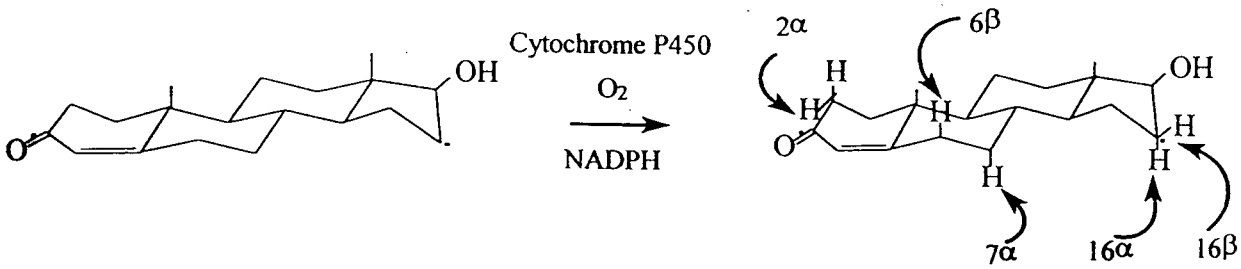

Figure 2.5. The cytochrome P450 mediated hydroxylation of testosterone at various positions

The reaction mixtures contained 50 μL of hepatic microsomal protein at a concentration of 6 mg/mL, 920 μL of 50 mM potassium phosphate buffer, pH 7.4, with 3 mM $MgCl_2$, 10 μL of 100mM NADPH and 20 μL of 12.5 mM testosterone to give a final volume of 1 mL. Reactions were initiated with the addition of 20 μL of 12.5 mM testosterone after preincubating the reaction mixture at room temperature for 10 min.

The reaction was allowed to proceed for 5 min at 37°C and was then halted with the addition of 6 mL of dichloromethane. Each sample was spiked with 2.5 nmol of the internal standard, 11β-hydroxytestosterone, and mixed vigorously for at least one min. The tubes were spun at 2,000 rpm for 1 min using a Jouan model CR3000 centrifuge to allow separation of the aqueous and organic phases. The aqueous layer was then aspirated and discarded, and the organic phase was evaporated under a gentle stream of nitrogen. The dried residues were reconstituted with 200 μL of HPLC-grade methanol, mixed for 1 min, and filtered through 13 mm, 0.45 μm syringe filters into autosampler vials fitted with conical inserts. Testosterone and its metabolites were separated and analyzed using a reverse phase C_{18} HPLC column at 40°C connected to a Shimadzu LC-6A binary gradient HPLC system equipped with an SIL-6B autosampler, SPD-6A variable wavelength UV detector (set at 254 nm) and a CTO-6A heater (Shimadzu Scientific Instruments). A Supelcosil LC-18 octyldecylsilane column (5 μm particle size, 15 cm x 4.6 mm) and a Supelcosil LC-18 guard column (40 μm particle size, 2 cm x 4.6 mm) (Supelco, Bellefonte, PA) were used. The column was eluted with 100% solvent A (methanol:water:acetonitrile, 35:64:1) from 0 to 10 min, followed by a linear gradient of solvent B (methanol:water:acetonitrile, 80:18:2) from 0 to 100 % from 10 to 29 min, then 100 % solvent B from 29 to 31 min, and a linear gradient to 100% solvent A from 31 to 32 min, followed by re-equilibration with 100 % solvent A from 32 to 34 min. The total flow rate was 2 mL/min. Testosterone and its metabolites, which include 2α–, 6β–, 7α–, 16α–, 16β-hydroxytestosterone, were identified by comparing retention time to those of authentic standards. Peak areas were integrated using a Shimadzu CR501 chromatography data processor. The quantity of each metabolite formed was calculated

from standard curves using the ratio of the peak area of the metabolite to that of the internal standard. Standard curves were prepared as part of every assay and incorporated four concentrations of each authentic standard. Calibration curves were generated for each metabolite by plotting the ratio of the peak area of the authentic standard to that of the internal standard versus the concentration of the authentic standard.

2.7. *IMMUNOBLOT ANALYSIS*

2.7.1. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis*

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Hoefer SE 600 vertical slab gel unit according to the procedure of Laemmli (1970). The SDS-polyacrylamide gel consisted of both a 3.0 % stacking gel and a 7.5 % separating gel. The stacking gel was 0.75 mm thick, 1 cm long, and contained 0.125 M Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 3% (w/v) acrylamide-BIS, 0.08% (w/v) ammonium persulphate, and 0.05% (w/v) TEMED. The separating gel was 0.75 mm thick, 12.5 cm long and contained 0.375 M Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 7.5% (w/v) acrylamide-BIS, 0.042% (w/v) ammonium persulphate, and 0.03% (w/v) TEMED. Hepatic microsomes were diluted in sample dilution buffer containing 0.062 M Tris-HCl (pH 6.8), 1% (w/v) SDS, 10% glycerol, 0.001% (w/v) bromophenol blue, and 5% (w/v) mercaptoethanol. After boiling the microsomal mixture for two min, 20 μL of the denatured microsomal samples were loaded into each well in the stacking gel. The samples were subjected to electrophoresis at constant current with cooling. An initial

current setting of 0.12 mA was used until the dye front had moved through the stacking gel followed by a current of 0.24 mA until the dye front reached the bottom of the separating gel.

2.7.2. *Immunoblots*

Proteins resolved by SDS-PAGE were transferred electrophoretically onto a 13 x 14 cm nitrocellulose membrane using a Hoefer Transphor Apparatus (model TE 52) at a setting of 0.4 A for 2 hours or 0.2 A for 16 hours at 4°C following the procedure described by Towbin *et al.* (1984). After the transfer, the nitrocellulose membranes were placed in blocking buffer (1% BSA, 3% skim milk powder, and modified PBS) at 4°C overnight. The next day, the nitrocellulose membranes were removed and washed using three ten minute incubations with wash buffer (0.05% Tween 20 in modified PBS) in a shaking water bath at 37°C. The nitrocellulose membranes were then incubated with primary antibody against rat cytochrome P450 1A1, cytochrome P450 2B1, or cytochrome P450 3A1 in antibody dilution buffer (1% BSA, 3% skim milk powder, 0.05% Tween 20, in modified PBS) at 37°C in a shaking water bath for two hours. After washing three times with wash buffer, the membranes were incubated with alkaline phosphatase conjugated goat anti-rabbit secondary antibody (1:3000 dilution) in antibody dilution buffer at 37°C for two hours in a shaking water bath. The membranes were again washed three times with wash buffer. Under dim light conditions, the nitrocellulose membranes were incubated with substrate solution consisting of 0.01% nitroblue tetrazolium chloride, 0.005% 5-bromo-4-chloro-3-indolyl phosphate disodium salt, in 0.1

M Tris-HCl, 0.5 mM $MgCl_2$, pH 9.5 at room temperature for periods of two to five min until bands were clearly visible. The substrate solution was discarded and the blot was rinsed with distilled water to stop the reaction. After this, the nitrocellulose membranes were air dried.

For the immunodetection of cytochrome P450 1A1, a concentration of monoclonal mouse anti-rat cytochrome P450 1A1 IgG of 1.0 μg/mL, and a 1:3000 dilution of the secondary antibody (goat anti-mouse IgG) were used. The reaction time for the alkaline phosphatase reaction varied from 2 to 5 minutes.

For the immunodetection of cytochromes P450 2B1 and P450 2B2, a concentration of backabsorbed polyclonal rabbit anti-rat cytochrome P450 2B1 IgG of 2.0 mg/mL, and a 1:3000 dilution of the secondary antibody (goat anti-rabbit IgG) were used. The reaction time for the alkaline phosphatase reaction varied from 2 to 5 minutes.

For the immunodetection of cytochrome P450 3A isozymes, a concentration of polyclonal rabbit anti-rat cytochrome P450 3A1 IgG of 10 μg/mL, and a 1:3000 dilution of the secondary antibody (goat anti-rabbit IgG) were used. The reaction time for the alkaline phosphatase reaction varied from 2 to 3 minutes.

2.7.3. *Immunoquantitation*

Densitometric quantitation of stained protein bands was determined using a PDI 420oe scanner interfaced with an IBM-compatible computer and using the PDI Quantity One software program. The amount of immunoreactive protein was determined from the integral of the optical density of the stained band. Values of integrated intensity were

converted into pmole quantities using calibration curves generated by loading various concentrations of purified cytochrome P450 standards on gels followed by immunoblotting and densitometric analysis as described above. A known concentration of purified cytochrome P450 was included on each blot as an internal standard. Calibration curves were prepared for each cytochrome P450 isozyme by plotting the ratio of the integrated intensity of the authentic standard to the integrated intensity of the internal standard versus the amount of purified cytochrome P450 (*i.e.* authentic standard) loaded. The pmole quantity corresponding to the stained band for each unknown sample was calculated from the slope of the calibration curve using the ratio of the integrated intensity of the immunoreactive sample band to that of the reference standard.

2.8. *PCB RESIDUE ANALYSIS*

2.8.1. *PCB tissue extraction*

Approximately 5 g of liver homogenate was spiked with 50 μL of a PCB internal standard mixture. The internal standard mixture contained seven $^{13}C_{12}$ labeled di- and tri-orthochlorinated PCBs (PCB-52, PCB-128, PCB-180, PCB-194, PCB-208, PCB-101, PCB-209) dissolved in toluene. Each $^{13}C_{12}$ labeled PCB isomer was present in the standard mixture at a concentration of 2000 pg/μL. The liver homogenates were dehydrated by grinding with anhydrous sodium sulfate, which had been baked overnight at 375°C. Lipid extracts were obtained by placing the the tissue-sodium sulfate mixtures into 500 mL fritted ceramic funnels and eluting with 400 mL of 1:1

dichloromethane:hexane (DCM:HEX). Each lipid extract was concentrated to less than 5 mL using a Buchi Rotavapor-R rotary evaporator at a maximum water bath temperature of 28°C, weighed, and then 10% of the total weight was removed for separate gravimetric determination of lipid content.

The remaining 90% of the lipid extract was loaded onto a silica gel column to separate the lipids from the PCBs. The silica gel was activated by baking overnight at 200°C. A glass wool plug was placed at the bottom of a 50 mL buret. Then, a 4 cm layer of basic silica gel (prepared by adding 3.5 g 1 M NaOH to 10 g SiO_2), followed by a 2 cm layer of neutral silica gel, a 8 cm layer acidic silica gel (prepared by adding 5.0 g concentrated H_2SO_4 to 10.0 g SiO_2), and a 2 cm layer of neutral silica gel were placed carefully into the buret. The silica gel column was conditioned by eluting with 25 mL of DCM:HEX (1:1). The sample was added to the column using 1.5 mL of DCM:HEX (1:1), followed by three rinses of the flask with a few mL of DCM:HEX (1:1). The column was then eluted with 60 mL of DCM:HEX (1:1). All of the eluate was collected in a clean 150 mL flat bottom flask. The eluate was concentrated to 1 mL or less using a Buchi rotary evaporator and then air dried to completeness. The sample was reconstituted in a few mL of hexane.

A second column was prepared as follows. A 50 mL buret was plugged with glass wool and then a 15 cm layer of activated alumina (prepared by baking overnight at 200°C) was added, followed by a 1 cm layer of anhydrous sodium sulfate on top. The column was conditioned by eluting with 25 mL of hexane. The reconstituted sample was added to the column and allowed to elute with hexane. The flask was rinsed three times with hexane and the hexane rinse volumes were added to the column to elute any residual

organochlorines in the flask. The first 10 mL of hexane eluant was used to elute polychlorinated diphenyl ethers (PCDEs) and other non-planar compounds. This fraction was discarded. The fraction of interest was eluted using 60 mL of DCM:HEX (1:1) and was collected in the previously used 150 mL flat bottom flask. The sample was concentrated using a Buchi rotary evaporator down to about 1 - 2 mL and transferred to a 15 mL centrifuge tube.

A carbon fiber column was prepared by filling a 7.5 cm (0.5 cm I.D.) stainless steel HPLC column with a mixture of activated carbon (Amoco PX-21) and filter paper (Nucleopore P100) (1:12 w/w). The column was part of a Millipore/Waters preparative liquid chromatography system. The solvents used, the elution volumes, and the identities of the eluates are described in Table 2.1.

Table 2.1. Solvents, elution volumes, and the identities of the eluates obtained in each fraction of the carbon-fibre chromatography column

Fraction	Solvent	Volume	Identity
I	3% DCM/hexane	20 mL	PCDEs, 2-4 di-ortho substituted PCBs
II	50% DCM/cyclohexane	44 mL	mono-ortho PCBs
III	50% ethyl acetate/benzene	50 mL	non-ortho PCBs
IV	toluene (reverse flow)	60 mL	PCDDs and PCDFs

Fraction I collected from the carbon fiber system was concentrated to less than 100 μL, transferred to a 200 μL amber chromocore vial, and then spiked with $^{13}C_{12}$-

labeled PCB-111, which serves as the external standard for the GC-MS analysis.

All glassware and utensils used during the extraction procedure were washed with 2-3% Decon detergent, rinsed with tap water and then distilled water, and finally baked overnight at 150°C. After cooling, the glassware was rinsed three times with each of the following solvents in the order listed: hexane; dichloromethane; toluene; hexane; dichloromethane. The glassware was stored in clean containers and covered with hexane rinsed aluminum foil until needed.

2.8.2. *Mass spectrometric analysis*

The extracts were sent to the Institute of Ocean Sciences located in Sidney, B.C. for analysis. The PCB analysis was conducted by the staff at the Institute of Ocean Sciences.

Extracts were analyzed for PCBs by gas chromatography/mass spectrometry (GC-MS) using the method outlined by Rantalainen (1997). The instrument was a VG-Autospec high resolution mass spectrometer (Micromass, Manchester, U.K.) equipped with a Hewlett-Packard model 5890 Series II gas chromatograph and a CTC A200S auto sampler (CTC Analytics, Zurich, Switzerland). The GC was operated in the splitless injection mode and the splitless injector purge valve was activated 2 min after sample injection. The volume injected was 1 μL of sample plus 0.5 μL of air. The analyses were conducted using a 60 m DB-5 fused silica capillary column (0.25 mm i.d. with 0.1 μm film thickness) from J&W Scientific (Folsom, CA), with UHP He as the carrier gas at a constant head pressure of 25 psi, which maintains a linear velocity of 35 cm/s. The

temperature program for the PCB analysis was as follows: the initial column temperature was held at 80°C for 2 min after injection and increased at a rate of 8°C/min to 150°C, and then increased at a rate of 4°C/min to 285°C, where it was held for 1 min. All sample injections were performed using the CTC A200S auto sampler. The MS was the only on-line detector attached to the GC system. The splitless injector port, direct GC/MS interface, and the MS ion source were maintained at 280°C, 290°C, and 290°C, respectively.

For PCB analysis, the MS was operated under positive electron ionization conditions with the filament in trap stabilization mode at 600 μA and an electron energy of 28 to 35 eV. The instrument was routinely resolving at a resolution setting of 10,000. Data were acquired in the Single Ion Resolving Mode for achieving maximum possible sensitivity. Two or more ions, M^+ and $(M+2)^+$ in most cases, of known relative abundance were monitored for each molecular ion cluster representing a group of isomers, as were two of each of the $^{13}C_{12}$-labeled surrogate standards. The criteria for analyte identification and quantification are as specified in the Environment Canada Protocol for PCDD/PCDF analysis and through analysis of prepared GC/MS performance standards (anonymous). The concentrations of identified compounds and their minimum detection limits were calculated by the internal standard method using mean relative response factors determined from the analysis of calibration standard solutions run before and after each batch of samples was analyzed.

2.9. *STATISTICAL ANALYSIS*

All data were analyzed by a one way analysis of variance (ANOVA) and the differences between pairs of mean values were tested using the Student Newman-Keuls test. Mean differences that had a p value of <0.05 were considered to be statistically significant.

Linear regression analysis was conducted using the regression equation from Microsoft Excel version 97.

3. RESULTS

The results components of the thesis is organized into three sections. Validation of the assays used in the project including both intra- and interassay variability is presented in the first section of the Results. Data from a study of the effect of varying doses of Aroclor 1260 on hepatic cytochrome P450 enzymes in adult male rats are presented in the second section. Adult male rats were treated with either 0.5, 5.0, 10, 20, 50 mg/kg Aroclor 1260 or control corn oil by oral gavage for seven consecutive days and then were killed three days after the last treatment. The third section involves a study of the effects of Aroclor 1260 treatment on hepatic cytochrome P450 enzymes in adult male rats at various times after treatment. Rats were treated with Aroclor 1260 at a dose of 10 mg/kg by oral gavage for seven consecutive days and were then killed at various times post-treatment. The effect of Aroclor 1260 treatment in rats was determined by measuring changes in liver and body weight, total hepatic cytochrome P450 content, various cytochrome P450 dependent enzyme activities, and hepatic levels of specific cytochrome P450 isozymes. Hepatic PCB concentrations were also measured for the study presented in the second section.

SECTION 1: ASSAY VALIDATION

The validation of quantitative analytical assays is essential in order to ensure that data acquired from the assays are both reliable and reproducible. Variability of data obtained from quantitative analytical assays is unavoidable and in order for assay results to be valid,

the variation in the data should not be greater than the calculated coefficient of variation. Variability is expressed as the coefficient of variation (CV), which is the standard deviation divided by the mean of the replicates. In general, a minimum of five replicate determinations is required for each sample to calculate the variability and the coefficient of variation should not exceed 15% at any concentration. Intraassay variation is defined as the variation of the assay within a single day. Interassay variation is defined as the variation of the assay between different days.

The sensitivity of the assay is an essential parameter for evaluating the validity of the results. The sensitivity of any assay is determined by the limit of quantitation (LOQ) and the limit of detection (LOD) of the assay. The LOQ is the lowest concentration on the standard curve that can be measured with a coefficient of variation of less than 20%. The LOD is the lowest concentration of analyte that can be detected by the instrument at a level that is at least three times greater than the background noise level.

To validate the enzyme and immunoblot assays used in the present study, standard curves were constructed and the coefficient of variability was calculated for each assay. The interassay and intraassay variabilities were calculated when possible. In addition, optimal conditions for the AHH assay were examined.

3.1. *VALIDATION OF THE EROD, BROD, AND PROD ASSAYS*

Figure 3.1 shows a standard curve constructed using six different concentrations of resorufin. The slope of the standard curve was calculated to be 1.04×10^{-4} with a LOQ of < 100 pmol resorufin/mL (CV of 9.4 %) and a LOD of < 100 pmol resorufin/mL. The interassay and intraassay coefficients of variation were calculated to be 11.5 % and 2.6 %, respectively, using a resorufin standard solution at a concentration of 1000 pmol/mL.

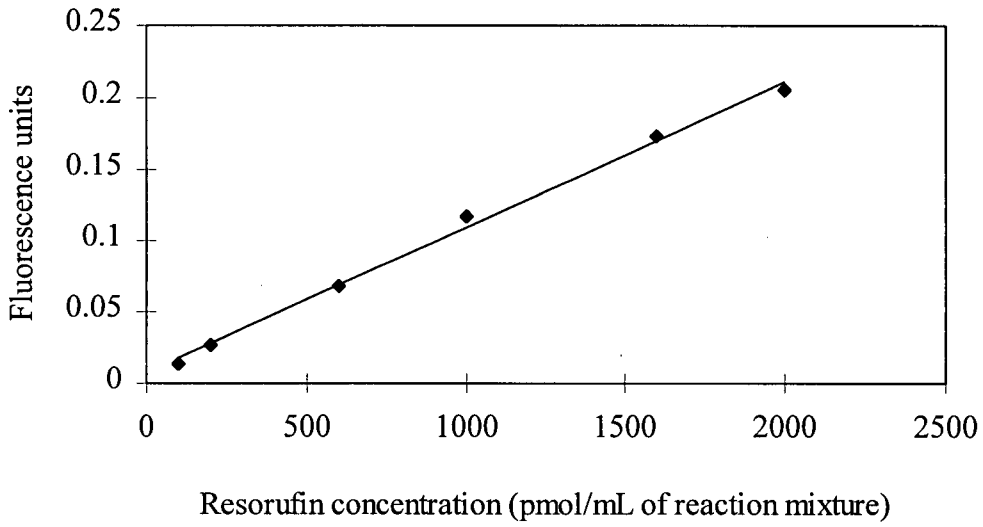

Figure 3.1. Resorufin standard curve (slope = 1.04×10^{-4})

3.2. *VALIDATION OF THE AHH ASSAY*

Figure 3.2. shows a standard curve constructed using ten different concentrations of 3-OH benzo[a]pyrene. The slope of the standard curve was calculated to be 3.87×10^{-3} with

a LOQ of 50 pmol 3-OH benzo[a]pyrene/mL (CV of 14.4 %) and a LOD of 50 pmol 3-OH benzo[a]pyrene/mL. The interassay coefficient of variation was calculated to be 13.7 % using a 3-OH benzo[a]pyrene standard solution at a concentration of 150 pmol/mL.

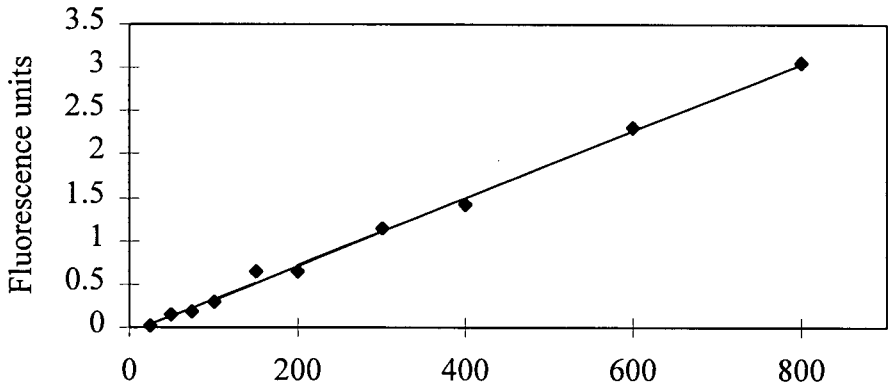

Figure 3.2. AHH assay standard curve (slope = 3.87×10^{-3})

3.3. *OPTIMAL CONDITIONS FOR THE AHH ASSAY*

Unexpectedly low values of microsomal AHH activity were obtained for rats treated with Aroclor 1260 at doses of 5.0 mg/kg and greater. To rule out the possibility of poor experimental design and to ensure that the assay was conducted using optimal and reproducible conditions, assay conditions were investigated. Previously prepared, hepatic microsomes from untreated and Aroclor 1254-treated rats were used for each assay, and the results are shown below.

The effect of varying microsomal protein concentration on product formation is shown in **Figure 3.3**. Formation of 3-OH benzo[a]pyrene was measured at various protein

concentrations using hepatic microsomes from untreated and Aroclor 1254-treated rats. Product formation was found to increase in a linear fashion at final protein concentrations between 0.025 and 0.1 mg/mL with microsomes from both untreated and Aroclor 1254-treated rats.

