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Accumulation and Metabolism of Halogenated Compounds in Sea Turtles

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by

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The text of this dissertation, in part, is a reprint of the material as it appears in "Polychlorinated Biphenyls and Biotransformation Enzymes in Three Species of Sea Turtles from the Baja California Peninsula of Mexico" published in Archives of Environmental Contamination and Toxicology, volume 58, pages 183-193, 2010. The

co-authors, Melania Lopez Castro and Susan C. Gardner, listed in the publication assisted with sample procurement and technical aspects related to the import of endangered sea turtles. The text of this dissertation, in part, is also a reprint of the material as it appears in "The Characterization of Cytosolic Glutathione Transferase From Four Species of Sea Turtles: Loggerhead (Caretta caretta), Green (Chelonia mydas), Olive Ridley (Lepidochelys olivacea), and Hawksbill (Eretmochelys imbricata)" published in Comparative Biochemistry and Physiology, Part C, volume 150, pages 279-284, 2009. The co-author, Gerardo Gold-Bouchot, listed in the publication assisted with sample procurement and technical aspects related to the import of endangered sea turtles. The co-author Daniel Schlenk listed in both of the publications directed and supervised the research which forms the basis for this dissertation.

Dedication

This dissertation is dedicated to my parents, Keith and Linda Richardson. Their endless support and encouragement in all stages of my education made all of this possible.

ABSTRACT OF THE DISSERTATION

Accumulation and Metabolism of Halogenated Compounds in Sea Turtles

by

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Doctor of Philosophy, Graduate Program in Environmental Toxicology
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Dr. Daniel Schlenk, Chairperson

All sea turtle populations face the risk of extinction. Of the threats to sea turtles, the effects of environmental chemicals are the least understood. Polychlorinated biphenyls (PCBs) are toxic, persistent, ubiquitous, anthropogenic halogenated organic contaminants (HOCs). While anthropogenic HOCs are notorious for their toxicity, exposure to naturally produced HOCs, many of which exist in the marine environment, may also cause adverse effects. The purpose of this dissertation was to gain insight into possible impacts of HOCs on the health of sea turtle populations by assessing biotransformation enzymes, as well as the accumulation and metabolism of HOCs in several species of sea turtles- loggerhead (*Caretta caretta*), green (*Chelonia mydas*), olive ridley (*Lepidochelys olivacea*), and hawksbill (*Eretmochelys imbricata*).

The results showed that sea turtle livers possess the biotransformation enzymes cytochrome P450 (CYP) and glutathione *S*-transferase (GST). Western blots revealed CYP2- and CYP3-like proteins, but do not CYP1A-like proteins.

Spectrophotometric assays indicated that sea turtles showed similar GST kinetic parameters, but inter- and intra-species variation in activities towards GST class-specific substrates. PCBs accumulated in the livers of sea turtles, with levels ranging from 5-25 ng/g, as measured by gas chromatography with electron capture detection. *In vitro* incubations of sea turtle liver microsomes with 2,2',5,5'-tetrachlorinated biphenyl (PCB 52) indicated the formation a hydroxylated metabolite by liquid chromatography/mass spectroscopy, while incubations with 3,3'4,4'-tetrachlorinated biphenyl (PCB 77) did not reveal metabolites. Taken together, these results supported a model in which rates of hepatic biotransformation may determine elimination and relative concentrations of PCBs in reptilian tissues.

Hawksbill sea turtles feed primarily on marine sponges, which produce natural HOCs, such as 4,5-dibromopyrrole-2-carboxylic acid (DBPC), as deterrents against predation. The lack of detectable *in vitro* metabolism of DBPC by hawksbill sea turtles, as measured by liquid chromatography with radioactivity detection, indicated that biotransformation may not be the primary mechanism of tolerance to natural dietary. Kinetic analysis of spectrophotmetric assays indicated non-substrate binding of DBPC by GST, which suggested potential protection from sponge HOCs via GST transport or sequestration. The information in this dissertation provides critical knowledge to connect toxic effects of HOCs to sea turtle population decline.

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Chapter 1: Introduction

Halogenated organic compounds (HOCs) can be produced through natural or anthropogenic processes. Anthropogenic HOCs, such as dichlorodiphenyl-trichloroethane, chlordane, toxaphene, lindane, polychlorinated dibenzo-p-dioxins, dibenzo-p-dioxin-furans, polybrominated diphenyl ethers, and polychlorinated biphenyls, are some of the most hazardous environmental contaminants documented by environmental toxicologists. A multitude of toxic effects in a variety of organisms has been reported for these compounds, and has resulted in a ban of use or production of most of these compounds. However, the physiochemical properties that made them useful, such as their chemical stability, have led to their persistence and ubiquity in the environment. Thousands of naturally produced HOCs are also known to exist in the environment (Gribble 1996, 1998, 2002, 2003). Recently, the persistence of these natural HOCs has been recognized and the possibility of adverse effects from exposure to these compounds has been suggested (Vetter et al. 2004).