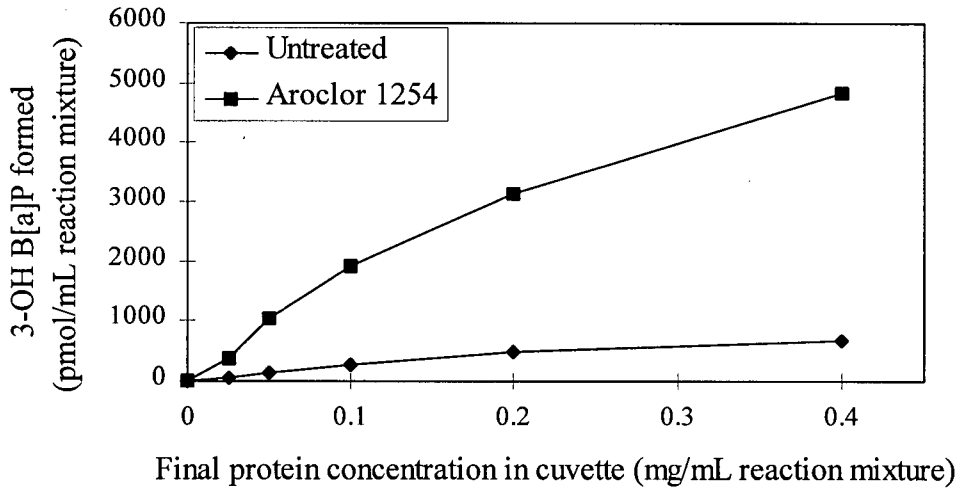

Figure 3.3. Effect of protein concentration on rate of product formation in microsomes from untreated and Aroclor 1254-treated rats

The effect of varying substrate concentration on AHH activity is shown in Figure 3.4. Formation of 3-OH benzo[a]pyrene was measured using a final protein concentration of 0.1 mg/mL with microsomes from untreated or Aroclor 1254-treated rats. AHH activity was found to be relatively constant at final substrate concentrations in the range of 100-400 μM.

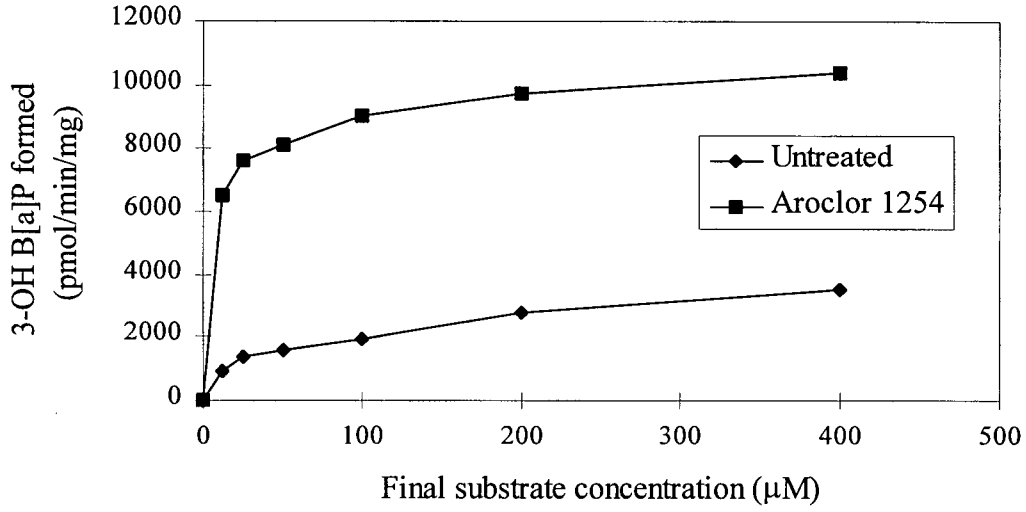

Figure 3.4. Effect of substrate concentration on AHH activity in microsomes from untreated and Aroclor 1254-treated rats

In summary, the results indicate that a final microsomal protein concentration of 0.1 mg/mL and a final substrate concentration of 100 μM were optimal for measurement of AHH activity with hepatic microsomes from untreated and Aroclor 1254-treated rats. Previous experiments from our laboratory had determined that a reaction time of 4 min was within the range where product formation was linearly related to time. All AHH assays were conducted using these optimal conditions.

3.4. *VALIDATION OF THE TESTOSTERONE HYDROXYLASE ASSAY*

Table 3.1 lists the mean slopes of the standard curves of six hydroxytestosterone metabolites. The slope of the standard curves of the different metabolites varied from 0.15 to 0.31. For all six standard curves, the interassay variability of the slope was less than 5%.

Table 3.1. Interassay variability of the testosterone hydroxylase assay

Metabolite	Mean Slope ± SEM	Coefficient of Variation (%)
2α-hydroxytestosterone	0.28 ± 0.003	1.94
2β-hydroxytestosterone	0.23 ± 0.002	1.85
6β-hydroxytestosterone	0.15 ± 0.003	2.99
7α-hydroxytestosterone	0.31 ± 0.005	2.92
16α-hydroxytestosterone	0.29 ± 0.004	2.61
16β-hydroxytestosterone	0.16 ± 0.002	1.77

Mean slopes were calculated from three separate assays.

3.5. *VALIDATION OF THE IMMUNOQUANTITATION OF CYTOCHROME P450 1A1*

Figure 3.5 represents a standard curve for the quantitation of cytochrome P450 1A1 on immunoblots. This curve was constructed using six different concentrations of cytochrome P4501A1 standard. The slope of the standard curve was calculated to be 1.11 x 10^1 with a LOQ of 0.2 pmol cytochrome P450 1A1 applied per lane (CV of 9.4 %) and a LOD of 0.01 pmol cytochrome P4501A1 applied per lane. The interassay coefficient of variation was determined to be 13.7% using a cytochrome P450 1A1 concentration of 0.2 pmol per lane and the intraassay coefficient of variation was calculated to be 1.3% using a cytochrome P450 1A1 concentration of 0.1 pmol per lane.

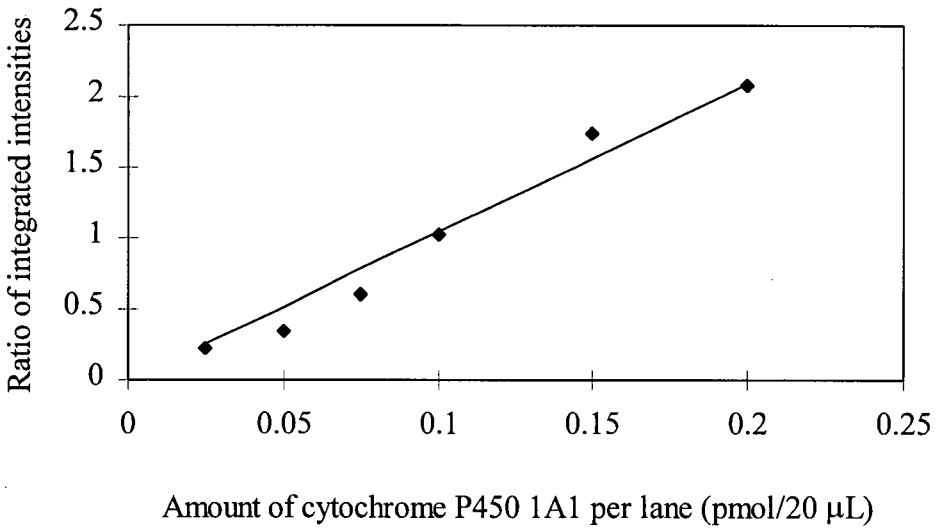

Figure 3.5. Standard curve for cytochrome P450 1A1 (slope = 1.11 x 10^1). Cytochrome P450 1A1 bands were quantified by densitometry and integrated intensity (optical density x mm^2) was measured. The ratio of the integrated intensity of the cytochrome P450 1A1 standard to the integrated intensity of an internal standard was calculated for each standard.

3.6. *VALIDATION OF THE IMMUNOQUANTITATION OF CYTOCHROMES P450 2B1 AND P450 2B2*

Figure 3.6 represents a standard curve for the quantitation of cytochrome P450 2B1 on immunoblots. This curve was constructed using five different concentrations of cytochrome P450 2B1 standard. The slope of the standard curve was calculated to be 1.058 with a LOQ of 0.035 pmol cytochrome P450 2B1 applied per lane (CV of 4.9 %) and a LOD of 0.025 pmol cytochrome P450 2B1 applied per lane. The interassay coefficient of variation was determined to be 11.5% using a cytochrome P450 2B1 concentration of 0.1 pmol per lane and the intraassay coefficient of variation was calculated to be 9.4 % using a cytochrome P450 2B1 concentration of 0.035 pmol per lane.

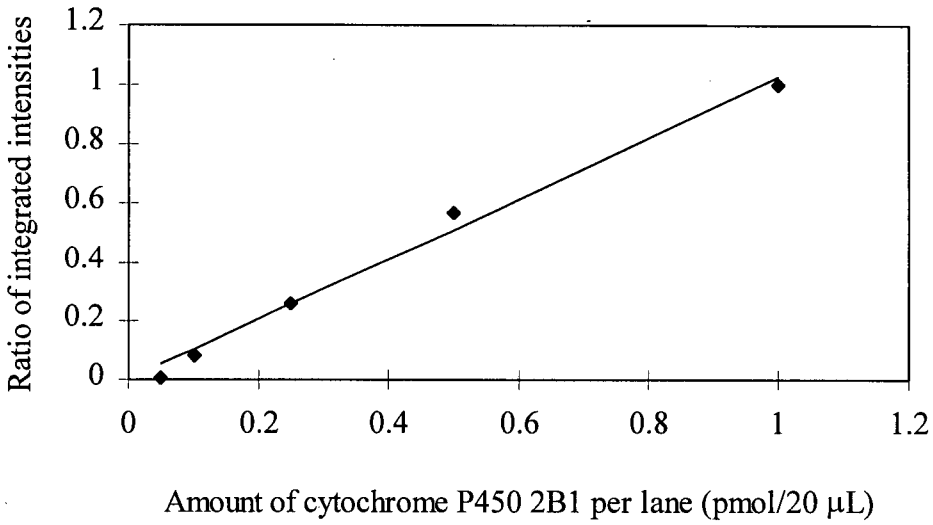

Figure 3.6. Standard curve for cytochrome P450 2B1 (slope = 1.058). Cytochrome P450 2B1 bands were quantified by densitometry and integrated intensity (optical density x mm^2) was measured. The ratio of the integrated intensity of the cytochrome P450 2B1 standard to the integrated intensity of an internal standard was calculated for each standard.

3.7. *VALIDATION OF THE IMMUNOQUANTITATION OF CYTOCHROME P450 3A*

Figure 3.7 represents a standard curve for the immunoquantitation of cytochrome P450 3A1 on immunoblots. The curve was constructed using four different concentrations of cytochrome P450 3A1 standard. The slope of the standard curve was calculated to be 4.259 with a LOQ of 0.0625 pmol cytochrome P450 3A1 applied per lane (CV of 6.36 %) and a LOD of 0.03125 pmol cytochrome P450 3A1 applied per lane. The interassay coefficient of variation was determined to be 6.1% using a cytochrome P450 3A1 concentration of 0.5 pmol per lane and the intrassay coefficient of variation was calculated to be 10.0 % using a cytochrome P450 3A1 concentration of 0.5 pmol per lane.

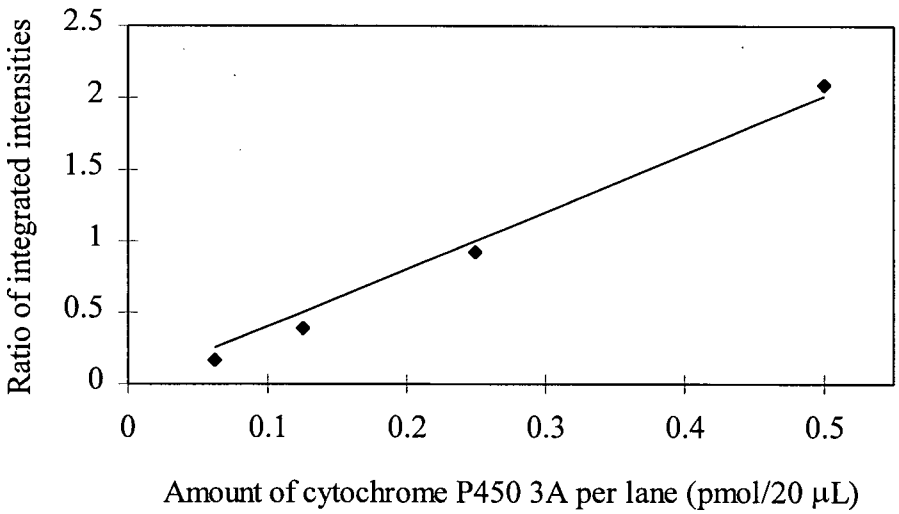

Figure 3.7. Standard curve for cytochrome P450 3A1 (slope = 4.259). Cytochrome P450 3A bands were quantified by densitometry and integrated intensity (optical density x mm^2) was measured. The ratio of the integrated intensity of the cytochrome P450 3A standard to the integrated intensity of an internal standard was calculated for each standard.

SECTION 2: EFFECTS OF INCREASING DOSES OF AROCLOR 1260

3.8. *EFFECTS ON BODY WEIGHT, LIVER WEIGHT, AND TOTAL CYOTOCHROME P450 CONTENT*

Mean values of body weight and liver weight for rats treated with various doses of Aroclor 1260 are presented in Table 3.2. No significant difference in body weight was observed between control and treatment groups. A significant increase in liver weight as a percent of body weight was noted for rats treated with Aroclor 1260 at doses of 10, 20 and 50 mg/kg. The increase in liver weight is suggestive of increased protein synthesis in rats treated with the larger doses of Aroclor 1260.

A significant increase in total cytochrome P450 content was observed for hepatic microsomes prepared from rats treated with Aroclor 1260 at doses of 5.0 mg/kg and greater, relative to the control. Total cytochrome P450 values remained relatively constant at doses greater than 5.0 mg/kg. The increase in total cytochrome P450 content indicates induction of hepatic cytochrome P450 by Aroclor 1260 treatment.

Table 3.2. Mean body and liver weights, and total cytochrome P450 content of male Long-Evans rats treated with various doses of Aroclor 1260

Aroclor 1260 Dose (mg/kg)	Body Weight (g)	Liver Weight (g)	Liver Weight as a % of Body Weight	Total Cytochrome P450 Content (nmol P450/mg protein)
Control	275.0 ± 7.0	12.7 ± 0.7	4.6 ± 0.2	1.5 ± 0.05
0.5	272.6 ± 13.3	12.8 ± 0.7	4.7 ± 0.2	1.6 ± 0.2
5.0	283.0 ± 9.6	15.3 ± 1.1	5.4 ± 0.2	3.3 ± 0.3* (2.2)
10.0	269.8 ± 9.7	15.3 ± 1.3	5.6 ± 0.3*	3.2 ± 0.1* (2.1)
20.0	288.4 ± 6.3	17.5 ± 0.4*	6.1 ± 0.1*	3.1 ± 0.2* (2.1)
50.0	266.4 ± 6.7	18.9 ± 1.2*	7.1 ± 0.3*	3.6 ± 0.2* (2.4)

Values represent mean ± SEM of five rats per treatment group, except for the control group where n=4. Corn oil vehicle was given to the control group. All rats were treated for seven consecutive days and killed three days after the last treatment.
Values in parentheses indicate the relative difference from the control group.
* Significantly different ($p<0.05$) from the control group (using one way ANOVA).

3.9. *EFFECTS ON CYTOCHROME P450-MEDIATED ENZYME ACTIVITIES*

3.9.1. *Effects on BROD, EROD, PROD, and AHH activities*

To determine the effects of treatment with Aroclor 1260 on cytochrome P450 isozymes, several enzyme activities that are commonly used as marker activities for individual cytochrome P450 isozymes were measured. The effects of treatment with varying doses of Aroclor 1260 on hepatic microsomal PROD, BROD, EROD, and AHH activities are shown in Table 3.3.

Microsomal PROD and BROD activities, which serve as indicators of cytochrome P450 2B (Harris *et al.* 1993), were increased significantly for rats treated with all doses of Aroclor 1260 compared to the control (vehicle-treated) rats. There was a 3-fold and 7-fold increase in mean PROD and BROD activities, respectively, at the lowest dose of Aroclor 1260, and both activities were increased by 20-fold or more at the next higher dose. However, these activities did not increase further at doses of Aroclor 1260 greater than 5.0 mg/kg.

Microsomal EROD activity increased with increasing dose for rats treated with Aroclor 1260 at doses of 10 to 50 mg/kg, while AHH activity was increased significantly only in rats treated with the highest dose of Aroclor 1260. Both EROD and AHH activities serve as indicators for cytochrome P450 1A (Lubet *et al.* 1991).

Table 3.3. Hepatic microsomal PROD, BROD, EROD, and AHH activities of male Long-Evans rats treated with various doses of Aroclor 1260

Aroclor 1260 Dose (mg/kg)	PROD (nmol/mg/min)	BROD (nmol/mg/min)	EROD (nmol/mg/min)	AHH (nmol/mg/min)
Control	0.27 ± 0.1	0.49 ± 0.04	0.85 ± 0.1	1.6 ± 0.03
0.5	0.80 ± 0.1* (3)	3.6 ± 0.6* (7)	1.8 ± 0.4	2.3 ± 0.2
5.0	5.4 ± 0.4* (20)	13.7 ± 0.4* (28)	2.1 ± 0.3	2.0 ± 0..2
10.0	6.2 ± 0.4* (23)	14.3 ± 0.3* (29)	3.0 ± 0.2* (3.5)	2.1 ± 0.2
20.0	5.6 ± 0.6* (21)	13.7 ± 0.4* (28)	3.9 ± 0.5* (4.5)	2.0 ± 0.1
50.0	5.1 ± 0.5* (19)	13.2 ± 0.5* (27)	8.0 ± 0.7* (9)	2.8 ± 0.2* (1.8)

Values represent mean ± SEM of five rats per treatment group, except for the control group where n=4. Corn oil vehicle was given to the control group. All rats were treated for seven consecutive days and killed three days after the last treatment.
Values in parentheses indicate the relative difference from the control group.
* Significantly different ($p<0.05$) from the control group (using one way ANOVA).

3.9.2. *Effects on testosterone hydroxylase activities*

The effects of treatment with various doses of Aroclor 1260 on hepatic microsomal testosterone hydroxylase activities are shown in Table 3.4. Treatment with Aroclor 1260 at a dose of 0.5 mg/kg resulted in significant increases in testosterone 6β- and 16β-hydroxylase activities. The most striking effect of treatment with Aroclor 1260 at a dose of 5.0 mg/kg was a 28-fold increase in testosterone 16β-hydroxylase activity, an indicator for cytochrome P450 2B (Waterman *et al.* 1991). At doses greater than 5.0 mg/kg this activity remained relatively high and unchanged. Likewise, the increase in testosterone 6β-hydroxylase activity, which is catalyzed by several isozymes including cytochrome P450 1A and P450 3A (Waterman *et al.* 1991), remained relatively unchanged at doses greater than 5.0 mg/kg. There was also a significant increase in testosterone 2β-hydroxylase activity, which is an indicator for both cytochromes P450 1A and P450 3A (Waterman *et al.* 1991), at doses of 5.0 mg/kg and greater. Significant increases in testosterone 7α- and 16α-hydroxylase activities were observed after treatment with Arolcor 1260 at doses of 5.0 mg/kg and greater. Microsomal testosterone 7α-hydroxylase activity is catalyzed by cytochrome P450 2A1, while microsomal testosterone 16α-hydroxylase activity is catalyzed primarily by cytochromes P450 2B1, P450 2B2 and P450 2C11 in rats. A significant reduction in testosterone 2α-hydroxylase activity, which is mediated solely by cytochrome P450 2C11, was noted at doses of 10 mg/kg and greater.

Table 3.1. Testosterone hydroxylase activities of hepatic microsomes from rats treated with varying doses of Aroclor 1260

	Testosterone Metabolites (nmol metabolite formed/mg protein/min)					
Dose (mg/kg)	2α	2β	6β	7α	16α	16β
Control	2.6 ± 0.3	0.26 ± 0.01	2.9 ± 0.1	0.24 ± 0.03	4.0 ± 0.5	0.26 ± 0.007
0.5	2.5 ± 0.2	0.39 ± 0.02	4.5 ± 0.3* (1.5)	0.33 ± 0.05	4.3 ± 0.2	1.0 ± 0.2* (4.0)
5.0	1.9 ± 0.2* (0.7)	0.63 ± 0.08* (2.4)	5.9 ± 0.6* (2.0)	0.52 ± 0.08* (2.0)	6.8 ± 0.5* (1.7)	7.2 ± 0.8* (28)
10.0	1.5 ± 0.2* (0.6)	0.70 ± 0.02* (2.7)	6.2 ± 0.3* (2.0)	0.51 ± 0.07* (2.0)	7.6 ± 0.8* (2.0)	8.9 ± 0.6* (34)
20.0	1.2 ± 0.1* (0.5)	0.73 ± 0.06* (2.8)	6.0 ± 0.6* (2.0)	0.61 ± 0.06* (2.5)	6.1 ± 0.6* (1.5)	7.0 ± 0.9* (27)
50.0	0.63 ± 0.1* (0.2)	0.98 ± 0.04* (3.8)	7.4 ± 0.3* (2.5)	0.92 ± 0.07* (3.8)	6.1 ± 0.2* (1.5)	8.6 ± 0.4* (33)

Values represent mean ± SEM of five rats per treatment group, except for the control group where n=4. Corn oil vehicle was given to the control group. All rats were treated for seven consecutive days and killed three days after the last treatment.
Values in parentheses indicate the relative difference from the control group.
* Significantly different ($p<0.05$) from the control group (using one way ANOVA).

3.10. *IMMUNOBLOT ANALYSIS*

Hepatic levels of cytochromes P450 1A1, P450 1A2, P450 2B1, P450 2B2, and P450 3A were measured by densitometric quantitation of immunoblots. Hepatic microsomal proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with antibodies against rat P450 1A, P450 2B, and P450 3A isozymes. The specificity of each antibody had been determined previously in our laboratory using enzyme-linked immunosorbent and immunoblot assays with purified rat cytochrome P450 isozymes and with different rat liver microsomal preparations.

3.10.1. *Immunoblot analysis of hepatic microsomes probed with anti-cytochrome P450 1A1 IgG*

A blot of hepatic microsomal samples prepared from rats treated with various doses of Aroclor 1260 and probed with monoclonal anti-cytochrome P450 1A1 IgG is shown in Figure 3.8. This monoclonal antibody recognizes both rat cytochrome P450 1A1 and P450 1A2. The upper band that can be seen in each lane containing hepatic microsomal samples represents cytochrome P450 1A1, and the lower more diffuse band represents cytochrome P450 1A2. Very little or no cytochrome P450 1A1 was found in lanes containing hepatic microsomes from control rats or rats treated with Aroclor 1260 at a dose of 0.5 mg/kg, while a darkly stained, diffuse band corresponding to cytochrome P450 1A2 was present in these same lanes. As the dose Aroclor 1260 increased, both the cytochrome P450 1A1 and P450 1A2 bands appeared more intensely stained.

Results of immunoquantitation of the cytochrome P450 1A1 and P450 1A2 isozymes are presented in Table 3.5. There was a significant increase in the hepatic level of cytochrome P450 1A1 for rats treated with Aroclor 1260 at a dose of 5.0 mg/kg. As the dose of Aroclor 1260 increased, cytochrome P450 1A1 content also increased. This is shown in Figure 3.9 as an approximately linear relationship between the Aroclor 1260 dose and the hepatic level of cytochrome P450 1A1.

A standard curve could not be generated for cytochrome P450 1A2 because of a lack of the purified isozyme. Hence, the hepatic level of this isozyme is given in densitometry units as integrated intensity per miligram of microsomal protein. Immunoquantitation of cytochrome P450 1A2 indicated no significant difference in the hepatic level of cytochrome P450 1A2 between the control group and groups treated with Aroclor 1260 at all doses except the highest dose. A significant increase above control values was observed for rats treated at a dose of 50 mg/kg.

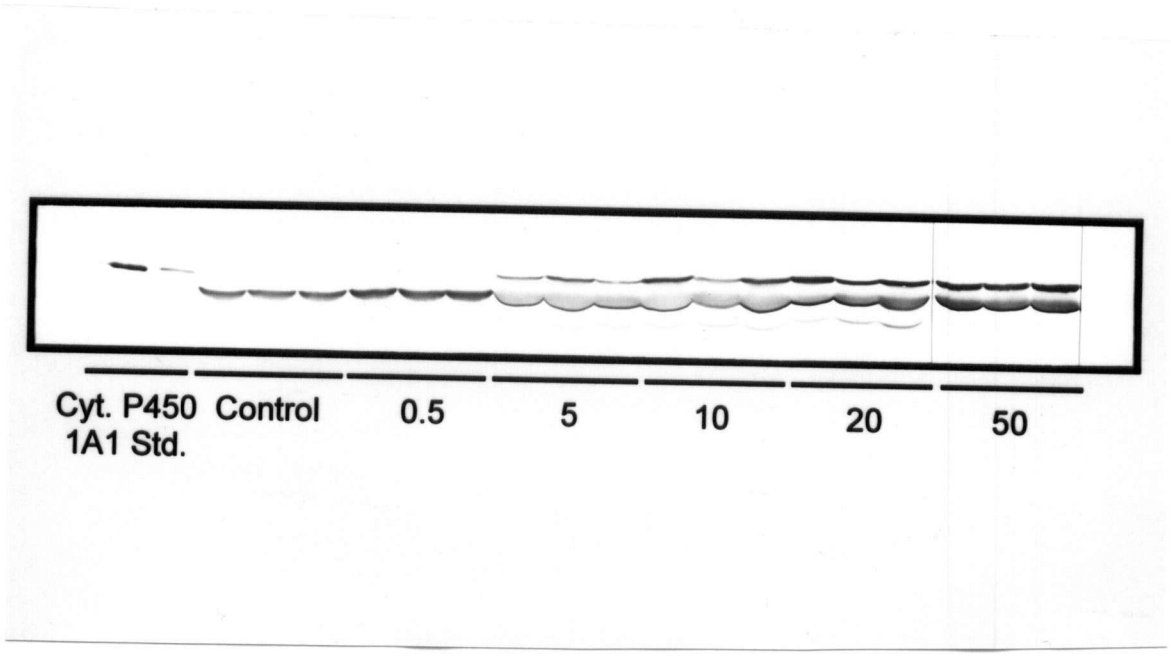

Figure 3.8. A representative immunoblot of rat hepatic microsomes probed with antibody against rat cytochrome P450 1A1. Microsomes from three individual rats from the control (C) and Aroclor 1260 treatment groups are shown. The numbers indicate the doses of Aroclor 1260 used and correspond to 0.5, 5.0, 10, 20, and 50 mg/kg. Final protein concentration was 20 μg per lane, except for the rats treated with doses of 20 and 50 mg/kg where the final concentration was 10 μg per lane. The concentration of primary antibody (monoclonal mouse anti-rat cytochrome P450 1A1) was 1.0 μg/mL and the secondary antibody (goat anti-mouse IgG) was used at a dilution of 1:3000. The concentrations of the purified rat cytochrome P450 1A1 standards are 0.2 and 0.05, pmol per lane, respectively.

Table 3.5. Hepatic levels of cytochrome P450 1A1 and cytochrome P450 1A2 in male rats treated with various doses of Aroclor 1260

Aroclor 1260 Dose (mg/kg)	Cytochrome P450 1A1 Content (pmol/mg protein)	Cytochrome P450 1A1 as a % of Total Cytochrome P450	Amount of Immunoreactive Cytochrome P450 1A2[§]
Control	< 0.5[†]	< 0.034 ± 0.001[†]	2.20 ± 0.1
0.5	0.51 ± 0.008[‡]	0.031 ± 0.002[‡]	2.92 ± 0.2 (1.3)
5.0	5.35 ± 1.2* (> 11)	0.19 ± 0.07* (> 6)	1.25 ± 0.2 (0.6)
10.0	8.85 ± 1.4* (> 18)	0.28 ± 0.04* (> 8)	1.62 ± 0.08 (0.7)
20.0	24.84 ± 3.2* (> 50)	0.79 ± 0.07* (> 23)	2.70 ± 0.8 (1.2)
50.0	58.90 ± 4.6* (> 118)	1.68 ± 0.2* (> 49)	6.72 ± 0.5* (3.1)

Values represent mean ± SEM of five rats per treatment group, except for the control group where n=4. Corn oil vehicle was given to the control group. All rats were treated for seven consecutive days and killed three days after the last treatment.

Values in parentheses indicate the relative difference from the control group.

* Significantly different ($p<0.05$) from the control group (using one way ANOVA).

† A faint band was visible in lanes containing liver microsomes from control rats, but the staining intensity of the band was below the limit of detection (0.01 pmol). The value presented above and used for statistical analysis is the limit of detection and represents the maximal concentration possible in these microsome samples.

‡ The band corresponding to cytochrome P450 1A1 was below the limit of detection for many microsome samples in the 0.5 mg/kg treatment group. For bands where no value was obtained, a concentration of 0.01 pmol was used in the calculation of the mean.

§ Each value was expressed as the integrated intensity of the band per milligram protein

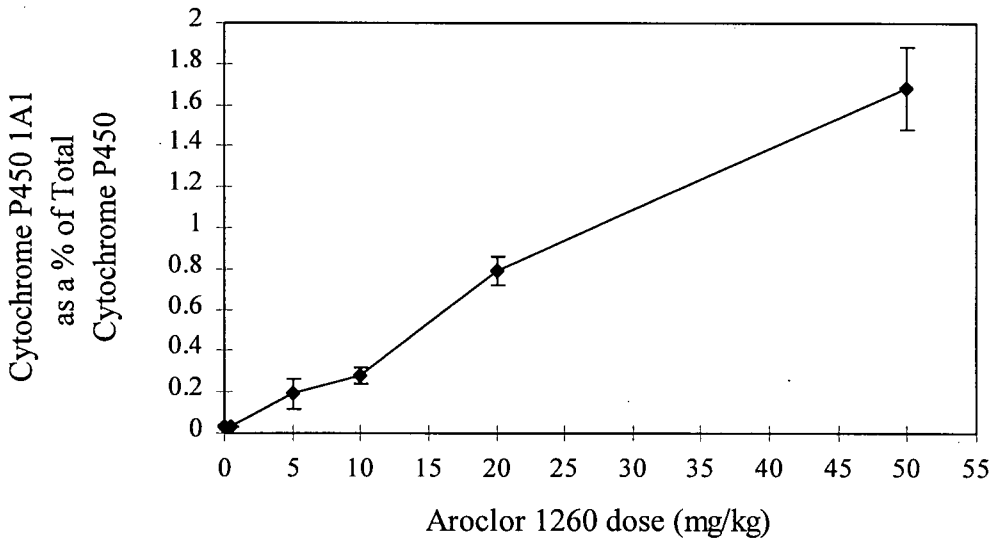

Figure 3.9. Dose response curve of cytochrome P450 1A1 in control and Aroclor 1260 Treated Rats

3.10.2. *Immunoblot analysis of hepatic microsomes probed with anti-cytochrome 450 2B1 IgG*

Figure 3.10 represents a blot of hepatic microsomal samples prepared from rats treated with various doses of Aroclor 1260 and probed with polyclonal anti-cytochrome P450 2B1 IgG. This antibody recognizes rat cytochrome P450 2B1 and P450 2B2 equally, which are 97% similar in amino acid sequence. Various concentrations of purified cytochrome P450 2B1 were applied to the gel to construct a standard curve. The same standard curve was used for both cytochrome P450 2B1 and cytochrome P450 2B2. The upper band in lanes containing hepatic microsomal samples corresponds to cytochrome P450 2B2 and the lower band corresponds to cytochrome P450 2B1. A faintly stained third band below cytochrome P450 2B1 is apparent in many of the hepatic microsomal samples and

corresponds to a related protein, possibly cytochrome P450 2B3.

The band corresponding to cytochrome P450 2B1 was faint in microsome samples from the control group but became more intensely stained in lanes containing microsomes from rats treated with Aroclor 1260 at a dose of 5.0 mg/kg. At doses larger than 5.0 mg/kg the staining intensity remained relatively unchanged. A similar staining pattern was observed for the upper band, corresponding to cytochrome P450 2B2.

Results of immunoquantitation of the cytochrome P450 2B1 and P450 2B2 isozymes are presented in Table 3.6. There was a significant increase in hepatic levels of both cytochromes P450 2B1 and P450 2B2 for rats treated with all doses of Aroclor 1260 relative to the control group. The extent of induction of cytochrome P450 2B1 was greater than that of cytochrome P450 2B2. For example, there was a 9-fold and 4-fold increase in cytochrome P450 2B1 and P450 2B2 content, respectively, relative to the control group, at a dose of 0.5 mg/kg and a further increase to 122-fold and 37-fold, respectively, relative to the control values at a dose of 5.0 mg/kg, but no further increases at higher doses. This is shown in Figure 3.11 whereby maximal induction of both cytochrome P450 2B1 and P450 2B2 appears to be reached at an Aroclor 1260 dose of 5.0 mg/kg.

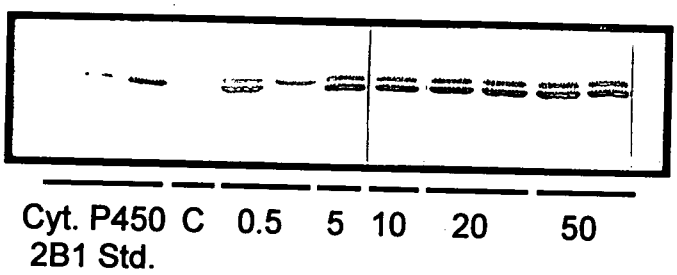

Figure 3.10. A representative mmunoblot of rat hepatic microsomes probed with backabsorbed polyclonal rabbit antibody against rat cytochrome P450 2B1. Microsomes of individual rats from the control (C) and Aroclor 1260 treatment groups are shown. The numbers indicate the doses of Aroclor 1260 used and correspond to 0.5, 5.0, 10, 20, and 50 mg/kg. Final protein concentration was 1 μg per lane, except for the control group and the group treated at a dose of 0.5 mg/kg where the final protein concentration was 10 μg per lane. The concentration of primary antibody (backabsorbed polyclonal rabbit anti-rat cytochrome P450 2B1) was 2.0 mg/mL and the secondary antibody (goat anti-mouse IgG) was used at a dilution of 1:3000. The concentrations of the purified rat cytochrome P450 2B1 standards are 0.25 and 1.0 pmol per lane, respectively.

Table 3.6. Hepatic levels of cytochrome P450 2B isozymes in male rats treated with various doses of Aroclor 1260

Aroclor 1260 Dose (mg/kg)	Cytochrome P450 2B1 Content (pmol/mg protein)	Cytochrome P450 2B1 as a % of Total Cytochrome P450	Cytochrome P450 2B2 Content (pmol/mg protein)	Cytochrome P450 2B2 as a % of Total Cytochrome P450
Control	7.62 ± 0.2	0.52± 0.02	14.47± 2.9	0.98 ± 0.2
0.5	65.08 ± 9.0* (9)	3.95 ± 0.6* (8)	64.15 ± 3.5* (4)	3.94 ± 0.4* (4)
5.0	926.0 ± 83.2* (122)	28.40 ± 1.7* (55)	529.46 ± 74.4* (37)	16.10 ± 1.7* (16)
10.0	924.37 ± 47.4* (121)	29.34 ± 0.9* (56)	675.60 ± 18.6* (47)	21.55 ± 0.7* (22)
20.0	995.50 ± 99.7* (131)	31.66 ± 1.9* (61)	701.25 ± 83.4* (49)	22.26 ± 2.0* (23)
50.0	1005.60 ± 90.9* (132)	28.17 ± 2.4* (54)	804.47 ± 42.6* (56)	22.65 ± 1.6* (23)

Values represent mean ± SEM of five rats per treatment group, except for the control group where n=4. Corn oil vehicle was given to the control group. All rats were treated for seven consecutive days and killed three days after the last treatment.
Values in parentheses indicate the relative difference from the control group.
* Significantly different ($p<0.05$) from the control group (using one way ANOVA).

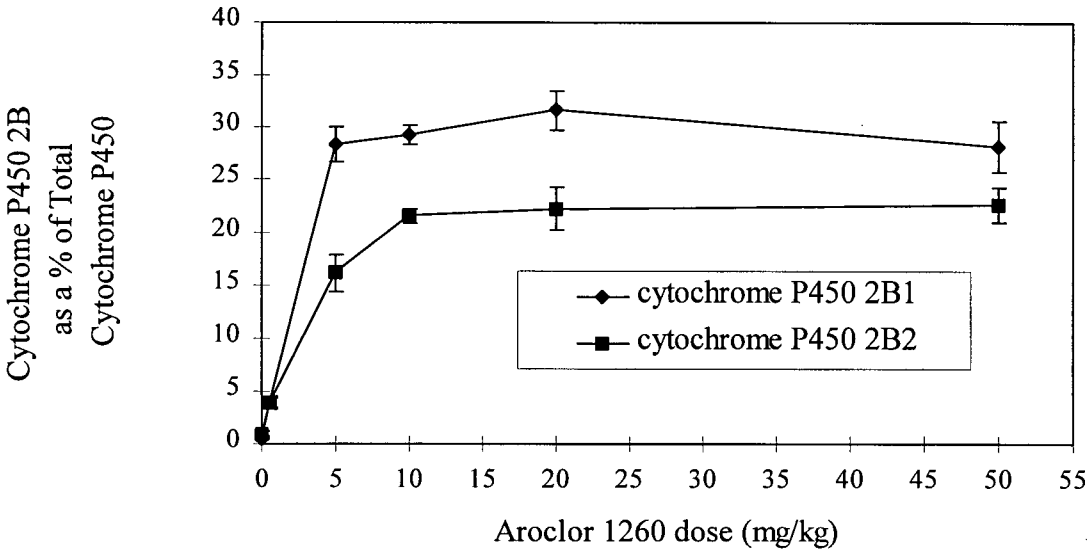

Figure 3.11. Dose response curve of cytochromesP450 2B1 and P450 2B2 in control and Aroclor 1260-treated rats

3.10.3. *Immunoblot analysis of hepatic microsomes probed with anti-cytochrome P450 3A1 IgG*

Figure 3.12 represents an immunoblot of hepatic microsomal samples prepared from rats treated with various doses of Aroclor 1260 and probed with anti-cytochrome P450 3A IgG. This antibody recognizes both cytochrome P450 3A1 and 3A2 and possibly other members of the cytochrome P450 3A subfamily (Cooper *et al.* 1993). These proteins cannot be resolved on SDS-PAGE gels. The weakly stained band in the lane containing a microsomal sample from the control group likely corresponds to cytochrome P450 3A2. However, the stained band in the lanes containing microsomes from Aroclor 1260-treated rats probably represents both cytochromes P450 3A1 and P450 3A2. As the dose of Aroclor 1260 was increased the apparent staining intensity of the band also increased.

Results of immunoquantitation of the cytochrome P450 3A isozymes are presented in Table 3.7. There was a significant increase in hepatic cytochrome P450 3A levels for Aroclor 1260-treated rats at doses of 5.0 mg/kg and greater. The relationship between the dose of Aroclor 1260 and hepatic levels of cytochrome P450 3A is parabolic as shown in Figure 3.13.

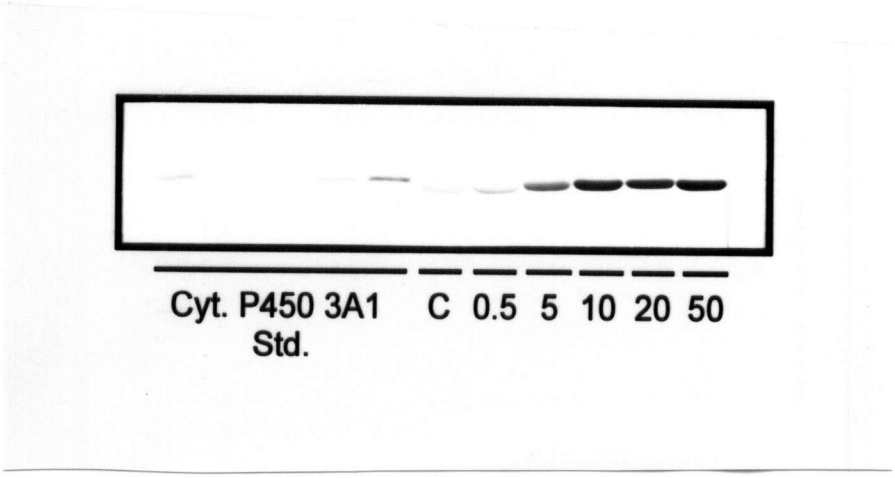

Figure 3.12. A representative immunoblot of rat hepatic microsomes probed with polyclonal rabbit antibody against rat cytochrome P450 3A1. Microsomes from individual rats from the control (C) and Aroclor 1260 treatment groups are shown. The numbers indicate the doses of Aroclor 1260 used and correspond to 0.5, 5.0, 10, 20, and 50 mg/kg. Final protein concentration was 10 μg per lane, except for the control group and the group treated at a dose of 0.5 mg/kg where the final protein concentration was 20 μg per lane. The concentration of primary antibody (polyclonal rabbit anti-rat cytochrome P450 3A1) was 10.0 μg/mL and the secondary antibody (goat anti-mouse IgG) was used at a dilution of 1:3000. The concentrations of the purified rat cytochrome P450 3A1 standards are 0.01, 0.0625, 0.125, 0.25, and 0.5 pmol per lane, respectively.

Table 3.7. Hepatic levels of cytochrome P450 3A in male rats treated with various doses of Aroclor 1260

Aroclor 1260 Dose (mg/kg)	Cytochrome P450 3A Content (pmol/mg protein)	Cytochrome P450 3A as a % of Total Cytochrome P450
Control	13.95 ± 0.9	0.95 ± 0.05
0.5	21.53 ± 1.3 (1.5)	1.30 ± 0.1 (1.3)
5.0	82.70 ± 8.9* (5.9)	2.54 ± 0.2* (2.6)
10.0	99.71 ± 6.9* (7.2)	3.17 ± 0.2* (3.2)
20.0	116.61 ± 12.6* (8.4)	3.70 ± 0.5* (3.9)
50.0	138.01 ± 13.7* (9.9)	3.90 ± 0.5* (4.1)

Values represent mean ± SEM of five rats per treatment group, except for the control group where n=4. Corn oil vehicle was given to the control group. All rats were treated for seven consecutive days and killed three days after the last treatment.
Values in parentheses indicate the relative difference from the control group.
* Significantly different ($p<0.05$) from the control group (using one way ANOVA).

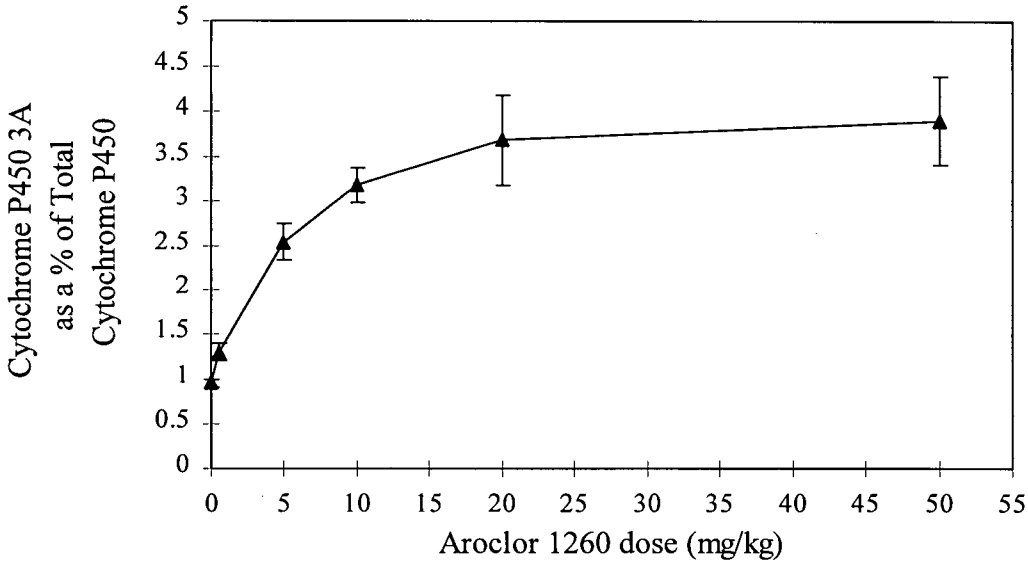

Figure 3.13. Dose response curve of cytochrome P450 3A in control and Aroclor 1260 treated rats

3.11. *PCB EXTRACTION AND GC-MS ANALYSIS*

PCBs were extracted from aliquots of liver homogenate from control and Aroclor 1260-treated rats, and were analyzed by GC-MS at the Institute of Ocean Sciences, Sidney, B.C. Both an internal standard cocktail consisting of ^{13}C-labeled purified PCB-52, -128, -180, -194, -208, -101, and -209 and an external standard of ^{13}C-labeled purified PCB-111 were used in the analysis. All samples were spiked with the internal standard cocktail at the start of the extraction process and spiked with the external standard before being analyzed by GC-MS. Selected ion monitoring was implemented to detect PCB isomers present in the tissue sample. PCB isomer identification and quantification were determined by analysis of prepared GC-MS performance standards.

3.11.1. *Analysis of a pure sample of Aroclor 1260*

As Aroclor 1260 mixtures vary from batch to batch it is essential to determine the PCB composition of the Aroclor 1260 mixture used in the present study. A 10 μL sample of a mixture of pure Aroclor 1260 and acetone was analyzed by GC-MS to determine the weight percentage of the PCB isomers in the Aroclor mixture. The PCB composition (reported as weight percentage) of the Aroclor 1260 mixture is presented in Table 3.8. Figure 3.14 shows the distribution of PCB isomers in Aroclor 1260.