Fundamental biological processes, such as gas exchange and nutrient uptake, as well as contact with the environment provide mechanisms for exposure to HOCs and other xenobiotics. Biotransformation evolved as a defense mechanism to protect organisms from potential toxic insult of such xenobiotics. The process of biotransformation allows an organism to eliminate xenobiotics. However, biotransformation is not perfect - the efficiency of elimination varies greatly depending

on factors such as the xenobiotic and the organism, and the potential exists for the transformation of xenobiotics to more toxic metabolites during the process.

Reptiles are one of the most ancient classes of animals. Sea turtles have existed for about one hundred million years, outliving even dinosaurs. Throughout this time, evolutionary mechanisms of natural selection and genetic drift have driven the adaptation of sea turtles for their environment and sea turtles fill important ecological niches in marine ecosystems. Yet little is known of the evolutionary adaptations of sea turtle biotransformation pathways. Today, all species of sea turtles face the threat of extinction and little knowledge exists to indicate the potential hazards of HOCs on sea turtle populations.

Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) are a group of 209 organic compounds, called congeners, with between 1 and 10 chlorine atoms substituted on the biphenyl ring system (Figure 1.1; see Appendix A for a list of PCB congener nomenclature). PCBs are characterized by non-flammability, electrical insulating properties, and extreme chemical and thermal stability, which made them desirable for industry and manufacturing. For example, PCBs have been used as adhesives, hydraulic lubricants, plasticizers, flame retardants, dielectric fluid in transformers and capacitors, and in carbonless copy paper (Fiedler 2001). The synthesis of PCBs resulted in complex mixtures containing different percentages of chlorination suitable for various applications and thus not all 209 congeners were produced in equal amounts (De Voogt and Brinkman 1989). In total,

worldwide production of PCBs is estimated at 1.5 million tons (Ivanov and Sandell 1992; Rantanen 1992).

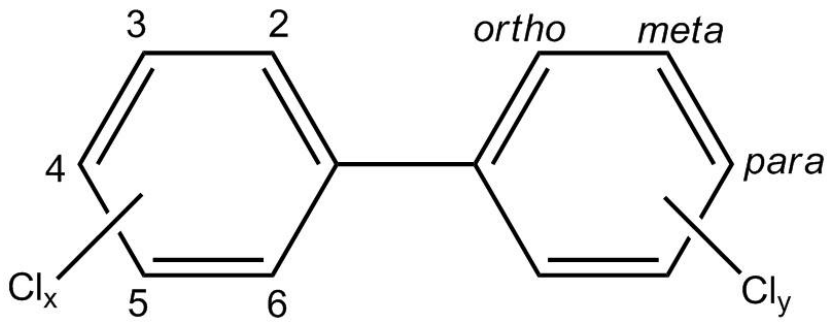

Figure 1.1. Structure of polychlorinated biphenyl. Numbering is indicated on the left ring, and positional nomenclature on the right. The number of chlorine atoms is represented by the following formula: $1 \leq x + y \leq 10$.

Various pathways have led to environmental contamination by PCBs including: accidental or intentional release into water, soil, or air; improper disposal or incineration; and leaching or volatilization from industrial and disposal sites (Erickson 2001). Once in the environment, the chemical properties which made PCBs useful are the same properties which cause problems. Because of their high thermodynamic stability, PCB molecules are difficult to degrade through physical, chemical, thermal, or metabolic processes. PCBs, which have been detected in the environment since 1966 (Jensen 1966), are therefore persistent, especially in soils and sediments (Risebrough et al. 1968). Additionally, the highly non-polar, lipophilic nature of PCBs results in accumulation and magnification through the food web (Hansen 1987; James 2001).

The first indications of toxicity related to PCBs emerged in 1936 when workers at Monsanto chemical manufacturing plants (the largest PCB producer in USA) presented with chloracne, fatigue, and symptoms of liver disease (Drinker et al. 1937). Since then, extensive research has documented diverse toxic effects of PCBs, which include adverse effects to the immune, endocrine, nervous, and reproductive systems, as well as probable carcinogenicity (Safe 1994). More recently, potential toxicological impacts have been demonstrated for stable metabolites of PCBs, namely hydroxylated (OH-) and methylsulfonated ($MeSO_2$-PCBs) PCBs; these impacts include thyroid and endocrine disruption (Letcher et al. 2000a). Because environmental PCBs always exist as mixtures of congeners, and usually as more complex mixtures with metabolites and other persistent environmental contaminants, it is difficult to assign discrete cause and effect relationships in ecological assessments. Thus, research on the effects of PCBs and their metabolites in wildlife continues and will be the focus of later discussion.

Biotransformation Enzymes

Organisms are continuously and inescapably exposed to foreign compounds, or xenobiotics, through their diet and environment. Xenobiotics vary dramatically in their chemical properties so the manner in which a compound is absorbed also varies; generally absorption is related to the lipophilicty of the compound. However, the elimination of a xenobiotic may be hindered because lipophilic compounds are readily stored in lipid and reabsorbed. Xenobiotic metabolism, or biotransformation evolved as a defense mechanism to protect organisms from toxic insult by transforming lipophilic

molecules into more polar compounds in order to increase hydrophilicity and subsequent elimination (Parkinson 2001). This highly conserved transformation process generally occurs in two distinct stages, phase I and phase II, although phase II is not always preceded by phase I. Phase I biotransformation pathways often involve increasing the polarity by catalyzing oxidation, reduction, or hydrolysis reactions (Parkinson 2001). Phase II biotransformation reactions, which include glucuronidation, sulfonation, methylation, acetylation, and conjugation with glutathione or amino acids, usually results in a large increase in hydrophilicity of the parent compound, thereby aiding its elimination. Thus, during biotransformation most non-polar compounds are converted to polar compounds. Organisms have also developed mechanisms to regulate biotransformation enzymes in response to exposure to a wide range of xenobiotics (Parkinson 2001). For example, exposure to coplanar HOCs causes induction of enzymes responsible for their biotransformation. Ultimately biotransformation tends to deter bioaccumulation of potentially toxic xenobiotics.

Many enzymes that contribute to xenobiotic biotransformation through a variety of pathways have been studied extensively. In multi-cellular organisms, these enzymes are distributed throughout the organism, but usually the highest concentrations occur in tissues more prone to exposure. In vertebrates, the liver generally has the highest concentration of biotransformation enzymes; within a liver cell, the cytoplasm and endoplasmic reticulum contain most enzymes but still others occur in additional sub-cellular compartments (Parkinson 2001).

Cytochrome P450

Of the phase I enzymes, the superfamily of cytochrome P450 (CYP) enzymes is of utmost importance because it has the greatest range of catalytic adaptability and substrate specificity (Nebert et al. 1987). Within vertebrates, CYP enzymes are present in most tissues, with the highest concentration in the endoplasmic reticulum of liver cells. In fact, CYP makes up 12-15% of the endoplasmic reticulum of a hepatocyte, indicating that up to 2% of the mass of an individual hepatocyte is CYP (Lewis 2001). Thus the amount of cellular resources dedicated to CYP suggests a critical role of this enzyme system.

Heme-containing CYP proteins were first characterized in the 1950's as a pigment present in rat (Klingenberg 1958) and pig (Garfinkel 1958) liver microsomes. The pigment, when treated with carbon monoxide, demonstrated maximum light absorption at 450 nm hence the term "cytochrome P450" was assigned to the pigment (Omura and Sato 1964) - *the term is now misleading because CYP is not an electron-transfer protein*. Originally scientists believed that CYP was a single unique cytochrome containing protein, but further research demonstrating differential induction patterns and unique catalytic activities led to the discovery of multiple CYP isoforms (Conney 2003). CYP proteins have since been found in many organisms ranging from bacteria to humans (Nebert and McKinnon 1994). According to D.R. Nelson's Cytochrome P450 Homepage (http://drnelson.uthsc.edu/cytochromeP450.html), as of August 2009, 11,294 *CYP* gene sequences (excluding variants) have been identified – 3,282 in animals, 4,266 in plants, 247 in protists, 2,570 in fungi, 905 in bacteria, 22 in archaea, and 2 in viruses.