Aroclor 1260 is composed primarily of the higher chlorinated PCBs, such as the hexa- and heptachlorobiphenyls. In particular, PCB-153, -180, and -138 are the three most predominant isomers present in Aroclor 1260

Table 3.8. Quantitative analysis of PCB isomers in Aroclor 1260

Isomer No.	% by weight in Aroclor 1260	Isomer No.	% by weight in Aroclor 1260
PCB-18	0.03	PCB-149	6.63
PCB-41	0.06	PCB-151	2.53
PCB-42	0.03	PCB-153	10.92
PCB-44	0.04	PCB-170	5.68
PCB-47	0.03	PCB-171	1.28
PCB-49	0.04	PCB-172	0.78
PCB-52	0.21	PCB-174	5.46
PCB-85	0.04	PCB-177	2.65
PCB-87	0.32	PCB-178	0.97
PCB-90+101‡	2.59	PCB-179	2.28
PCB-91	0.04	PCB-180	10.79
PCB-95	1.99	PCB-182+187‡	5.97
PCB-97	0.09	PCB-183	2.91
PCB-99	0.04	PCB-185	0.76
PCB-110	1.14	PCB-194	2.60
PCB-128	0.63	PCB-196+203‡	2.96
PCB-130	0.44	PCB-197	0.14
PCB-131	0.08	PCB-199	0.41
PCB-137	0.06	PCB-200	0.66
PCB-138	8.45	PCB-205	0.13
PCB-141	2.53	PCB-206	0.54
PCB-144	1.32	PCB-208	0.17
PCB-146	1.05	PCB-209	0.02

‡ % weight for the indicated isomers are combined as these isomers co-elute

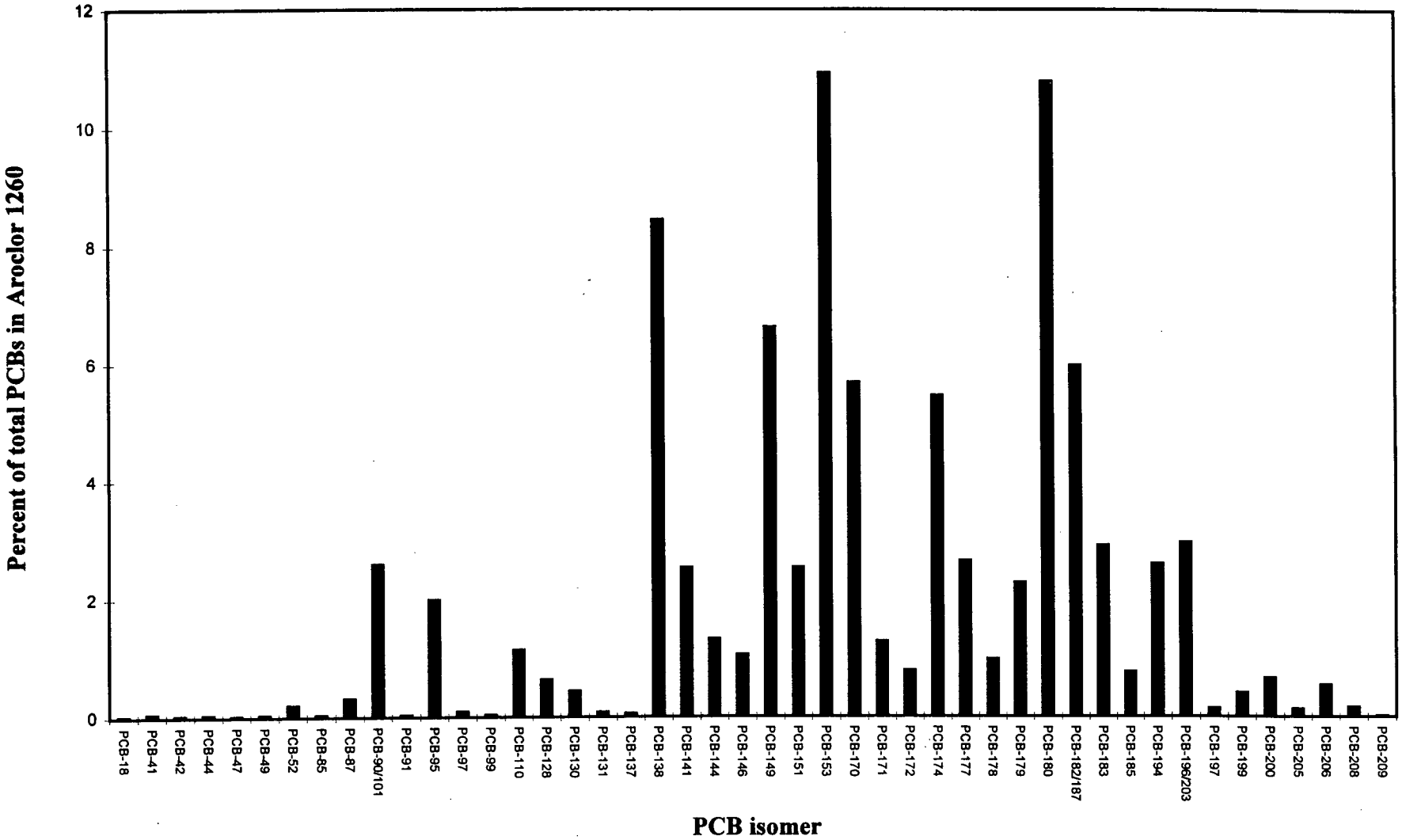

Figure 3.14. Total weight percentage of PCB isomers in pure Aroclor 1260 used in the present study. An aliquot of Aroclor 1260 was taken from the batch used for the present study and analyzed by GC-MS and the weight percentage of each PCB isomer in the total sample was calculated.

3.11.2. *Effects on hepatic PCB levels*

Liver samples from all of the rats of the dose response study were analyzed for hepatic PCB levels. The mean total PCB concentration of each treatment group is listed in Table 3.9. Mean PCB levels of each of the isomers were calculated for each treatment group and are presented in Table 3.10 as ng PCB per g lipid. Differences in hepatic PCB levels between control and Aroclor 1260-treated rats are shown in Figure 3.15 through Figure 3.20.

As shown in Table 3.9, total hepatic PCB levels, measured as either mg/g liver or mg/g lipid in rats, increased in a dose-related fashion as dose of Arolcor 1260 increased. A ten-fold increase in the dose of Aroclor 1260 from 0.5 to 5.0 mg/kg resulted in an approximate ten-fold increase in hepatic PCB levels.

Table 3.9. Total hepatic PCB concentrations of male rats treated with various doses of Aroclor 1260

Aroclor 1260 Dose	Total PCB (mg PCB/g liver)	Total PCB (mg PCB/g lipid)
Control	0.053 ± 0.009	1.37 ± 0.4
0.5 mg/kg	0.81 ± 0.2* (16)	21.27 ± 3.1* (16)
5.0 mg/kg	8.47 ± 1.2* (170)	166.32 ± 33.9* (121)
10 mg/kg	13.79 ± 1.2* (276)	236.70 ± 19.9* (173)
20 mg/kg	23.18 ± 2.3* (464)	440.71 ± 42.4* (322)
50 mg/kg	50.39 ± 2.5* (1008)	1016.62 ± 88.7* (742)

Values represent mean ± SEM of five rats per treatment group, except for the control group where n=4. Corn oil vehicle was given to the control group. All rats were treated for seven consecutive days, and killed three days after the last treatment.
Values in parentheses indicate the relative difference from the control group
* Significantly different ($p<0.05$) from the control group (using one way ANOVA

Table 3.7. Quantitative analysis of PCB isomers in male rats treated with various doses of Aroclor 1260

	Treatment Group					
Congener No.	Control (ng PCB/g lipid)	0.5 mg/kg (ng PCB/g lipid)	5 mg/kg (ng PCB/g lipid)	10 mg/kg (ng PCB/g lipid)	20 mg/kg (ng PCB/g lipid)	50 mg/kg (ng PCB/g lipid)
PCB-4	5.81 ± 1.9	13.15 ± 7.7	4.42 ± 0.8	5.29 ± 1.0	3.90 ± 1.2	5.53 ± 1.0
PCB-18	36.52 ± 5.3	69.05 ± 33.3	27.13 ± 5.5	24.00 ± 8.8	28.02 ± 2.8	30.90 ± 3.8
PCB-24+27‡	4.25 ± 0.7	7.21 ± 3.2	3.13 ± 0.7	2.81 ± 1.0	2.95 ± 0.9	4.02 ± 0.5
PCB-41	28.04 ± 2.7	54.18 ± 19.9	20.84 ± 3.8	20.37 ± 6.6	22.84 ± 4.2	29.78 ± 5.8
PCB-42	8.91 ± 0.9	16.85 ± 5.9	7.12 ± 1.3	7.09 ± 2.3	8.46 ± 1.2	9.84 ± 1.7
PCB-44	25.69 ± 2.4	50.42 ± 19.5	19.47 ± 3.5	23.41 ± 2.8	22.03 ± 4.8	27.85 ± 5.7
PCB-45	4.26 ± 0.4	7.24 ± 2.6	3.19 ± 0.7	3.28 ± 1.2	2.91 ± 1.0	4.34 ± 0.7
PCB-46	2.05 ± 0.3	3.36 ± 1.2	1.52 ± 0.4	1.46 ± 0.5	1.29 ± 0.4	1.93 ± 0.3
PCB-47	8.40 ± 1.1	17.73 ± 5.6	12.25 ± 2.0	11.30 ± 3.3	15.40 ± 0.7	27.31 ± 3.1
PCB-49	31.08 ± 3.7	65.67 ± 20.5	16.23 ± 2.6	14.23 ± 4.5	15.45 ± 2.8	18.52 ± 3.3
PCB-52	43.77 ± 4.4	81.22 ± 31.4	29.61 ± 5.1	35.53 ± 4.5	34.67 ± 6.7	42.04 ± 9.2
PCB-82	3.15 ± 1.4	5.92 ± 1.9	4.96 ± 0.9	4.10 ± 1.2	4.71 ± 1.6	2.84
PCB-84	9.74 ± 5.0	-	2.60	-	3.10	-
PCB-85	12.55 ± 4.5	19.04 ± 5.6	22.72 ± 6.6	23.05 ± 1.2	20.83 ± 1.9	33.41 ± 5.3
PCB-87	23.20 ± 3.6	29.86 ± 8.9	23.72 ± 4.1	29.27 ± 2.1	31.17 ± 2.5	49.04 ± 9.5
PCB-90+101‡	67.13 ± 10.0	239.80 ± 74.2	235.94 ± 80.0	221.18 ± 47.7	164.81 ± 13.3	267.96 ± 44.8
PCB-91	5.52 ± 1.1	4.91 ± 1.4	3.61 ± 0.9	4.10 ± 0.4	3.72 ± 1.4	5.86 ± 1.6
PCB-95	38.68 ± 3.9	42.34 ± 12.1	28.48 ± 5.0	28.17 ± 9.0	34.73 ± 5.0	50.81 ± 13.3
PCB-97	10.51 ± 1.6	12.77 ± 3.7	12.55 ± 2.3	14.16 ± 1.2	14.18 ± 1.4	20.77 ± 4.9
PCB-99	56.67 ± 14.9	164.92 ± 55.1	521.68 ± 150	785.92 ± 24.5	942.98 ± 145	1746.36 ± 180
PCB-110	38.98 ± 5.8	50.50 ± 16.2	32.54 ± 5.8	41.47 ± 5.3	38.43 ± 5.6	51.20 ± 14.5
PCB-119	1.71 ± 0.4	2.68 ± 0.8	2.68 ± 0.8	2.17 ± 0.6	1.95	4.05 ± 2.6
PCB-128	14.61 ± 7.6	102.51 ± 31.7	576.67 ± 109	663.43 ± 60.7	806.94 ± 117	1196.02 ± 144

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	Treatment Group					
Congener No.	Control (ng PCB/g lipid)	0.5 mg/kg (ng PCB/g lipid)	5 mg/kg (ng PCB/g lipid)	10 mg/kg (ng PCB/g lipid)	20 mg/kg (ng PCB/g lipid)	50 mg/kg (ng PCB/g lipid)
PCB-130	11.02 ± 5.8	51.22 ± 15.8	464.16 ± 54.0	14622.81 ± 13891	1598.18 ± 163	5009.19 ± 390
PCB-131	-	7.18 ± 3.5	39.12	77.70 ± 43.1	137.93	549.43 ± 412
PCB-137	3.82 ± 2.8	12.03 ± 3.5	76.36 ± 19.0	95.58 ± 27.2	139.40 ± 42.6	228.70 ± 121
PCB-138	130.70 ± 96.2	3022.81 ± 1027	32703.10 ± 6485	43350.21 ± 12191	81257.73 ± 9399	2.04×10^5 ± 13370
PCB-141	6.17 ± 2.5	34.57 ± 10.5	13.23	-	-	-
PCB-144	5.00 ± 1.0	11.70 ± 3.3	11.03 ± 1.8	9.13 ± 1.1	15.81 ± 1.7	21.77 ± 2.9
PCB-146	16.81 ± 13.6	191.92 ± 61.8	1646.35 ± 195	2160.44 ± 633	5839.61 ± 582	18293.20 ± 1559
PCB-149	26.63 ± 11.6	92.03 ± 28.1	75.88 ± 16.9	53.72 ± 15.2	89.65 ± 9.8	143.97 ± 19.5
PCB-151	5.32 ± 2.1	14.94 ± 4.3	20.36 ± 3.5	12.96 ± 3.7	28.50 ± 3.7	56.45 ± 7.9
PCB-153	136.02 ± 100.2	2798.73 ± 942	33133.48 ± 6355	47950.18 ± 13552	1.0×10^5 ± 13436	2.78×10^5 ± 21155
PCB-170	50.57 ± 25.0	1735.29 ± 583	20607.69 ± 4212	27283.01 ± 7821	55204.76 ± 5611	93187.19 ± 11764
PCB-171	7.45 ± 2.3	214.92 ± 67.5	1392.36 ± 265	4438.43 ± 2677	3187.86 ± 298	5201.40 ± 712
PCB-172	6.67 ± 2.8	142.32 ± 43.6	984.78 ± 141	1020.12 ± 303	3215.69 ± 330	6753.36 ± 933
PCB-174	9.13 ± 0.9	30.90 ± 9.6	5.20	-	13.76	-
PCB-175	0.40	-	45.45	528.50	-	-
PCB-177	16.80 ± 6.9	470.26 ± 154	4453.31 ± 629	4773.10 ± 1241	13301.58 ± 1223	24740.70 ± 3150
PCB-178	5.78 ± 4.4	90.20 ± 27.1	837.57 ± 95.3	707.25 ± 205	3298.92 ± 430	5726.72 ± 1657
PCB-179	1.78 ± 0.7	6.28 ± 2.4	-	-	-	-
PCB-180	90.98 ± 42.7	2952.82 ± 758	27650.56 ± 5534	32406.60 ± 8263	75609.62 ± 7627	1.4×10^5 ± 17738
PCB-182+187[‡]	39.51 ± 28.7	1309.88 ± 411	9637.08 ± 1702	11340.28 ± 2390	29265.14 ± 2995	57228.62 ± 7240
PCB-183	20.00 ± 12.2	1190.39 ± 389	9012.05 ± 2389	15166.26 ± 4320	17462.86 ± 1681	25392.49 ± 3570

... continued on next page

	Treatment Group					
Congener No.	Control (ng PCB/g lipid)	0.5 mg/kg (ng PCB/g lipid)	5 mg/kg (ng PCB/g lipid)	10 mg/kg (ng PCB/g lipid)	20 mg/kg (ng PCB/g lipid)	50 mg/kg (ng PCB/g lipid)
PCB-174	9.13 ± 0.9	30.90 ± 9.6	5.20	-	13.76	-
PCB-175	0.40	-	45.45	528.50	-	-
PCB-177	16.80 ± 6.9	470.26 ± 154	4453.31 ± 629	4773.10 ± 1241	13301.58 ± 1223	24740.70 ± 3150
PCB-178	5.78 ± 4.4	90.20 ± 27.1	837.57 ± 95.3	707.25 ± 205	3298.92 ± 430	5726.72 ± 1657
PCB-179	1.78 ± 0.7	6.28 ± 2.4	-	-	-	-
PCB-180	90.98 ± 42.7	2952.82 ± 758	27650.56 ± 5534	32406.60 ± 8263	75609.62 ± 7627	1.4×10^5 ± 17738
PCB-182+187‡	39.51 ± 28.7	1309.88 ± 411	9637.08 ± 1702	11340.28 ± 2390	29265.14 ± 2995	57228.62 ± 7240
PCB-183	20.00 ± 12.2	1190.39 ± 389	9012.05 ± 2389	15166.26 ± 4320	17462.86 ± 1681	25392.49 ± 3570
PCB-185	0.67 ± 0.3	3.56 ± 1.1	-	-	-	-
PCB-193	-	55.71	-	-	-	-
PCB-194	63.40 ± 30.0	1241.91 ± 395	7453.89 ± 1073	10576.75 ± 1075	17777.30 ± 5557	74233.34 ± 12844
PCB-196+203‡	43.38 ± 15.0	1411.01 ± 426	5211.12	10491.48 ± 376	15710.83 ± 4738	29779.21 ± 16236
PCB-197	0.40 ± 0.2	6.90 ± 4.7	27.22	25.89 ± 2.1	14.35	138.11
PCB-199	0.22 ± 0.09	0.77 ± 0.5	0.45	-	-	-
PCB-200	1.70 ± 0.7	36.15 ± 24.8	141.84	98.62 ± 33.6	105.30 ± 62.8	235.39
PCB-205	12.65 ± 14.3	7.74	1793.09	-	-	-
PCB-206	17.82 ± 6.7	2945.89 ± 2292	4183.88 ± 648	4917.72 ± 506	10392.64 ± 1196	30037.38 ± 3397
PCB-208	3.11 ± 1.2	81.05 ± 24.9	558.62 ± 89.2	1812.36 ± 1119	1715.16 ± 190	5433.42 ± 601
PCB-209	2.45 ± 1.0	34.59 ± 10.8	367.14 ± 57.7	817.12 ± 102	2807.88 ± 515	6654.28 ± 756

Values represent mean ± SEM of five rats per treatment group, except for the control group where n=4. Corn oil was given to the control group.

‡ Values represent both PCB isomers as they co-elute and cannot be distinguished

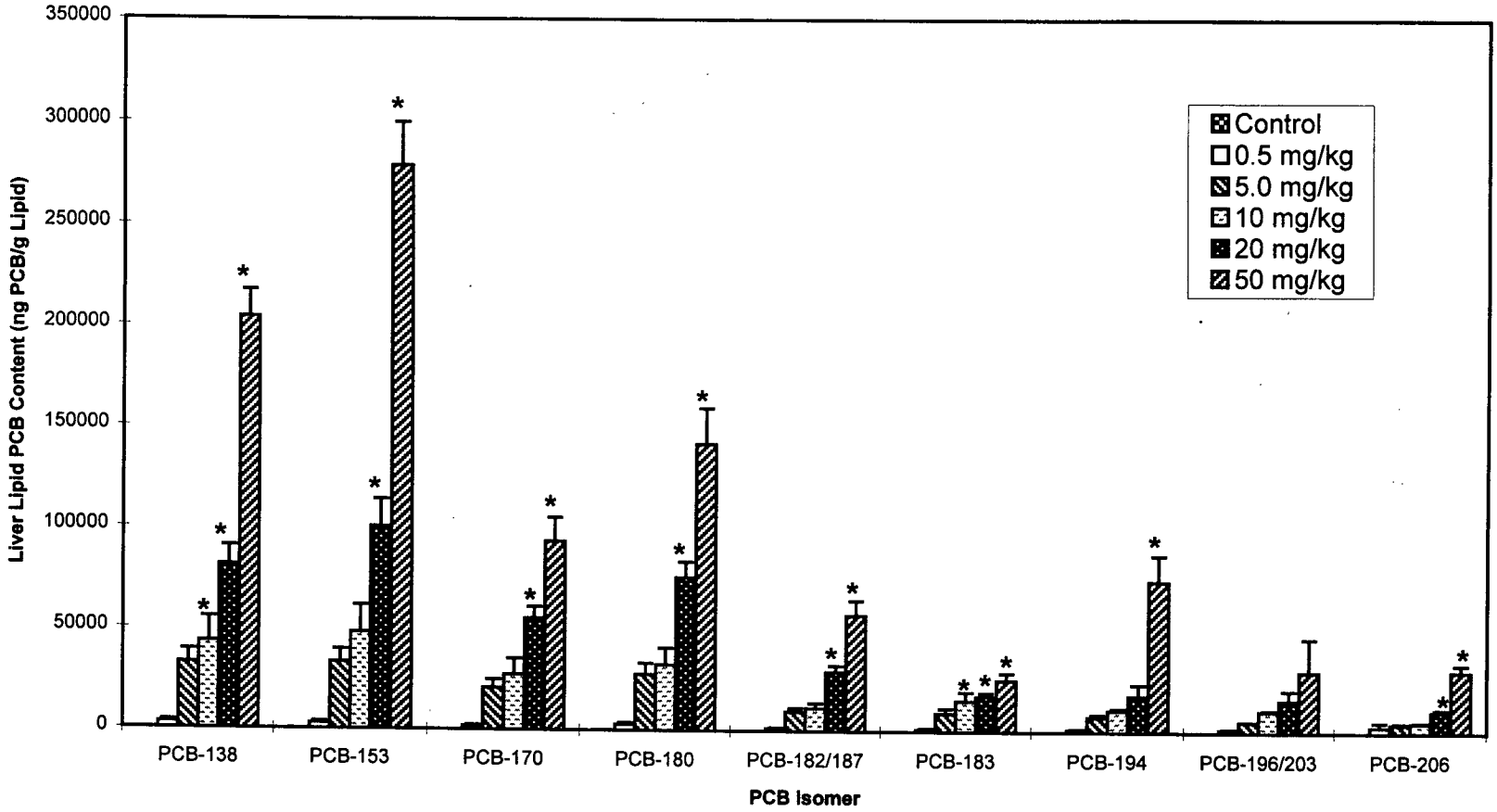

Figure 3.15. Hepatic PCB isomer levels in control and Aroclor 1260-treated rats. Values represent mean ± SEM of five rats per treatment group except for the control group and the 5.0 mg/kg Aroclor 1260 treatment group where n=4. Corn oil was given to the control group. All rats were treated for seven consecutive days and killed three days after the last treatment. * Significantly different ($p<0.05$) from the control group (using one way ANOVA).

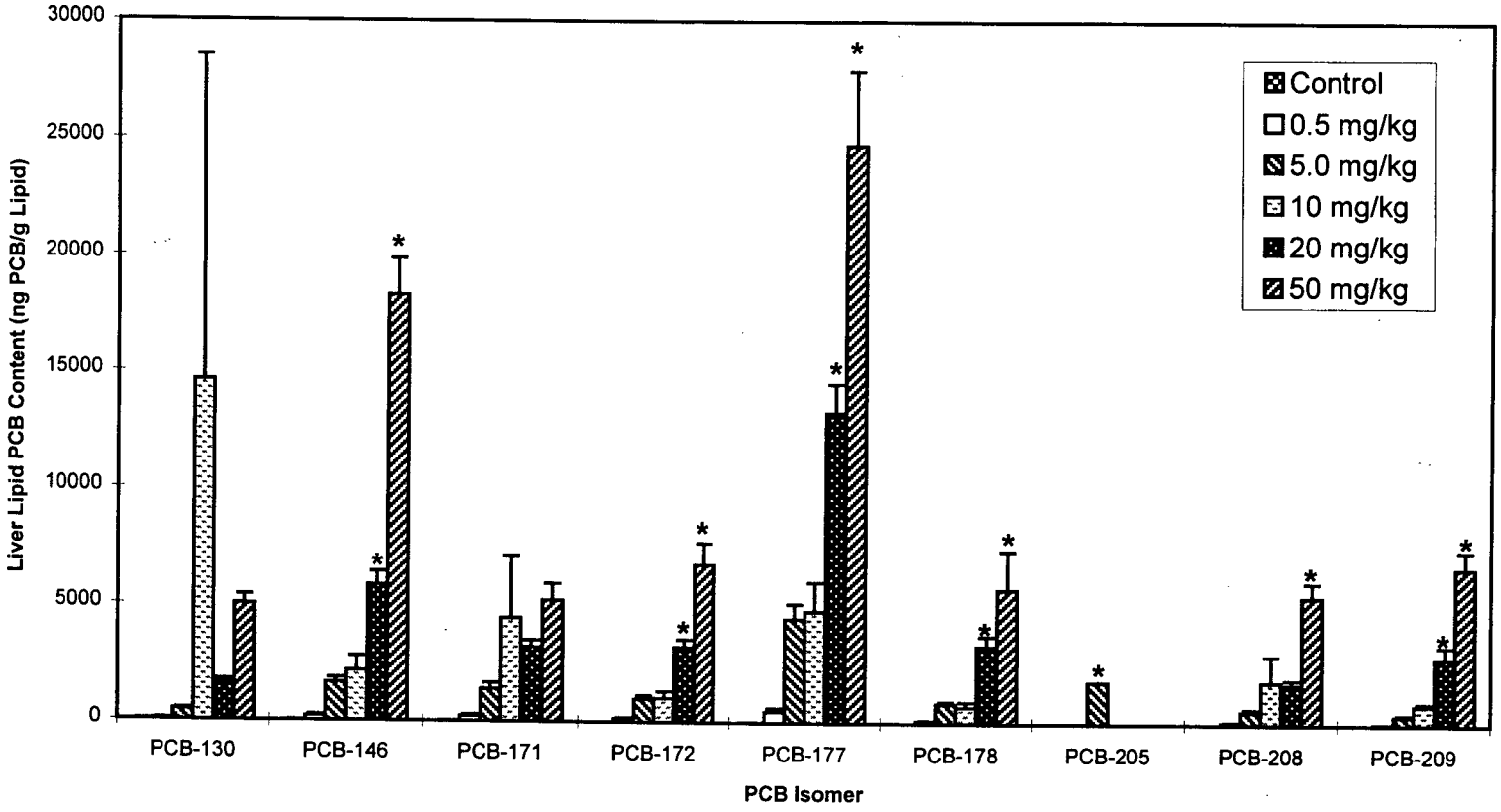

Figure 3.16. Hepatic PCB isomer levels in control and Aroclor 1260-treated rats. Values represent mean ± SEM of five rats per treatment group except for the control group and the 5.0 mg/kg Aroclor 1260 treatment group where n=4. Corn oil was given to the control group. All rats were treated for seven consecutive days and killed three days after the last treatment. * Significantly different ($p<0.05$) from the control group (using one way ANOVA).

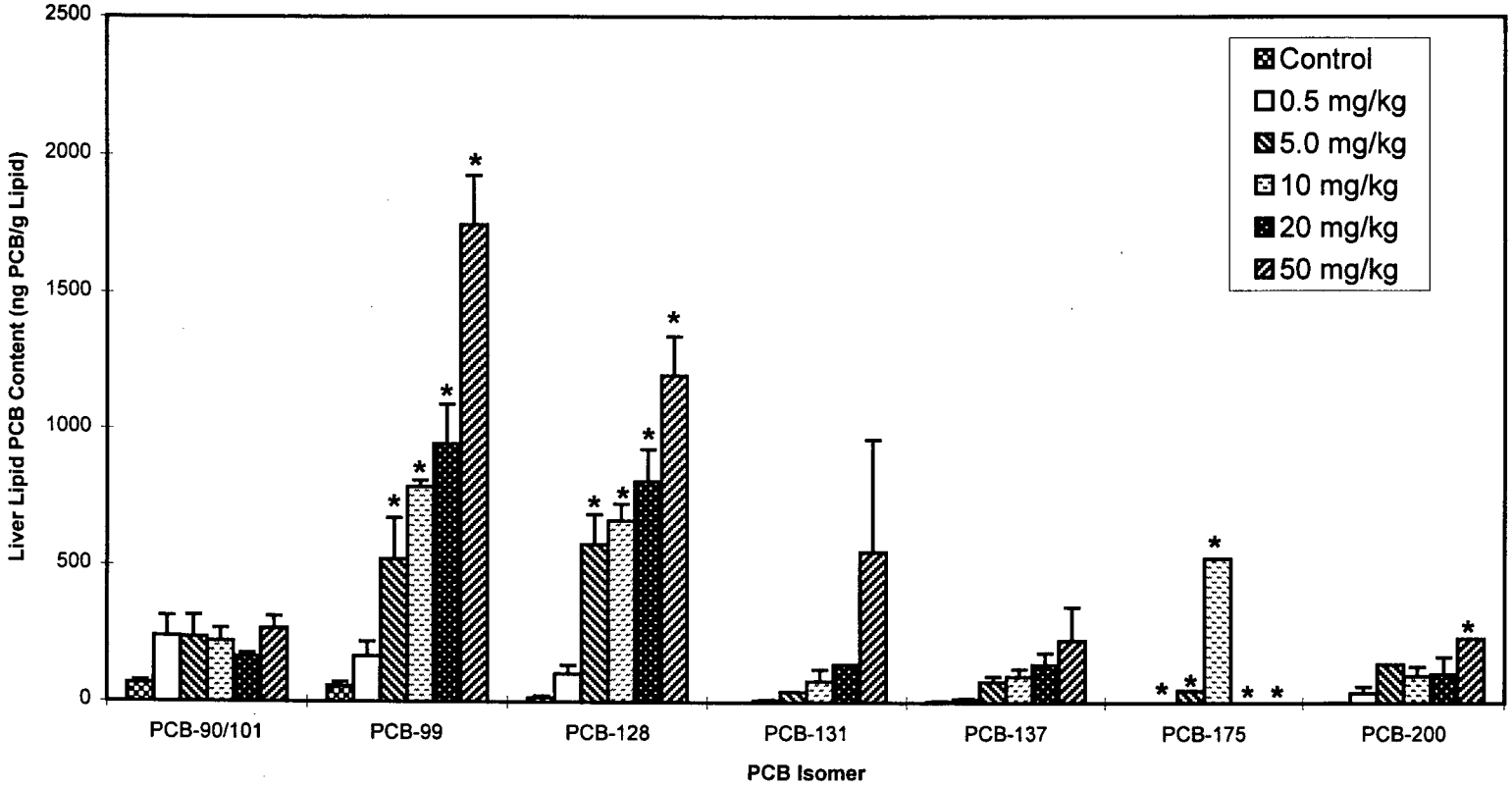

Figure 3.17. Hepatic PCB isomer levels in control and Aroclor 1260-treated rats. Values represent mean ± SEM of five rats per treatment group except for the control group and the 5.0 mg/kg Aroclor 1260 treatment group where n=4. Corn oil was given to the control group. All rats were treated for seven consecutive days and killed three days after the last treatment. * Significantly different (p<0.05) from the control group (using one way ANOVA).

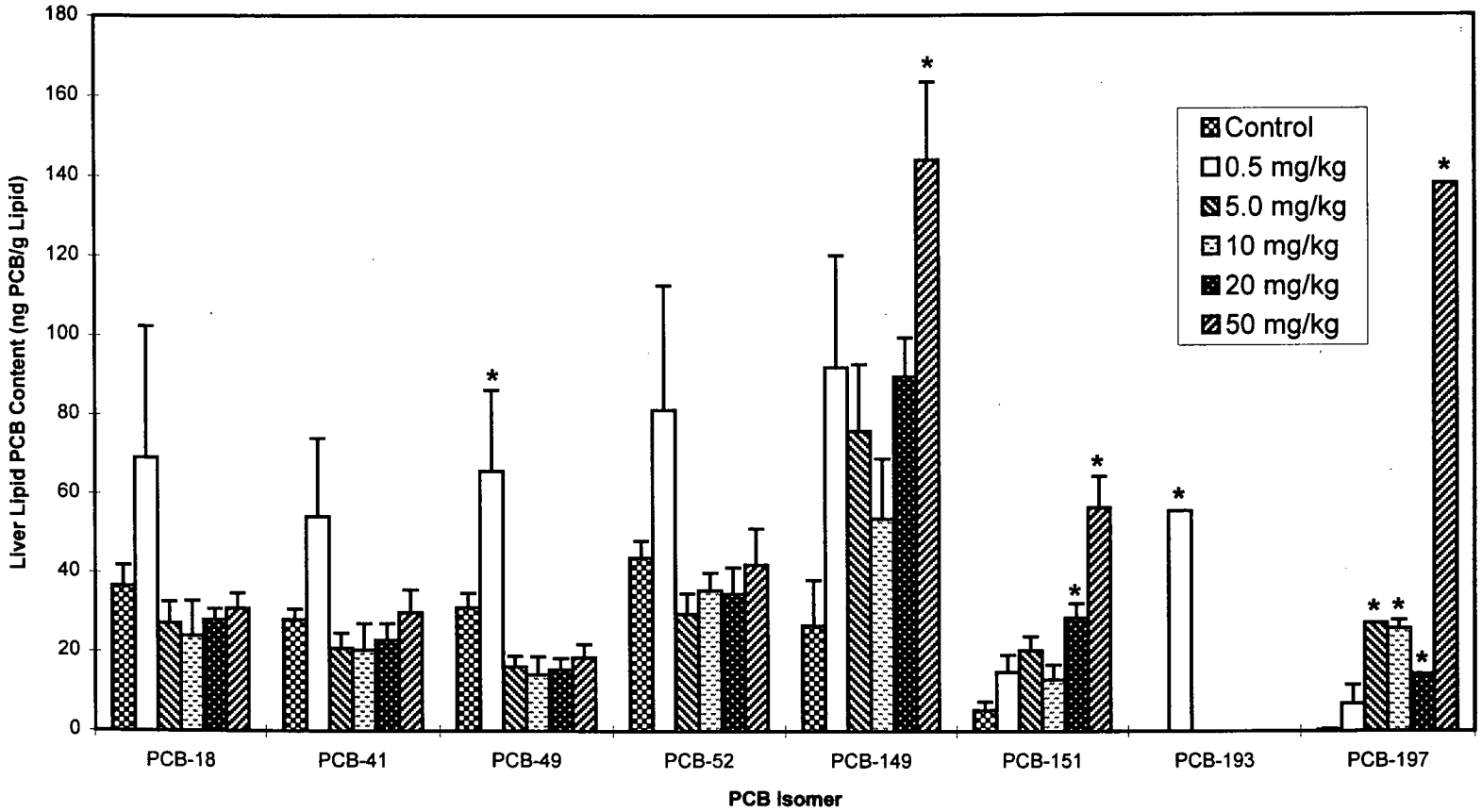

Figure 3.18. Hepatic PCB isomer levels in control and Aroclor 1260-treated rats. Values represent mean ± SEM of five rats per treatment group except for the control group and the 5.0 mg/kg Aroclor 1260 treatment group where n=4. Corn oil was given to the control group. All rats were treated for seven consecutive days and killed three days after the last treatment. * Significantly different ($p<0.05$) from the control group (using one way ANOVA).

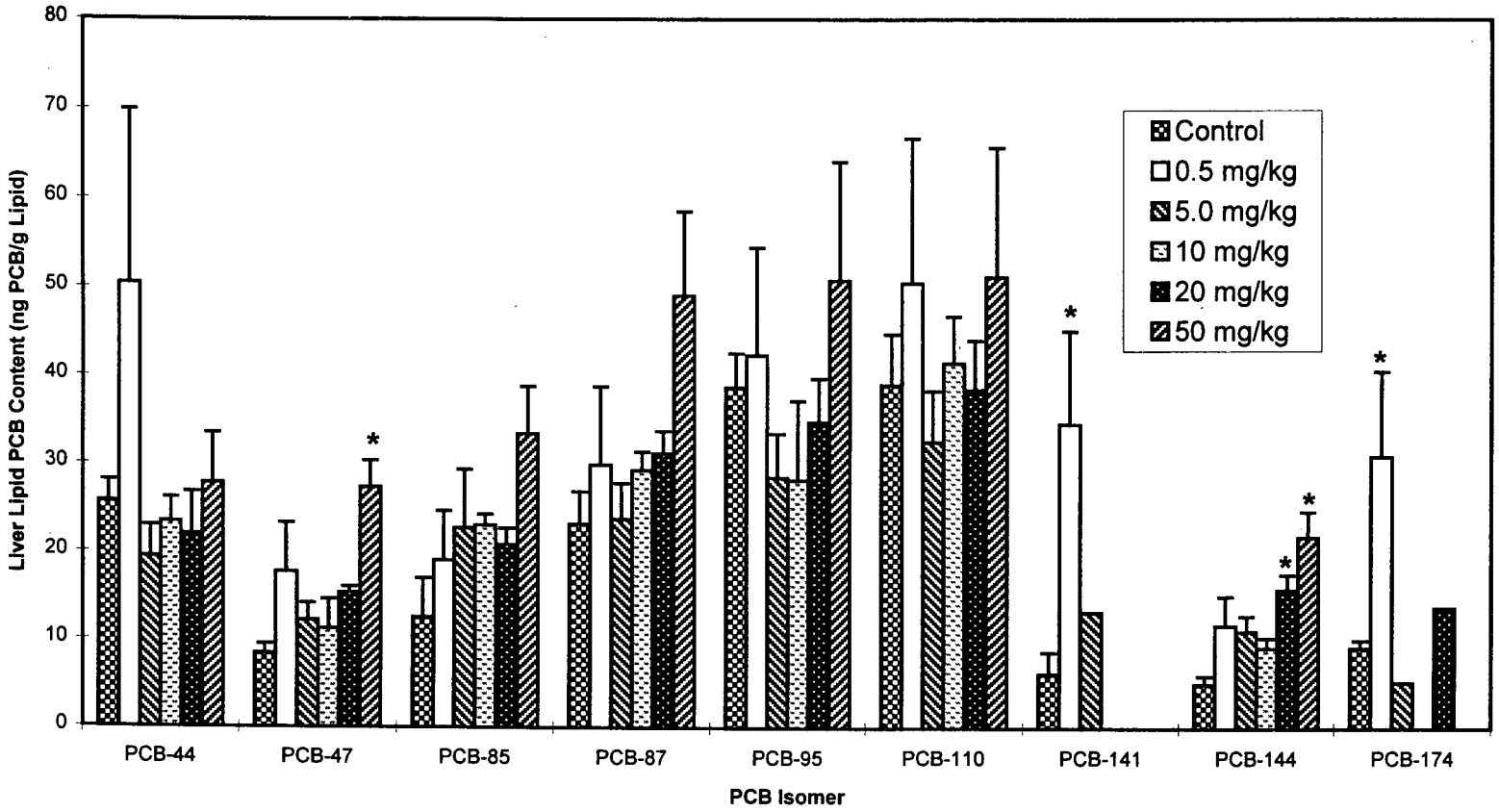

Figure 3.19. Hepatic PCB isomer levels in control and Aroclor 1260-treated rats. Values represent mean ± SEM of five rats per treatment group except for the control group and the 5.0 mg/kg Aroclor 1260 treatment group where n=4. Corn oil was given to the control group. All rats were treated for seven consecutive days and killed three days after the last treatment. * Significantly different (p<0.05) from the control group (using one way ANOVA).

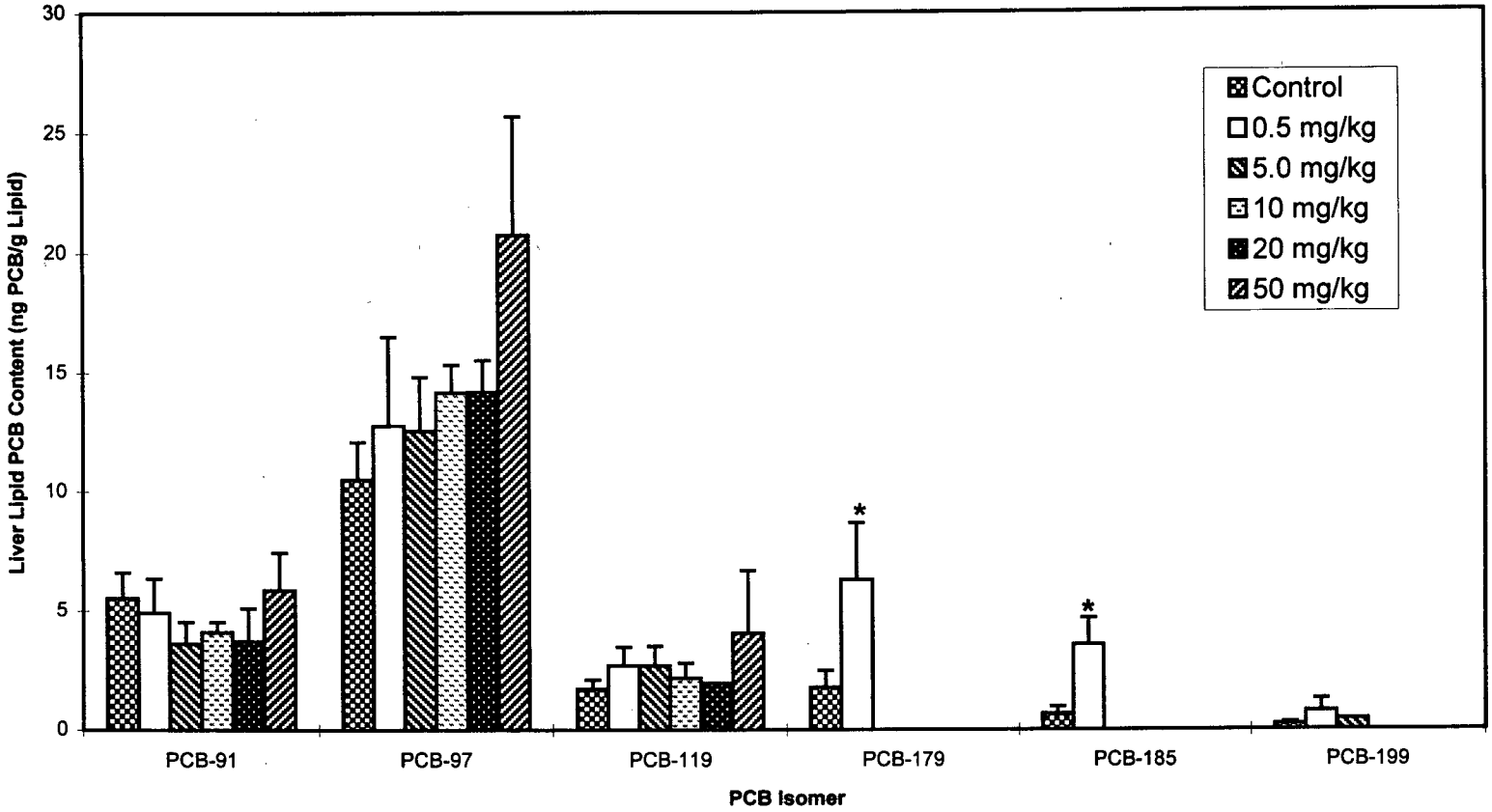

Figure 3.20. Hepatic PCB isomer levels in control and Aroclor 1260-treated rats. Values represent mean ± SEM of five rats per treatment group except for the control group and the 5.0 mg/kg Aroclor 1260 treatment group where n=4. Corn oil was given to the control group. All rats were treated for seven consecutive days and killed three days after the last treatment. * Significantly different ($p<0.05$) from the control group (using one way ANOVA).

3.11.3. *Correlations between hepatic PCB levels and specific cytochrome P450 isozymes*

The PCB isomers identified in rats treated with increasing doses of Aroclor 1260 were classified into Type I, Type II, and Mixed Type inducers (Table 3.11). No Type I PCB isomers were present in the control or Aroclor 1260-treated rats. The relationship between hepatic cytochrome P450 1A1, cytochrome P450 2B1, and cytochrome P450 3A contents and hepatic concentrations of the different types of PCB inducers was examined. As shown in Figure 3.21, a positive linear relationship was found between cytochrome P450 1A1 content and hepatic levels of PCBs classed as Mixed Type inducers (r^2=0.987, $p < 0.05$). Cytochrome P450 1A1 content was also found to correlate with hepatic levels of total PCBs, PCB isomers classed as Type II inducers, and PCB isomers classed as Type II + PCB isomers classed as Mixed Type inducers (r^2=0.987, $p < 0.05$; r^2=0.993, $p < 0.05$; and r^2=0.992, $p < 0.05$, respectively). A relatively straight line was obtained in each case, and all data points fell close to the regression line.

A parabolic relationship was observed between cytochrome P450 2B1 content and hepatic levels of total PCBs, PCB isomers classed as Type II inducers, PCB isomers classed as Mixed Type inducers, and PCB isomers classed as Type II + PCB isomers classed as Mixed Type inducers (Figure 3.22).

A parabolic relationship was also observed between cytochrome P450 3A content and hepatic levels of total PCBs, PCB isomers classed as Type II inducers, PCB isomers classed as Mixed Type inducers, and PCB isomers classed as Type II + PCB isomers classed as Mixed type inducers suggesting that the increase in cytochrome P450 3A content is not dose-dependent on the dose of Aroclor 1260 (Figure 3.23).

Table 3.11. Type I, Type II, and Mixed Type PCB Isomers in Aroclor 1260

Type I (3-MC like inducer)	Type II (PB like inducer)		Mixed Type (3-MC and PB like inducers)	
none	PCB-47	PCB-153	PCB-119	PCB-138
	PCB-49	PCB-180	PCB-128	PCB-170
	PCB-52	PCB-183	PCB-137	
	PCB-99	PCB-194		
	PCB-101			

The indicated PCB isomers were present in Aroclor 1260 used for this study

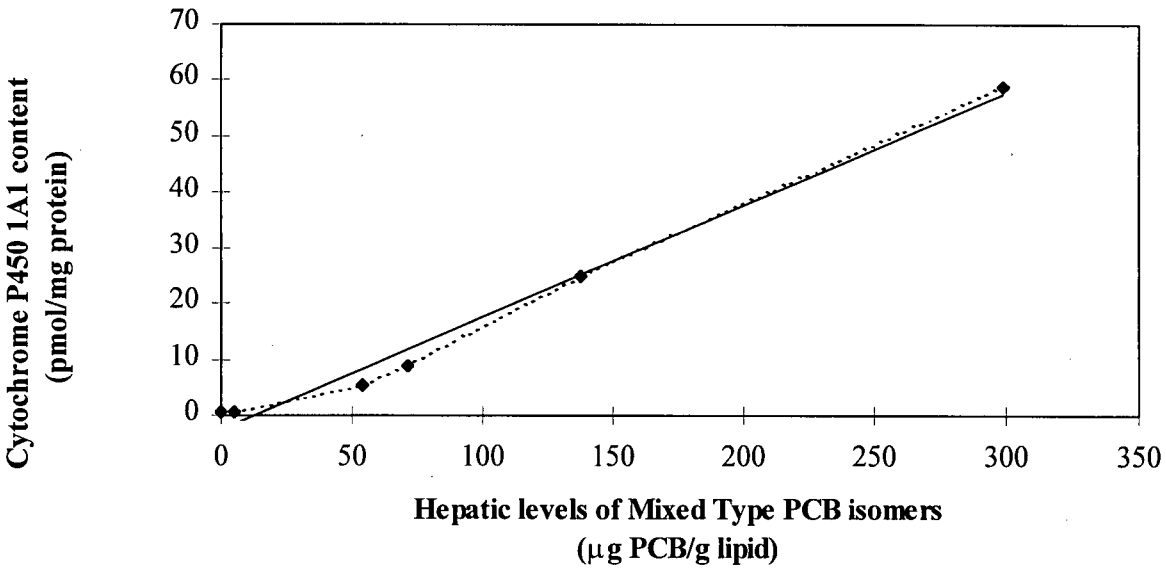

Figure 3.21. Correlation between cytochrome P450 1A1 content and Mixed Type PCB inducers in livers of rats treated with Aroclor 1260. The solid line represents the regression line and the dotted line connects the data points.

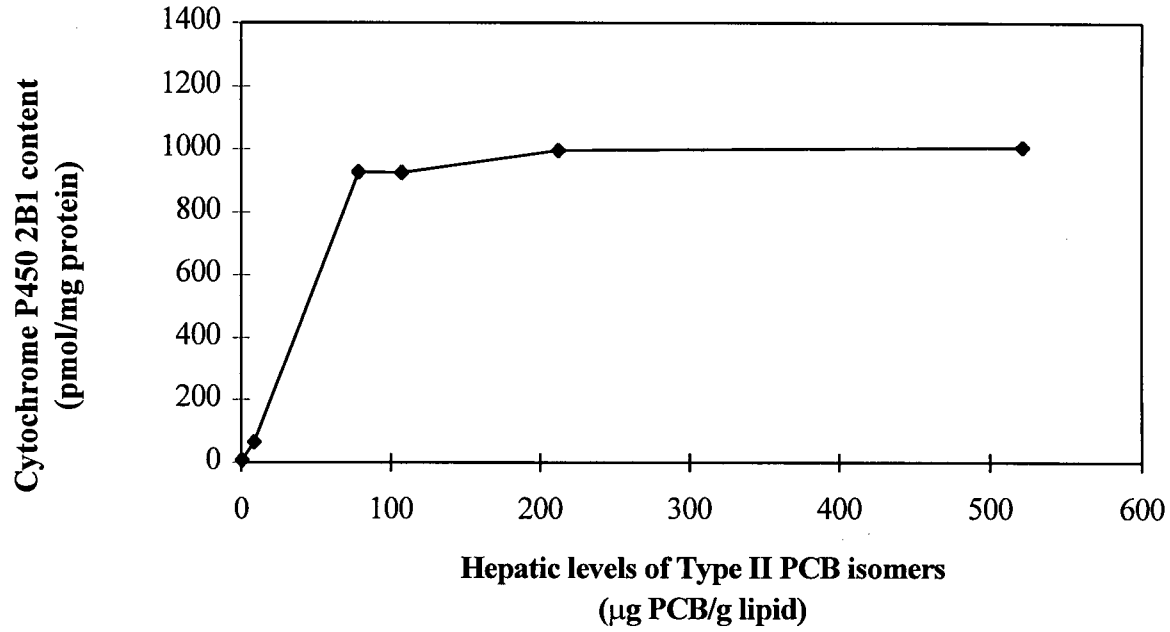

Figure 3.22. Relationship between cytochrome P450 2B1 content and Type II PCB inducers in livers of rats treated with Aroclor 1260.

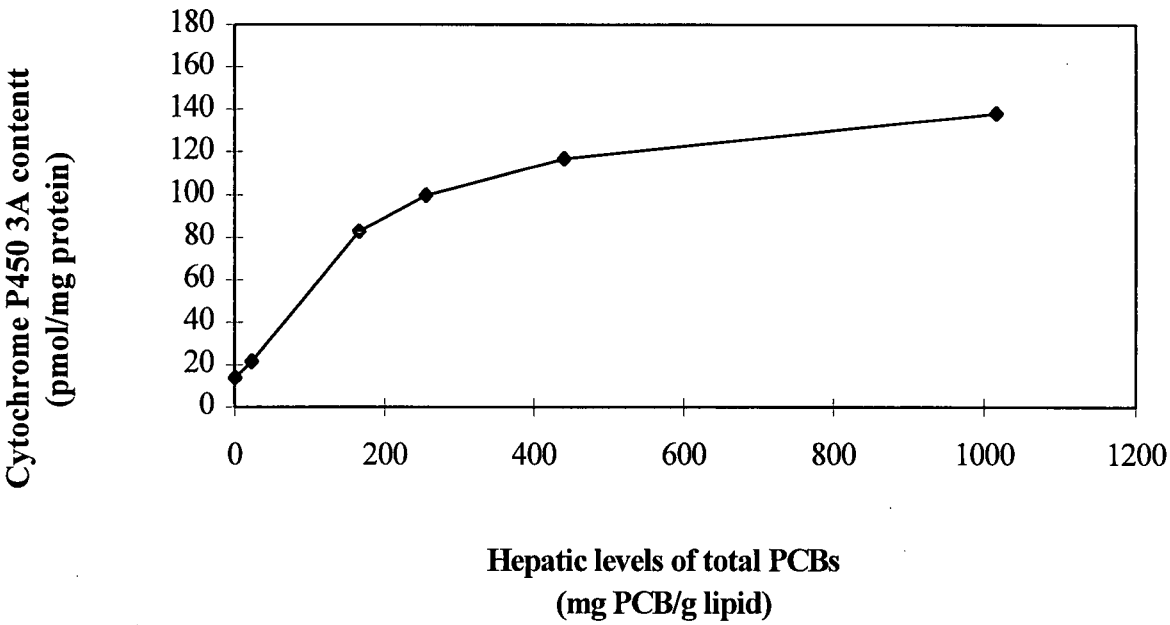

Figure 3.23. Relationship between cytochrome P450 3A content and total PCB in livers of rats treated with Aroclor 1260.

SECTION 3: EFFECTS OF TIME AFTER TREATMENT WITH AROCLOR 1260

To determine the duration of cytochrome P450 induction after low dose treatment with Aroclor 1260, groups of rats treated with a dose of 10 mg/kg by oral gavage for seven consecutive days were killed at various times after treatment.

3.12. *EFFECTS ON BODY WEIGHT, LIVER WEIGHT, AND TOTAL CYTOCHROME P450 CONTENT IN RATS*

As shown in Table 3.12, both body and liver weights of rats treated with Aroclor 1260 at a dose of 10 mg/kg increased with time after treatment. This is expected as the rats in this study were 9-10 weeks old at the time of treatment and were gaining weight on a daily basis during at this age. Liver weight as a percentage of body weight was increased significantly relative to the mean value of the control group, for at least six days post-treatment. By twelve days post-treatment, liver weight as a percentage of body weight had returned to the control value.

A significant increase in total cytochrome P450 content was observed on the first day post-treatment and this effect persisted for at least 24 days post-treatment. By 48 days post-treatment, total cytochrome P450 content had returned to control values.

Table 3.12. Mean body and liver weights and total cytochrome P450 content of male Long-Evans rats at various times after treatment with Aroclor 1260

Days after Treatment	Body Weight (g)	Liver Weight (g)	Liver Weight as a % of Body Weight	Total Cytochrome P450 Content (nmol P450/mg protein)
Control	275.0 ± 7.0	12.7 ± 0.7	4.6 ± 0.2	1.5 ± 0.05
1 Day	260.3 ± 7.8	15.5 ± 1.4	5.9 ± 0.4[a]	3.3 ± 0.2[a] (2.2)
3 Day	269.8 ± 9.7	15.3 ± 1.3	5.6 ± 0.3[a]	3.2 ± 0.3[a] (2.1)
6 Day	330.0 ± 7.5	19.3 ± 0.6	5.9 ± 0.3[a]	3.0 ± 0.4[a] (2.0)
12 Day	315.0 ± 4.0	16.1 ± 0.4	5.1 ± 0.1	2.5 ± 0.1[a] (1.7)
24 Day	359.0 ± 8.7	16.0 ± 0.5	4.5 ± 0.2[bcd]	2.4 ± 0.2[a] (1.7)
48 Day	420.3 ± 11.3	18.1 ± 0.7	4.3 ± 0.1[bcd]	1.6 ± 0.04[bcd]

Values represent mean ± SEM of three rats per treatment group, except for the 3 Day group where n=5 and the control group where n=4. Rats were treated for seven consecutive days with a 10 mg/kg dose of Aroclor 1260 except the control group which received corn oil. The control group was killed three days after treatment.
Values in parentheses indicate the relative difference from the Control group.
[a] Significantly different ($p<0.05$) from the Control group (using one way ANOVA).
[b] Significantly different ($p<0.05$) from the 1 Day group (using one way ANOVA).
[c] Significantly different ($p<0.05$) from the 3 Day group (using one way ANOVA).
[d] Significantly different ($p<0.05$) from the 6 Day group (using one way ANOVA).

3.13. *EFFECTS ON CYTOCHROME P450-CATALYZED ENZYME ACTIVIITES*

3.13.1. *Effects on BROD, EROD, PROD, and AHH activities*

The effects of length of time after treatment with Aroclor 1260 on hepatic microsomal PROD, BROD, EROD, and AHH activities are shown in Table 3.13. Microsomal PROD and BROD activities were 24-fold and 31-fold greater than those of the control group on the first day post-treatment and remained increased and relatively unchanged for up to 24 days post-treatment. A decrease in microsomal PROD and BROD activities was observed at 48 days post-treatment, however both activities were still significantly greater relative to the control group. Microsomal EROD activity was approximately five-fold greater than that of the control group by one day post-treatment, but was not significantly different than control by six days after treatment. There was no difference between the AHH activity of the control group and that of each of the treatment groups.

Table 3.13. Hepatic microsomal PROD, BROD, EROD, and AHH activities of male rats at various times after treatment with Aroclor 1260

Days after Treatment	PROD (nmol/mg/min)	BROD (nmol/mg/min)	EROD (nmol/mg/min)	AHH (nmol/mg/min)
Control	0.27 ± 0.1	0.49 ± 0.04	0.85 ± 0.1	1.6 ± 0.06
1 day	6.4 ± 0.4[a] (24)	15.0 ± 0.1[a] (31)	4.0 ± 1.0[a] (4.5)	1.8 ± 0.3
3 day	6.2 ± 0.4[a] (23)	14.3 ± 0.3[a] (29)	3.0 ± 0.3[a] (3.5)	2.1 ± 0.3
6 day	5.5 ± 1.2[a] (20)	13.5 ± 1.2[a] (27)	2.2 ± 0.4[b] (2.6)	1.8 ± 0.4
12 day	5.7 ± 0.6[a] (21)	13.8 ± 0.7[a] (28)	2.3 ± 0.1[b] (2.7)	2.4 ± 0.5
24 day	5.5 ± 0.4[a] (20)	13.0 ± 0.4[a] (26)	2.1 ± 0.3[b] (2.5)	2.1 ± 0.1
48 day	2.1 ± 0.3[abcdef] (8)	9.5 ± 0.8[abcdef] (19)	1.9 ± 0.2[b] (2.2)	1.3 ± 0.2

Values represent mean ± SEM of three rats per treatment group, except for the 3 Day group where n=5 and the control group where n=4. Rats were treated for seven consecutive days with a 10 mg/kg dose of Aroclor 1260 except the control group which received corn oil. The control group was killed three days after treatment.
Values in parentheses indicate the relative difference from the Control group.
[a] Significantly different ($p<0.05$) from the Control group (using one way ANOVA).
[b] Significantly different ($p<0.05$) from the 1 Day group (using one way ANOVA).
[c] Significantly different ($p<0.05$) from the 3 Day group (using one way ANOVA).
[d] Significantly different ($p<0.05$) from the 6 Day group (using one way ANOVA).
[e] Significantly different ($p<0.05$) from the 12 Day group (using one way ANOVA).
[f] Significantly different ($p<0.05$) from the 24 Day group (using one way ANOVA).

3.13.2. *Effects on testosterone hydroxylase activities*

Mean microsomal testosterone hydroxylase activities of rats killed at various times after treatment with Aroclor 1260 are presented in Table 3.14. Testosterone 16β-hydroxylase activity was increased by almost 30-fold relative to the control group by the first day post-treatment. This activity remained increased and relatively constant for up to 24 days post-treatment, followed by a 50% reduction in activity by 48 days post-treatment. Testosterone 2β-hydroxylase activity remained constant for up to 48 days after treatment with Aroclor 1260. Testosterone 6β-hydroxylase activity was increased and remained at this level for up to 24 days post-treatment, and then decreased to control values by 48 days following treatment. A similar trend was observed with testosterone 7α-hydroxylase activity, but this activity decreased to control values by 12 days post-treatment. A significant reduction in testosterone 2α-hydroxylase activity was observed from the first to the sixth day following treatment with a return to control values by 12 days following treatment.

Table 3.11. Hepatic microsomal testosterone hydroxylase activities of male rats at various times after treatment with Aroclor 1260

	Testosterone Metabolites (nmol metabolite formed/mg protein/min)					
Days after Treatment	2α	2β	6β	7α	16α	16β
Control	2.6 ± 0.3	0.26 ± 0.01	2.9 ± 0.1	0.24 ± 0.03	4.0 ± 0.5	0.26 ± 0.007
1 day	1.1 ± 0.1[a] (0.4)	0.85 ± 0.07 (3.3)	7.4 ± 0.6[a] (2.6)	0.39 ± 0.05 (1.6)	6.1 ± 0.2 (1.5)	7.4 ± 0.4[a] (29)
3 day	1.5 ± 0.2 (0.6)	0.70 ± 0.02 (2.7)	6.2 ± 0.3[a] (2.0)	0.51 ± 0.07[a] (2.0)	7.6 ± 0.8 (2.0)	8.9 ± 0.6[a] (34)
6 day	1.5 ± 0.2 (0.6)	0.72 ± 0.2 (2.8)	4.8 ± 1.1[ab] (1.7)	0.43 ± 0.1 (1.8)	6.5 ± 1.7 (1.6)	6.8 ± 2.3[a] (26)
12 day	2.4 ± 0.5 (0.9)	0.58 ± 0.03 (2.2)	5.5 ± 0.3 [ab] (1.9)	0.33 ± 0.02 (1.4)	8.0 ± 1.3 (2.0)	7.4 ± 1.1[a] (29)
24 day	3.2 ± 0.3[bcd] (1.2)	1.2 ± 0.4 (4.6)	5.0 ± 0.3 [ab] (1.7)	0.25 ± 0.01[b] (1.0)	8.0 ± 0.5 (2.0)	5.8 ± 0.6[a] (22)
48 day	2.0 ± 0.3 (0.8)	0.60 ± 0.3 (2.3)	3.3 ± 0.2 [bce] (1.1)	0.20 ± 0.01 [b] (1.0)	4.4 ± 0.2 (1.1)	2.7 ± 0.3[bcdef] (10)

Values represent mean ± SEM of three rats per treatment group, except for the 3 day group where n=5 and the control group where n=4. Rats were treated for seven consecutive days with a 10 mg/kg dose of Aroclor 1260 except the control group which received corn oil. The control group was killed three days after treatment. Values in parentheses indicate the relative difference from the Control group. Symbols used in this table are the same as those in Table 3.13.

3.14. *IMMUNOBLOT ANALYSIS*

3.14.1. *Immunoblot analysis of hepatic microsomes probed with anti-cytochrome P450 1A1 IgG*

An immunoblot of hepatic microsomes prepared from rats at various times after treatment and probed with monoclonal anti-cytochrome P450 1A1 IgG is presented in Figure 3.24. Very little or no cytochrome P450 1A1 was found in lanes containing hepatic microsomes from the control group and the group killed 48 days post-treatment. A darkly stained band corresponding to cytochrome P450 1A1 is observed in the lane containing hepatic microsomes from rats killed one day post-treatment. The cytochrome P450 1A1 band appeared to be less intensely stained as time after treatment increased. At all times after treatment, there did not appear to be any change in staining intensity of the band corresponding to cytochrome P450 1A2 relative to the control microsomal sample.

Results of immunoquantitation of cytochrome P450 1A1 and P450 1A2 isozymes are presented in Table 3.15. There was a significant increase in hepatic cytochrome P450 1A1 levels, relative to the control group, from one day to three days post-treatment. Thereafter, cytochrome P450 1A1 levels were not significantly different from control values. A gradual decrease in hepatic cytochrome P450 1A1 levels was observed over time following 1 day post-treatment (Figure 3. 25).

A standard curve could not be constructed for cytochrome P450 1A2 because of a lack of purified cytochrome P450 1A2. Hence, the hepatic level of this isozyme is reported as integrated intensity per milligram of microsomal protein. Results of immunoquantitation of cytochrome P450 1A2 indicated no significant difference in the

integrated intensity of the band corresponding to cytochrome P450 1A2 between the control group and the group killed one day post-treatment. A significant decrease relative to the control value was observed at three days post-treatment. Furthermore, due to the high value obtained at one day post-treatment cytochrome P450 1A2, values for all later treatment groups were significantly lower.

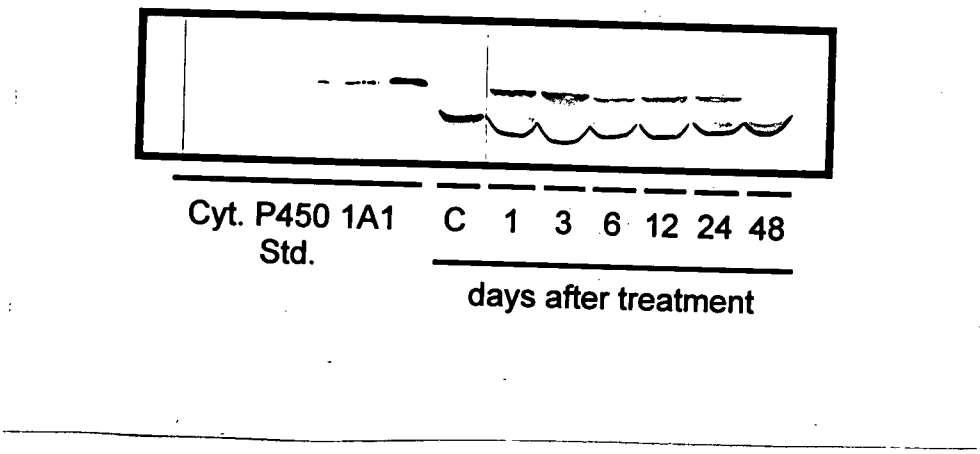

Figure 3.24. A representative immunoblot of rat hepatic microsomes probed with antibody against rat cytochrome P450 1A1. Microsomes from individual rats from the control (C) and Aroclor 1260 treatment group are shown. The numbers indicate the number of days after treatment the rats were killed and correspond to 1, 3, 6, 12, 24, and 48 days. Final concentration of microsomal protein was 20 μg per lane. The concentration of primary antibody (monoclonal mouse anti-rat cytochrome P450 1A1) was 1.0 μg/mL and the secondary antibody (goat anti-mouse IgG) was used at a dilution of 1:3000. The concentration of the purified rat cytochrome P450 1A1 standards are 0.05, 0.075, 0.1, 0.15 and 0.2, pmol per lane, respectively.

Table 3.15. Hepatic levels of cytochrome P450 1A1 and P450 1A2 of male rats at various times after treatment with Aroclor 1260

Days after Treatment	Cytochrome P450 1A1 Content (pmol/mg protein)	Cytochrome P450 1A1 as a % of Total Cytochrome P450	Amount of Immunoreactive Cytochrome P450 1A2[§]
Control	$< 0.5^{\dagger}$	$< 0.034 \pm 0.001^{\dagger}$	2.20 ± 0.1
1 Day	16.27 ± 2.9^{a} (> 33)	0.48 ± 0.06^{a} (> 14)	2.69 ± 0.1 (1.2)
3 Day	8.85 ± 1.4^{ab} (> 18)	0.28 ± 0.04^{ab} (> 8)	1.62 ± 0.1^{ab} (0.7)
6 Day	3.80 ± 0.3^{abc} (> 8)	0.11 ± 0.004^{bc} (> 3)	1.41 ± 0.1^{b} (0.6)
12 Day	4.72 ± 0.9^{abc} (> 9)	0.19 ± 0.03^{b} (> 6)	1.71 ± 0.1^{b} (0.8)
24 Day	2.97 ± 1.0^{bc} (> 6)	0.13 ± 0.06^{bc} (> 4)	1.87 ± 0.3^{b} (0.9)
48 Day	$0.54 \pm 0.03^{\ddagger bc}$ (> 1)	$0.035 \pm 0.002^{\ddagger bc}$ (> 1)	1.72 ± 0.3^{b} (0.8)

Values represent mean ± SEM of three rats per treatment group, except for the 3 day group where n=5 and the control group where n=4. Rats were treated for seven consecutive days with a 10 mg/kg dose of Aroclor 1260, except the control group which received corn oil. The control group was killed three days after treatment.

Values in parentheses indicate the relative difference from the Control group.

† A faint band was visible in lanes containing liver microsomes from control rats, but the staining intensity of the band was below the limit of detection (0.01 pmol). The value presented above and used for statistical analysis is the limit of detection and represents the maximal concentration possible in these microsome samples.

‡ The band corresponding to cytochrome P450 1A1 was below the limit of detection for many microsome samples in the 0.5 mg/kg treatment group. For bands where no value was obtained, a concentration of 0.01 pmol was used in the calculation of the mean.

§ Each value was expressed as the integrated intensity of the band per milligram protein

[a] Significantly different ($p<0.05$) from the Control group (using one way ANOVA).

[b] Significantly different ($p<0.05$) from the 1 Day group (using one way ANOVA).

[c] Significantly different ($p<0.05$) from the 3 Day group (using one way ANOVA).

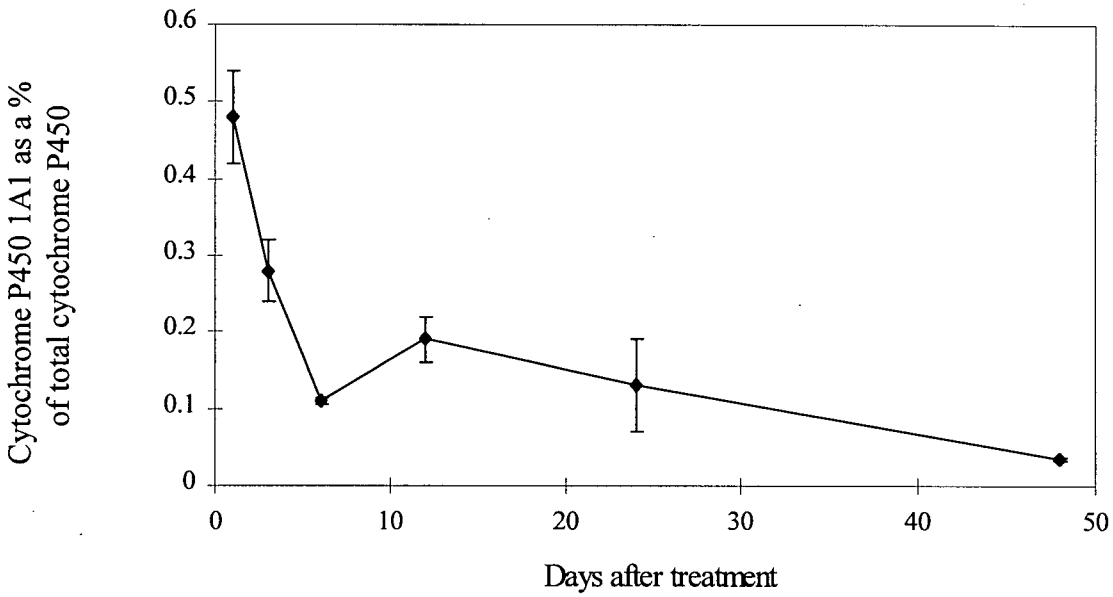

Figure 3. 25. Hepatic cytochrome P450 1A1 levels in Aroclor 1260-treated rats at various times after treament

3.14.2. *Immunoblot analysis of hepatic microsomes probed with anti-cytochrome P450 2B1 IgG*

An immunoblot of hepatic microsomal samples prepared from rats at various times after treatment with Aroclor 1260 and probed with monoclonal anti-cytochrome P450 2B1 IgG is shown in Figure 3.26. Two darkly stained bands in lanes containing hepatic microsomes from rats treated with Aroclor 1260 are apparent. These bands represent cytochrome P450 2B1 and cytochrome P450 2B2. The staining intensity of both bands decreased as time after treatment increased, however, both are darker than the bands of the control microsome sample.

Results of immunoquantitation of the cytochrome P450 2B1 and P4502B2 isozymes are presented in Table 3.16. Hepatic cytochrome P450 2B1 and cytochrome P450 2B2 levels were significantly greater than control values at all times after treatment. Hepatic levels of both cytochrome P450 2B isozymes were highly induced by one day post-treatment. Levels of cytochrome P450 2B1 and cytochrome P450 2B2, expressed a percentage of total cytochrome P450, remained constant for up to 48 days post-treatment (Figure 3.27), but a decrease in cytochrome P450 2B1 and cytochrome P450 2B2 content starting at 24 days post-treatment was noted.

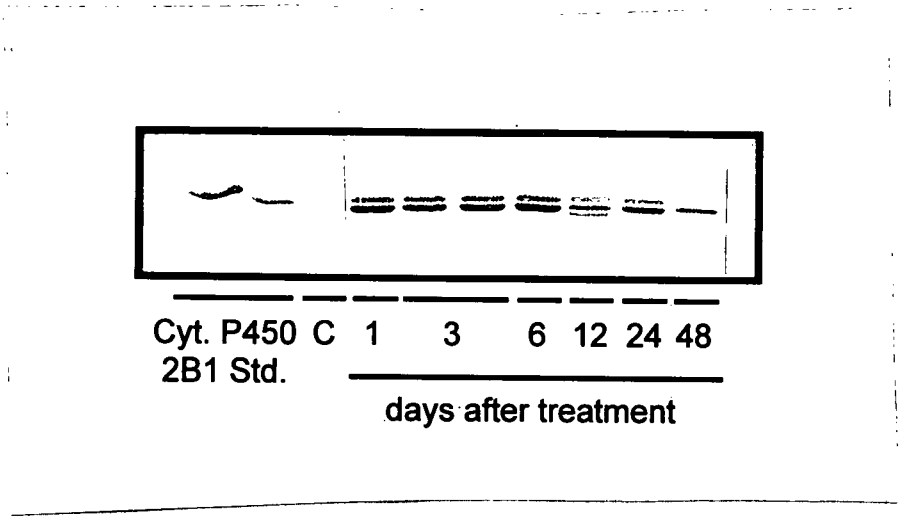

Figure 3.26. A representative immunoblot of rat hepatic microsomes probed with backabsorbed polyclonal rabbit antibody against rat cytochrome P450 2B1. Microsomes from individual rats from the control (C) and Aroclor 1260 treatment group are shown. The numbers indicate the number of days after treatment the rats were killed and correspond to 1, 3, 6, 12, 24, and 48 days. Final concentration of microsomal protein was 1 μg per lane except for the control-treated group and the Aroclor 1260 treated groups treated with 10.0 mg/kg and killed at 24 and 48 days post-treatment where the final concentration of microsomal protein was 10 μg per lane. The concentration of primary antibody (backabsorbed polyclonal rabbit anti-rat cytochrome P450 2B1) was 2.0 mg/mL and the secondary antibody (goat anti-mouse IgG) was used at a dilution of 1:3000. The concentrations of the purified rat cytochrome P450 2B1 standards are 1.0 and 0.25 pmol per lane, respectively.

Table 3.16. Hepatic levels of cytochrome P450 2B isozymes of male rats at various times after treatment with Aroclor 1260

Days after Treatment	Cytochrome P450 2B1 Content (pmol/mg protein)	Cytochrome P450 2B1 as a % of Total Cytochrome P450	Cytochrome P450 2B2 Content (pmol/mg protein)	Cytochrome P450 2B2 as a % of Total Cytochrome P450
Control	7.62 ± 0.2	0.52± 0.02	14.47± 2.9	0.98 ± 0.2
1 Day	1028.0 ± 73.0[a] (135)	30.90 ± 2.9[a] (59)	664.06 ± 37.6[a] (46)	19.90 ± 1.4[a] (21)
3 Day	924.37 ± 47.4[a] (121)	29.34 ± 0.9[a] (56)	675.60 ± 18.6[a] (47)	21.55 ± 0.7[a] (22)
6 Day	752.54 ± 238.9[a] (99)	37.97 ± 9.0[a] (73)	539.85 ± 139.8[a] (37)	21.80 ± 0.4[a] (23)
12 Day	874.09 ± 198.8[a] (115)	34.74 ± 7.4[a] (67)	547.07 ± 38.5[a] (38)	21.80 ± 0.4[a] (23)
24 Day	720.40 ± 52.1[a] (95)	30.74 ± 1.6[a] (59)	409.24 ± 32.5[abc] (28)	17.41 ± 0.5[a] (18)
48 Day	519.29 ± 86.2[a] (68)	33.02 ± 4.7[a] (64)	281.99 ± 64.7[abcde] (20)	18.25 ± 4.5[a] (19)

Note. Values represent mean ± SE of three rats per treatment group, except for the 3 day group where n=5 and the control group where n=4. Rats were treated for seven consecutive days with a 10 mg/kg dose of Aroclor 1260 except the control group which received corn oil. The control group was killed three days after treatment.
Values in parentheses indicate the relative difference from the Control group.
[a] Significantly different ($p<0.05$) from the Control group (using one way ANOVA).
[b] Significantly different ($p<0.05$) from the 1 Day group (using one way ANOVA).
[c] Significantly different ($p<0.05$) from the 3 Day group (using one way ANOVA).
[d] Significantly different ($p<0.05$) from the 6 Day group (using one way ANOVA).
[e] Significantly different ($p<0.05$) from the 12 Day group (using one way ANOVA).
[f] Significantly different ($p<0.05$) from the 24 Day group (using one way ANOVA).

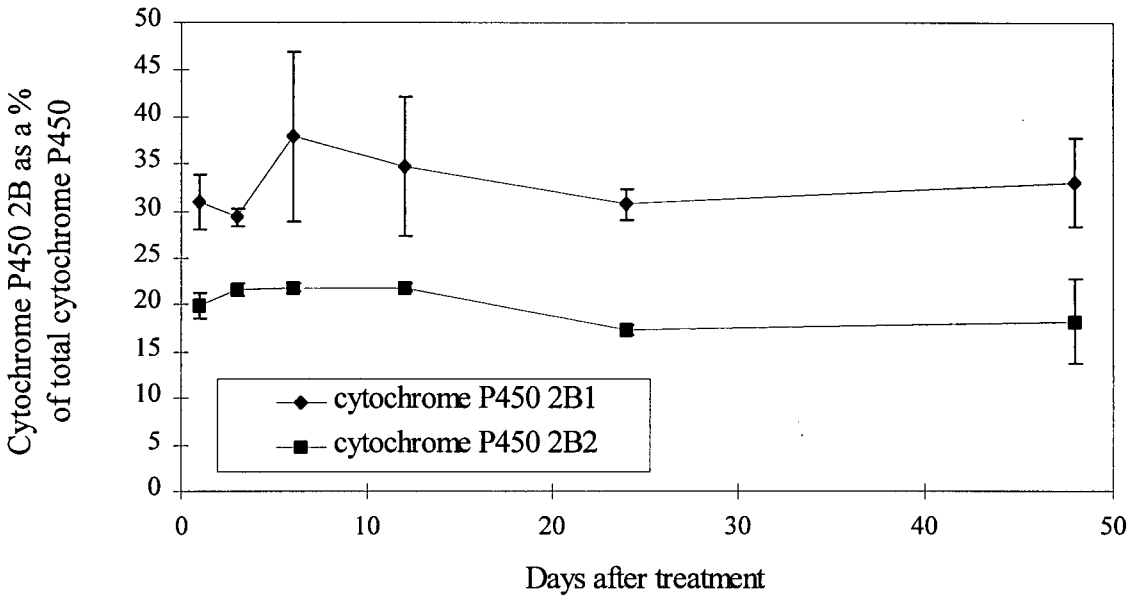

Figure 3.27. Hepatic cytochrome P450 2B levels in Aroclor 1260 treated rats at various times after treament

3.14.3. *Immunoblot analysis of hepatic microsomes probed with anti-cytochrome P450 3A IgG*

An immunoblot of hepatic microsomal samples prepared from rats at various times after treatment with Aroclor 1260 and probed with monoclonal anti-cytochrome P450 3A1 IgG is shown in Figure 3.28. A darkly stained band corresponding to cytochrome P450 3A isozymes is visible in lanes containing hepatic microsomes from rats killed at all time points post-treatment. The staining intensity of the cytochrome P450 3A band appeared to decrease with increasing time after treatment.

Results of immunoquantitation of the cytochrome P450 CYP3A isozymes are represented in Table 3.17. Hepatic levels of cytochrome P450 3A were increased significantly relative to control values at one day post-treatment and remained increased for up to 48 days post-treatment. Hepatic cytochrome P450 3A content was significantly lower at 6, 12, 24, and 48 days post-treatment than at one day post-treatment. In fact, cytochrome P450 3A content declined significantly between 24 days post-treatment and 48 days post-treatment. Hepatic levels of cytochrome P450 3A, expressed as a percentage of total cytochrome P450, was observed decreased more gradually (Figure 3.29).

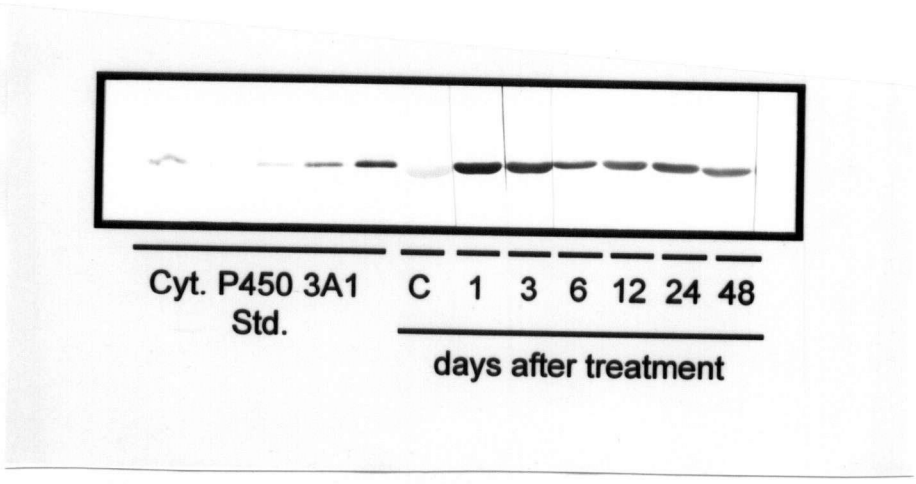

Figure 3.28. A representative immunoblot of rat hepatic microsomes probed with polyclonal rabbit antibody against rat cytochrome P450 3A1. Microsomes from individual rats from the control (C) and Aroclor 1260 treatment group are shown. The numbers indicate the number of days after treatment the rats were killed and correspond to 1, 3, 6, 12, 24, and 48 days. Final protein concentration was 10 μg per lane, except for the control group and the groups killed at 24 and 48 days post-treatment where the final protein concentration was 20 μg per lane. The concentration of primary antibody (polyclonal rabbit anti-rat cytochrome P450 3A1) was 10.0 μg/mL and the secondary antibody (goat anti-mouse IgG) was used at a dilution of 1:3000. The concentration of the purified rat cytochrome P450 3A1 standards are 0.01, 0.0625, 0.125, 0.25, and 0.5 pmol per lane, respectively.

Table 3.17. Hepatic levels of cytochrome P450 3A in male rats at various times after treatment with Aroclor 1260

Days after Treatment	Cytochrome P450 3A Content (pmol/mg protein)	Cytochrome P450 3A as a % of Total Cytochrome P450
Control	13.95 ± 0.9	0.95 ± 0.05
1 Day	133.32 ± 6.4[a] (9.6)	4.01 ± 0.3[a] (4.1)
3 Day	99.71 ± 6.9[ab] (7.2)	3.17 ± 0.2[a] (3.2)
6 Day	75.93 ± 10.3[abc] (5.4)	2.23 ± 0.3[ab] (2.3)
12 Day	64.80 ± 7.6[abc] (4.6)	2.57 ± 0.2[ab] (2.6)
24 Day	70.02 ± 5.5[abc] (5.0)	3.03 ± 0.4[a] (3.0)
48 Day	34.33 ± 7.6[bcdef] (2.5)	2.20 ± 0.5[ab] (2.2)

Values represent mean ± SEM of three rats per treatment group, except for the 3 day group where n=5 and the control group where n=4. Rats were treated for seven consecutive days with a 10 mg/kg dose of Aroclor 1260, except the control group which received corn oil for seven days. The control group was killed three days after treatment. Values in parentheses indicate the relative difference from the Control group.

[a] Significantly different ($p<0.05$) from the Control group (using one way ANOVA).
[b] Significantly different ($p<0.05$) from the 1 Day group (using one way ANOVA).
[c] Significantly different ($p<0.05$) from the 3 Day group (using one way ANOVA).
[d] Significantly different ($p<0.05$) from the 6 Day group (using one way ANOVA).
[e] Significantly different ($p<0.05$) from the 12 Day group (using one way ANOVA).
[f] Significantly different ($p<0.05$) from the 24 Day group (using one way ANOVA).

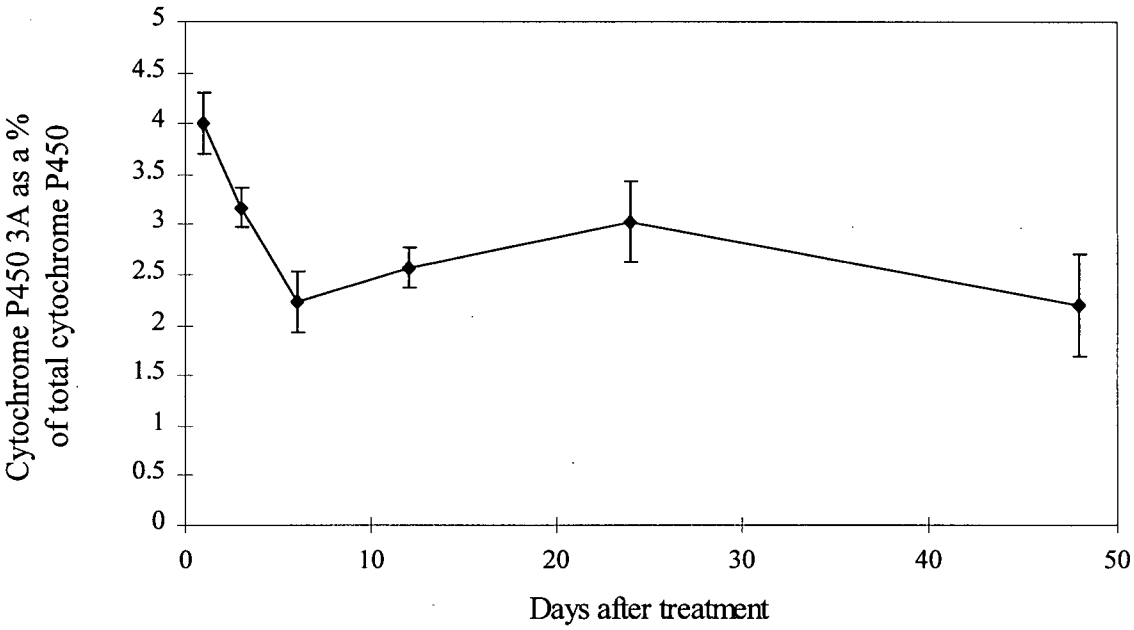

Figure 3.29. Hepatic cytochrome P450 3A levels in Aroclor 1260-treated rats at various times after treament

4. DISCUSSION

SECTION 1: EFFECTS OF INCREASING DOSES OF AROCLOR 1260

The effects of increasing doses of Aroclor 1260 on hepatic microsomal cytochrome P450-mediated activities, cytochrome P450 protein levels, and hepatic PCB levels in male Long-Evans rats was examined in the first part of the present study. In addition, the correlation between residual hepatic PCB levels and induction of cytochromes P450, namely cytochromes P450 1A1, P450 2B1, P450 2B2, and P450 3A, was investigated.

4.1. *EFFECTS OF TREATMENT ON BODY WEIGHT, LIVER WEIGHT, AND TOTAL CYTOCHROME P450 CONTENT*

Treatment with Aroclor 1260 for seven consecutive days at doses up to 50 mg/kg had no effect on rat body weight. However, an increase in liver weight as a percentage of body weight was observed with increasing doses of Aroclor 1260 and significant increase above control values was observed for rats treated with doses of 10 mg/kg and greater. It has been shown in previous studies that repeated PB treatment at doses of 100 mg/kg results in liver enlargement and proliferation of smooth endoplasmic reticulum in rats (Waxman & Azaroff 1992). The increase in liver weight is thought to be due to hypertrophy, an enlargement of the liver as a result of an increase in hepatocyte size, rather than an increase in hepatocyte number. An increase in liver weight as a percentage

of body weight has also been observed in rats treated with Aroclor 1254 at a concentration of 100 ppm in their diet (Lubet *et al.* 1991).

Total cytochrome P450 content was maximally increased in rats treated with Aroclor 1260 at a dose of 5.0 mg/kg for seven days. A ten-fold increase in Aroclor 1260 treatment did not further increase cytochrome P450 content over that of the 5.0 mg/kg treatment group. These results are in agreement with previous studies, which showed that total cytochrome P450 content was increased by several fold in rats treated with PCB (Ikegwuonu *et al.* 1996).

4.2. *THE EFFECTS ON CYTOCHROME P450-MEDIATED ENZYME ACTIVITIES*

The dealkylation of alkoxyresorufins, such as benzyloxy-, ethoxy-, and pentoxyresorufin are widely used as indicators of specific cytochrome P450 isozymes in induced animals. However, care must be taken when interpreting alkoxyresorufin dealkylase activity data as more than one cytochrome P450 isozyme may be contributing to each activity (Burke *et al.* 1994, Okey 1990).

In untreated rats, BROD activity is catalyzed primarily by cytochromes P450 2B1 and P450 2C11, and to a lesser extent by cytochromes P450 1A2, P450 2C6, and P450 3A (Burke *et al.* 1994). In PB-treated rats, BROD activity is catalyzed mostly by cytochrome P450 2B1 (Burke *et al.* 1994). Similarly, cytochromes P450 2C6, P450 2C11, and P450 3A are the major catalysts of PROD activity in untreated rats, however cytochrome P450 2B1 is the primary catalyst of PROD activity in PB-treated animals (Burke *et al.* 1994).

In the present study, BROD activity was increased 28-fold in rats treated with Aroclor 1260 at a dose of 5.0 mg/kg and this increase remained relatively unchanged with increasing doses of Aroclor 1260. A similar pattern was observed with PROD activity, whereby PROD activity was increased 20-fold in rats treated with Aroclor 1260 at dose of 5.0 mg/kg and did not increase further with increasing doses. In comparison, Harris *et al.* (1993) found no significant difference in PROD activity of male Wistar rats treated with Aroclor 1260 by single i.p. injection at doses of 40 to 2000 mg/kg and killed 14 days later. In another study, Connor *et al.* (1995) reported that Aroclor 1260 administered i.p. at a dose of 5.0 mg/kg to female Sprague-Dawley rats and killed three days later was the minimum dose required to increase PROD activity significantly above control values. A dose-dependent increase was observed with Aroclor 1260 treatment up to a dose of 150 mg/kg. The discrepancy in results among the studies is likely due to differences in the route of administration, number of doses, sex and strain of animal.

EROD activity is indicative of cytochrome P450 1A1 induction in rats treated with 3-MC and other Type I inducers (Burke *et al.* 1994, Okey 1990). A small but significant increase in EROD activity has also been reported in PB-treated rats (3- to 6-fold) (Burke *et al.* 1994). Antibody inhibition studies have shown that the majority of EROD activity is catalyzed by cytochrome P450 1A1 in hepatic microsomes from animals treated with 3-MC and by cytochrome P450 2B1 in microsomes from rats treated with PB (Burke *et al.* 1994). In untreated rats, several cytochrome P450 isozymes contribute to EROD activity, primarily cytochrome P450 2C6 and a combination of cytochromes P450 1A1, P450 2C11, and P450 3A (Burke *et al.* 1994, Kelley *et al.* 1987).

In the present study, EROD activity increased as the dose of Aroclor 1260

increased. The results suggest that increases in EROD activity might be due to increases in cytochrome P450 1A1 content. Harris *et al.* (1993) reported a dose-dependent increase in EROD activity in rats treated with Aroclor 1260 i.p. at doses ranging from 10 to 2000 mg/kg. A dose-dependent increase in cytochrome P450 1A1 has also been reported for rats treated with Aroclor 1254 by Lubet *et al.* (1991) and Nims *et al.* (1992).

As with EROD activity, hepatic AHH activity may be used as an indicator of cytochrome P450 1A1 levels. However, the results show that increasing doses of Aroclor 1260 did not increase AHH activity above control levels. Harris *et al.* (1993) reported that AHH activitiy was not increased significantly above control values when Aroclor 1260 was administered by a single i.p. injection at doses less than 160 mg/kg. Thus, induction of cytochrome P450 1A1 by Aroclor 1260 may not have been sufficiently great so as to be reflected in increased AHH activity in the current study.

In addition to EROD, BROD, PROD, and AHH activities, the effect of Aroclor 1260 on testosterone hydroxylase activity was examined. There are few studies that examined the effect of Aroclor 1260 treatment on testosterone hydroxylase activity. Testosterone 16β-hydroxylase activity, which is catalyzed primarily by cytochromes P450 2B1 and P450 2B2 (Sonderfan *et al.* 1987), was increased significantly over control levels in rats treated with Aroclor 1260 at a dose of 5.0 mg/kg and remained relatively unchanged at higher doses. This pattern parallels the change in BROD and PROD activities with increasing doses of Aroclor 1260. The testosterone 16β-hydroxylase data suggests that maximal induction of cytochrome P450 2B1 and P450 2B2 isozymes by Aroclor 1260 treatment occurs at a dose of 5.0 mg/kg. A dose-dependent increase in testosterone 16β-hydroxylase, BROD, and PROD activities was not obvious in the

present study but would be expected to occur in rats treated with Aroclor 1260 at doses less than 5.0 mg/kg.

Treatment with Aroclor 1260 at doses of 5.0 mg/kg and greater resulted in reduced testosterone 2α-hydroxylase activity, indicating a suppression of cytochrome P450 2C11. Previous studies have reported that cytochrome P450 2C11 expression is reduced in hepatic microsomes following treatment with compounds that are strong inducers of cytochromes P450 1A, P450 2B, or P450 3A (Yeowell *et al.* 1987). Testosterone 2α-hydroxylase activity is typically reduced to half the value measured with microsomes from untreated rats after treatment with 3-MC or PB, however, the mechanism responsible for enzyme suppression is unknown.

Testosterone 16α-hydroxylase activity is catalyzed by cytochromes P450 2B1, P450 2B2 and P450 2C11 (Sonderfan *et al.* 1987). A significant increase in 16α-hydroxylase activity was observed with Aroclor 1260 at doses of 5.0 mg/kg and greater, but the extent of the increase was less than that observed for testosterone 16β-hydyroxylase activity. This may by due to suppression of cytochrome P450 2C11. Previous studies have shown that only small increases of testosterone 16α-hydroxylase activity are observed after treatment with PB (Sonderfan *et al.* 1987). Thus, suppression of cytochrome P450 2C11 coupled with increases in cytochrome P450 2B1 and P450 2B2 may contribute to the smaller increase in testosterone 16α-hydroxylase activity relative to testosterone 16β-hydroxylase activity.

Significant increases in testosterone 2β-, 7α-, and 6β-hydroxylase activities were also noted in rats treated with Aroclor 1260. Treatment at doses of 0.5 mg/kg and greater significantly increased testosterone 6β-hydroxylase indicating induction of cytochrome

P450 3A. Treatment at doses of 5.0 mg/kg and greater significantly increased testosterone 7α- and 2β- hydroxylase activities, indicating induction of cytochrome P450 2A1, and induction of cytochromes P450 1A1 and P450 3A, respectively.

4.3. *IMMUNOBLOT ANALYSIS*

The enzyme activity data suggested that treatment with increasing doses of Aroclor 1260 for seven consecutive days increased cytochromes P450 1A-, P450 2B-, and P450 3A-mediated monooxygenase activities. To determine if Aroclor 1260 was inducing expression of the various cytochrome P450 isozymes at the protein level, immunoblot analysis was conducted.

Densitometric quantitation of immunoblots probed with anti-cytochrome P450 1A1 IgG showed that hepatic cytochrome P450 1A1 levels increased with increasing doses of Aroclor 1260. Thus, the increase observed with EROD activity can be assigned to induction of cytochrome P450 1A1. Although no previous studies examined the effects of Aroclor 1260 on cytochrome P450 1A1 protein levels, Dragnev *et al.* (1994), Lubet *et al.* (1991), and Nims *et al.* (1992) reported dose-dependent increases in cytochrome P450 1A1 protein levels after treatment with Aroclor 1254. A significant increase in integrated intensity of bands corresponding to cytochrome P450 1A2 was observed in rats treated with Aroclor 1260 at a dose of 50 mg/kg. However, there was no increase in the cytochrome P450 1A2 levels at doses below 50.0 mg/kg, which may be due to the diffuse bands corresponding to cytochrome P450 1A2. Band diffusion created a large amount of error in calculating integrated intensity and the results may not be a

good representation of cytochrome P450 1A2 content.

Densitometric quantitation of immunoblots probed with anti-cytochrome P450 2B1 IgG indicated a marked increase in hepatic cytochrome P450 2B1 and P450 2B2 levels in rats treated with the lowest dose of Aroclor 1260. However, hepatic cytochrome P450 2B levels remained relatively constant in rats treated with larger doses up to 50 mg/kg. The results indicate that maximal induction of cytochrome P450 2B1 was reached by a dose of 5.0 mg/kg and maximal induction of cytochrome P450 2B2 was reached by a dose of 5.0 to 10 mg/kg.

A discrepancy between the fold increase in the BROD and PROD activities and the fold increase in the cytochrome P450 2B protein levels is apparent. For example, there was a 28-fold increase in BROD activity at a dose of 20.0 mg/kg, while there was a 61-fold increase in the level of cytochrome P450 2B1 and a 23-fold increase in the level of cytochrome P450 2B2, expressed as a percent of total cytochrome P450. This discrepancy has been reported previously by Ikegwuonu *et al.* (1996) and Ronis *et al.* (1994). Ronis *et al.* (1994) suggested that the lack of similarity in the fold increase in enzyme activity and protein level may be due to complex formation between the cytochrome P450 enzyme and the inducing chemicals. In addition, the relative increases in both monooxygenase activities and protein levels are dependent upon the control values. Control values of enzyme activity are often the result of contributions from several cytochrome P450 isozymes and thus, may not be a good measure of the basal (constitutive) level of a specific enzyme.

Densitometric quantitation of immunoblots probed with anti-cytochrome P450 3A1 IgG indicated that treatment with Aroclor 1260 induced expression of the

cytochrome P450 3A isozymes. Maximal induction of cytochrome P450 3A was reached at a dose of 10 mg/kg. The same pattern was also seen with testosterone 2β- and 6β-hydroxylase activities, which are indicative of cytochrome P450 3A activity.

On the basis of enzyme activities, it was reported previously that Aroclor 1260 is a strong inducer of cytochromes P450 1A1 and P450 2B (Parkinson *et al.* 1980, 1981, 1983, Safe 1994). Results from the present study demonstrate that Aroclor 1260 also induces cytochrome P450 3A. When comparing the relative induction of cytochromes P450 1A1, P450 2B1, P450 2B2, and P450 3A, cytochrome P450 3A was induced the least. For example, a 3.9-fold increase in cytochrome P450 3A protein, expressed as a percent of total cytochrome P450 content, was observed for rats treated at a dose of 20 mg/kg while increases of > 23-, 61-, and 23-fold in cytochrome P450 1A1, P450 2B1, and P450 2B2 isozymes, respectively, were observed. The extent of induction of cytochrome P450 2B1 cannot be compared with that of cytochrome P450 1A1 because of the difficulty in measuring cytochrome P450 1A1 levels in control rats. However, hepatic cytochrome P450 2B1 levels were higher than those of cytochrome P450 1A1 and P450 3A at all doses of Aroclor 1260.

4.4. *PCB LIVER EXTRACTION AND GC-MS ANALYSIS*

4.4.1. *Hepatic PCB levels*

Analysis of the Aroclor 1260 preparation used in this study revealed that the

following isomers, PCB-138, -149, -153, -170, -174, -180, -182, and -187, made up 54% of the total PCBs in Aroclor 1260. Among the major PCB isomers in Aroclor 1260, PCB-153 was the most abundant (10.92%), followed by PCB-180 (10.79%) and PCB-138 (8.45%). Both PCB-153 and PCB-180 are considered to be Type II inducers in that they elicit PB-like induction. PCB-138 is considered a Mixed Type inducer, as it has both 3-MC and PB-like inductive characteristics. Therefore, it is likely that Aroclor 1260 would elicit a strong PB-like induction overall.

The five major isomers found in hepatic tissue of rats treated with Aroclor 1260 were, from highest to lowest concentration, PCB-153, -138, -180, -170 and -194. Interestingly, PCB-180 was more abundant than PCB-138 in the Aroclor 1260 preparations used. The change in the order of PCB isomer abundance in hepatic tissue relative to the original Aroclor 1260 sample may be a result of preferential excretion of PCB-180, or faster distribution of PCB-180 to adipose tissue.

Four of the five most abundant PCBs present in hepatic tissue from rats treated with Aroclor 1260 are among the five most abundant PCB isomers found in free ranging polar bears from the Canadian Arctic (PCB-138, -153, -170, and -180) (Letcher *et al.* 1996). At the lowest dose of Aroclor 1260, hepatic levels of these PCB isomers were similar to levels reported in polar bear liver (Letcher *et al.* 1996). Aroclor 1260 at doses of 5.0 mg/kg and greater resulted in hepatic concentrations of the four PCB isomers that are much greater than those arising from environmental exposure. The hepatic level of PCB-99, the other major PCB isomer found in free ranging polar bears, was present at a lower level in rats than in polar bear liver because PCB-99 makes up a very small percentage of PCBs in Aroclor 1260. Thus, exposure with Aroclor 1260 at a dose of 0.5

mg/kg would be indicative of environmental exposure for four of the major PCB isomers.

Results from the present study indicated a dose-dependent increase in total hepatic PCB levels. Dragnev *et al.* (1994) observed a dose-dependent increase in total hepatic PCB levels with increasing doses of Aroclor 1254. In the same study, PCB concentrations in adipose tissue were much greater than those seen in liver, most likely due to the relatively great lipophilicity of the PCB isomers, as well as the much longer apparent half-life of PCBs in the adipose tissue relative to the liver.

However, it is apparent that hepatic levels of several PCB isomer levels are neither dose dependent nor significantly different from control group levels. Interestingly, some PCB isomers that are not present in Aroclor 1260 were identified in the rat liver, possibly due to outside contamination. In general, levels of the lower chlorinated PCB isomers tend not increase in liver tissue with increasing doses of Aroclor 1260 while hepatic levels of the higher chlorinated PCB isomers tend to be dose-dependent. The higher chorinated PCB isomers are only slowly biodegradable and thus bioaccumulate easily in tissue (van Birgelen *et al.* 1995, Parkinson *et al.* 1980). Possible explanations as to why there is a dose-dependent increase in some PCB isomers and not in others may be preferential metabolism of the lower chlorinated PCB isomers and preferential distribution of the lower chlorinated isomers to other tissues such as adipose and skin due to differences in lipophilicity. For example, in a recent study by Dragnev *et al.* (1995), PCB-169 was reported to preferentially accumulate (30 times greater) in liver relative to PCB-105 and -118 in rats given dietary doses of 2 or 8 mg/kg of the individual PCB isomers. Dragnev *et al.* (1995) suggested the finding was due, in part, to the ability of cytochrome P450 1A proteins to bind to PCB 169. In another study by Dragnev *et al.*

(1994), it was reported that PCB-105 and PCB-118 were preferentially lost in animals fed Aroclor 1254 discontinuously. These results suggest preferential metabolism and clearance of PCB isomers -105 and -118. In a study by Chu *et al.* (1995), low levels of PCB-77 were found in tissues, most likely as a result of rapid metabolism of this isomer. As well, Chu *et al.* (1995) reported that the level of PCB-118 was 10 times higher in fat than in liver or any other tissue. Clevenger *et al.* (1989) demonstrated that different PCB isomers have different partition coefficients and this would determine how the isomers are distributed. From the studies mentioned, it is apparent that many factors are involved in tissue distribution and clearance of PCB isomers in the body. Other explanations include complex formation between some PCB isomers and hepatic proteins, and instrument sensitivity in measuring very slight changes with very low PCB concentrations. In addition, the lower chlorinated PCB isomers make up a small percentage of Aroclor 1260 and increases in dose of 100-fold may not be large enough to render a significant increase in the accumulation of these isomers in hepatic tissue.

In the present study, all of the PCBs classed as Mixed type inducers and most of the PCBs classed as Type II inducers show a dose-dependent increase. The increase in hepatic levels of PCBs classed as Mixed type inducers parallels the increase in cytochrome P450 1A1 levels.

4.4.2. *Effects of Type I, Type II, and Mixed Type PCB inducers on specific rat cytochrome P450 isozymes in rats treated with increasing doses of Aroclor 1260*

There was a positive linear relationship between cytochrome P450 1A1 protein

levels and total hepatic PCB levels. Letcher *et al.* (1996), using liver from polar bears, correlated hepatic cytochrome P450 1A levels with hepatic concentrations of PCBs known to be either Type I or Mixed type inducers and found a strong correlation (r^2 = 0.875, $p < 0.0001$). In the same study, a strong correlation was also observed using total hepatic PCBs ($r^2 = 0.953$, $p < 0.00005$). In another study in which rats were treated with Aroclor 1254, a r^2 value of 0.99 was determined when total hepatic PCB levels were correlated with EROD activity (Lubet *et al.* 1991). Both the current and previous studies strongly suggest that an increase in total hepatic PCB levels mediates an increase of hepatic cytochrome P450 1A1 protein levels.

Surprisingly, when PCBs classed as Type II inducers were correlated to cytochrome P450 1A1 levels, a strong correlation was observed also. The strong correlation is most likely a result of "coincidence". Because cytochrome P450 1A1 protein levels and hepatic levels of total PCBs, as well as of Type II PCB inducers, increased with increasing dose, a correlation between any two will result in a linear correlation with a large r^2 value. However, the correlation cannot be used as evidence of "cause and result".

In the present study, a parabolic relationship between total hepatic PCBs and hepatic cytochrome P450 2B protein levels was observed. Maximal induction of cytochrome P450 2B by Aroclor 1260 at a dose of 5.0 mg/kg is suggested because cytochrome P450 2B protein levels did not increase as total hepatic PCB levels increased with increasing doses of Aroclor 1260. However, there is a possibility of a dose-dependent increase in cytochrome P450 2B levels with increasing doses of Aroclor 1260 in the 0 to 5.0 mg/kg range. A previous study using liver tissue from polar bear observed

a moderate correlation between PCBs classed as Type II inducers and Mixed type inducers and hepatic cytochrome P450 2B levels ($r^2 = 0.419$, $p < 0.03$) (Letcher *et al.* 1996).

With respect to the increase in cytochrome P450 3A levels there have been no studies that report which PCB isomers are responsible for cytochrome P450 3A induction. It is possible that PCB Type II inducers cause an increase in cytochrome P450 3A expression because it has been reported that that cytochrome P450 3A can be induced by PB and PB-like compounds (Cooper *et al.* 1992). A parabolic relationship between cytochrome P450 3A and total hepatic PCB concentration was observed. This suggests that maximal induction of cytochrome P450 3A occurs at higher doses of Aroclor 1260. It is also possible there is a dose-dependent increase of cytochrome P450 3A with doses of Aroclor 1260 in the 0 to 5.0 mg/kg range.

SECTION 2: EFFECTS OF TIME AFTER TREATMENT WITH AROCLOR 1260

The effects of time after treatment with Aroclor 1260 at a dose of 10 mg/kg on hepatic microsomal cytochrome P450-mediated activities and cytochrome P450 protein levels in male Long-Evans rats was examined in the second part of the present study.

4.5. *EFFECTS ON BODY WEIGHT, LIVER WEIGHT, AND TOTAL CYTOCHROME P450 CONTENT*

For rats treated with Aroclor 1260 at a dose of 10.0 mg/kg for seven consecutive days and killed at different times after treatment, body and liver weights were observed to increase over time. The increase in both body and liver weight was expected as eight to ten week old male Long-Evans rats are still gaining weight at a rate of approximately 3 g/day. However, treatment with Aroclor 1260 causes an increase liver weight as a percentage of body weight for up to six days post-treatment. By 12 days post-treatment, the liver to body weight ratio had returned to near control values.

Treatment with Aroclor 1260 at a dose of 10 mg/kg for seven consecutive days significantly induce total hepatic cytochrome P450 content for at least 24 days post-treatment followed by a return to control levels by 48 days post-treatment. The decrease in total cytochrome P450 content may be due to a decline in hepatic PCB levels resulting in reduced induction of cytochrome P450.

4.6. *EFFECTS ON CYTOCHROME P450 ISOZYME LEVELS AND CYTOCHROME P450-MEDIATED ENZYME ACTIVITIES*

On the basis of the results of the microsomal testosterone 16β-hydroxylase, BROD, and PROD assays, maximal induction of cytochrome P450 2B1 appeared to have been reached by one day post-treatment and was maintained for up to 24 days post-treatment. Testosterone 16β-hydroxylase, BROD and PROD activities were still significantly greater than control values at 48 days post-treatment, but the activities were no longer maximally induced. Similarly, cytochrome P450 2B1 and P450 2B2 protein content, as determined by immunoquantitation, remain significantly increased and relatively unchanged up to 24 days post-treatment. The immunoquantitation data support the widely accepted view that cytochrome P450 2B1 and P450 2B2 are responsible for the large increases in testosterone 16β-hydroxylase, BROD and PROD activities. Although other studies have not reported similar results using Aroclor 1260, a study by Lubet *et al.* (1991) observed that BROD activity rapidly reached a maximum value by three days in male F344 rats fed low level concentrations of DDT or α-hexachlorocyclohexane. Values of BROD activity did not change between nine and 27 days post-treatment (Lubet *et al.* 1991).

Total cytochrome P450 content returned to control levels by 48 days post-treatment while hepatic cytochrome P450 2B1 and P450 2B2 levels remained highly induced for up to 48 days post-treatment. This discrepancy suggests that the cytochrome P450 2B enzymes are not responsible for all of the increase in total cytochrome P450 content from Aroclor 1260 exposure. It has been reported that cytochromes P450 2A1, P450 2C7, P450 2C6, and P450 3A are highly induced in PB-treated male rats (Ryan and

Levin 1990) . These cytochrome P450 isozymes may also be highly induced in Aroclor 1260-treated rats and thus would account for the large increase in total cytochrome P450 content. However, the decrease in cytochrome P450 2B content between 24 and 48 days post-treatment parallels the decrease in total cytochrome P450 content.

The microsomal EROD activity and cytochrome P450 1A1 protein levels suggest that induction of cytochrome P450 1A1 is relatively short lived. It may be interpreted that PCB isomers that exhibit Mixed Type induction are cleared from the body by 12 days post-treatment, metabolised to non-active metabolites, or distributed to adipose tissue whereby they do not cause increased expression of hepatic cytochrome P450 1A1. Lubet *et al.* (1991) reported a rapid decline in EROD activity post-treatment in female F344 rats fed low concentrations (1 to 10 ppm) of Aroclor 1254 in the diet for seven consecutive days.

The lack of change in AHH activity from control levels observed at all times up to 48 days post-treatment suggest the low sensitivity of the AHH assay. It has been shown that EROD activity is increased to a greater extent than AHH activity in rats treated with a single equivalent dose of 3-MC thus indicating that EROD activity is a more sensitive measure of exposure (Burke *et al.* 1977).

The significant increases in cytochrome P450 3A protein levels, tesosterone 2β- and 6β-hydroxylase activities were maintained for at least 48 days post-treatment, but both cytochrome P450 3A content and testosterone 6β-hydroxylase activity decreased gradually with time. This may be a result of a more rapid loss of those PCB isomers from liver that are responsible for the rapid increase in cytochrome P450 3A expression. The result suggest that a number of PCBs with differing chemical properties may be

responsible for cytochrome P450 3A induction.

The reduction in testosterone 2α-hydroxylase activity was short lived. By three days post-treatment testosterone 2α-hydroxylase activity had returned to near control values and was no longer significantly decreased. By 12 days post-treatment testosterone 2α-hydroxylase activity had returned to control values suggesting that cytochrome P450 2C11 is not suppressed for a long period of time.

An increase in testoserone 7α-hydroxylase activity was observed followed by a gradual decrease in activity. The results suggest that induction of cytochrome P450 2A1 by Aroclor 1260 treatment is increased over control values for up to three days post-treatment.

4.7. *THE EFFECT OF THE PCB HALF-LIFE ON CYTOCHROME P450 ENZYME LEVELS*

Although the PCBs from the liver tissue of the time course study were not extracted and analyzed, the fate of PCBs in liver can be interpreted from previous studies that investigated the half-life of PCBs in adipose and liver tissue.

The half-life of PCBs in adipose and liver tissue has been calculated to be approximately seven weeks and two to three weeks, respectively (Dragnev *et al.* 1994). Other studies reported the half-life of PCBs in liver to be approximately two weeks after dietary exposure to Aroclor 1254 for seven days (Lubet *et al.* 1991) and from 12 to 13 weeks in adipose tissue (Allen *et al.* 1976, Tanabe *et al.* 1981). In summary, it can be concluded that half-life of PCBs in liver is much shorter than in adipose tissue. The short

half-life in liver may account for some of the decrease in protein levels of cytochromes P450 1A1 and P450 3A seen in the present study.

On the other hand, a PCB half-life of two to three weeks in liver does not seem to explain the relatively unchanged cytochrome P450 2B protein levels in rats treated with Aroclor 1260 for seven days at a dose of 10.0 mg/kg for up to 48 days post-treatment. It has been suggested that although half-life is from 2-3 weeks for PCBs in liver tissue, the liver may have the ability to reequilibrate with the large pool of PCBs that are found in adipose tissue (Dragnev *et al.* 1994). In the present study, the combination of large concentrations of PCB Type II inducers present in Aroclor 1260 and the high lipophilicity of these PCB isomers may account for a large pool of Type II inducers in adipose tissue. Over time, this large pool may continuously re-equilibrate between the liver and adipose tissue, thus maintaining the high protein levels of cytochrome P450 2B that is observed in the present study.

4.8. *CONCLUSIONS*

1. Cytochrome P450 1A, P450 2B, P450 3A and P450 2A (possibly) isozymes were induced in male rats treated with Aroclor 1260 for seven days at doses of 0.5 to 50 mg/kg. Hepatic levels of cytochrome P450 2B1 were higher than levels of the other isozymes examined at all doses of Aroclor 1260. Cytochrome P450 3A was induced to a lesser extent relative to cytochrome P450 1A1, P450 2B1, and P450 2B2 at all doses of Aroclor 1260. Cytochrome P450 2B and P450 3A levels were maximally induced at a dose of 5.0 mg/kg and 20 mg/kg, respectively. However, the results suggest that there is dose dependent induction of cytochrome P450 1A1 at doses of Aroclor 1260 up to 50 mg/kg.

2. A dose dependent increase in total hepatic PCB levels was observed in the Aroclor 1260-treated rats. The major PCB isomers found in hepatic tissue are PCB-138, -153, -170, and -180. A positive linear correlation was obtained between total hepatic PCB concentrations and hepatic cytochrome P450 1A1 protein.

3. Cytochrome P450 2B and P450 3A isozymes were induced for at least 48 days after exposure to Aroclor 1260 at a dose of 10 mg/kg. Levels of cytochrome P450 1A1 returned to control values by six days after treatment suggesting that the duration of cytochrome P450 1A1 induction is shorter than other cytochrome P450 isozymes after Aroclor 1260 exposure.

4. Residual hepatic levels of PCB isomers measured in environmentally exposed polar bears were similar to levels measured in Aroclor 1260 treated rats at doses ranging from 0.5 to 10 mg/kg. Thus, treatment of rats with Aroclor 1260 at doses ranging from 0.5 to 10 mg/kg represents a reasonable model for achieving PCB concentrations and a PCB isomer profile that resembles those found in the polar bear.

4.9. *FUTURE STUDIES*

The results of the study implied that a dose-dependent increase in cytochrome P450 2B and P450 3A occurred at lower doses and that once saturation of hepatic PCBs was obtained no further increase in cytochrome P450 2B and P450 3A were observed. Doses of Aroclor 1260 ranging from 0 and 5.0 mg/kg should be used in order to obtain a dose-dependent increase in cytochrome P450 2B and doses ranging from 0 and 10.0 mg/kg should be used for cytochrome P450 3A.

Cytochrome P450 1A1 is widely used as a biomarker of exposure to PCBs and PCDDs. Cytochromes P450 2B and P450 3A may also be useful biomarkers for PCBs at low concentrations, thus more studies should be undertaken to determine the effects of PCDDs on cytochromes P450 2B and P450 3A.

Analysis of hepatic PCB levels in rats treated with Aroclor 1260 and killed at various times post-treatment would further aid the interpretation of the fate of hepatic cytochrome P450 isozyme levels. Due to restrictions of time and expense the PCBs from the liver tissue of the time course study were not extracted and analyzed.

A long-term dose-response study using free ranging animals under controlled conditions would be ideal, although most likely not possible. Rather, a long-term study involving a laboratory animal model is possible. The continuous treatment of rats with Aroclor 1260 would be important in interpretting the effects of long-term PCB exposure in the environment. As seen in the present study, Aroclor 1260 is a good substitute for environmental PCB mixtures as many of the highly chlorinated PCB isomers present in Aroclor 1260 are found in the environment.

In addition, future research should include identifying and measuring the levels of cytochrome P450 enzymes in various species found in the wild. With this information, we will be able to better assess the environmental impact of organochlorine contamination in a number of species and niches.

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