Initially, CYP enzymes were named according to their catalytic activity or other characteristics, e.g., aromatase is a CYP enzyme that catalyzes the aromatization of androgens to estrogens (now known as CYP19). However, as the number of CYPs discovered grew and overlapping catalytic activities became apparent, a systematic method of nomenclature became necessary. First proposed by Nebert and colleagues in 1987, the enzymes were categorized by protein sequence similarity (Nebert et al. 1987; Nebert et al. 1989a; Nebert et al. 1989b; Nebert and Nelson 1991; Nebert et al. 1991; Nelson et al. 1993; Nelson et al. 1996). This nomenclature system abbreviates the protein cytochrome P450 as CYP, or *CYP* when referring to the gene. An alphanumeric descriptor follows CYP, indicating the family, subfamily, and individual isoform. For example, CYP1A1 indicates a cytochrome P450 of the "1" family, "A" subfamily, and is the first protein of this subfamily. Enzymes within a family must share >40% sequence homology, while members of a subfamily share >55% homology.

Research on CYP was fueled by interest in metabolism of steroids, drugs, and carcinogens in the early 20th century (Guengerich 2008). Early studies of difficult oxidations, such as ω-hydroxylation of lauric acid, allowed for the isolation of a cell-free extract capable of performing NAD(P)H-dependent oxidation reactions (Coon 2005). This reaction was later found to be an example of "mixed-function oxidase" chemistry (Hayaishi et al. 1955; Mason 1957). Mixed-function oxidation was also shown to occur with steroids (Axelrod 1955), drugs (Brodie et al. 1958), and carcinogens (Mueller and Miller 1953). More accurately, CYPs are mono-oxygenases, inserting one atom of molecular oxygen into the substrate while the other atom is reduced to water, yet

products are not restricted to alcohols or phenols due to rearrangement (Guengerich 1991). The basic stoichiometry of most CYP reactions is:

$$NAD(P)H + O_2 + RH + H^+ \rightarrow NAD(P)^+ + H_2O + ROH$$

CYP dependent biotransformation generally involves the oxidation of substrates, however reduction of substrates may happen, particularly under anaerobic conditions (Potter and Reed 1983). In addition to xenobiotic biotransformation, CYP proteins are responsible for the metabolism of various endogenous substrates including steroids, fatty acids, and prostaglandins (Guengerich 1977). Reactions catalyzed by CYP proteins are listed in Table 1.1.

Table 1.1. CYP mediated reactions (adapted from Parkinson 2001).

Aliphatic hydroxylation & epoxidation	Dehalogenation
Aromatic hydroxylation & epoxidation	Azoreduction
N-hydroxylation	Deamination
Heteroatom oxygenation	Desulfuration
Heteroatom dealkylation	Denitration
Oxidative group transfer	Amide hydrolysis
Cleavage of esters	Ester hydrolysis
Dehydrogenation	Peroxidation
Aldehyde oxidation	Halothane oxidation & reduction
Androgen aromatization	Cholesterol side-chain cleavage

While most CYP activities are detoxifying, some CYPs are infamous for producing more toxic compounds - a process termed bioactivation. Examples of CYP bioactivation reactions include the oxidative group transfer of parathion to the corresponding paraoxon, the dehydrogenation of acetaminophen, the epoxidation of chlorobenzene, or the multi-step formation of diolepoxides in the bay-regions of polyaromatic hydrocarbons (PAHs) (Parkinson 2001). However, the toxicity of these CYP-dependent reactive intermediates can be mitigated, at least in part, by the activities of phase II enzymes. For example, the phase II enzyme glutathione *S*-transferase (GST) catalyzes the addition of glutathione (GSH) to the toxic, CYP-produced 3,4-oxide intermediate of chlorobenzene (Parkinson 2001). Without phase II detoxification, reactive intermediates produced by CYPs may lead to cellular toxicity (Kadlubar et al. 1976).

A complete cytochrome P450 system consisting of CYP and NADPH-cytochrome P450 reductase (alternatively cytochrome b_5, ferroredoxin or flavoprotein can be used in other systems) in membrane-bound form is required for catalytic activity (Lu and Coon 1968; Lu et al. 1969). In eukaryotic microsomal CYPs, electrons for the reduction of molecular oxygen are supplied by NAD(P)H in two consecutive steps via the flavoprotein, NADPH-cytochrome P450 reductase (CPR) (Degtyarenko 1995). CPR contains two redox cofactors: flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). Thus, within the CYP system, electrons flow as follows:

$$\text{NADPH} \rightarrow \text{FAD} \rightarrow \text{FMN} \rightarrow \text{CYP} \rightarrow O_2$$

CYP uses the chemical potential of oxygen for organic reactions by controlling, via the heme group, the transfer of electrons to create reactive oxygen intermediates (Hayaishi et al. 1955). The heme is bound to a cysteine in the conserved C-terminal region of all CYP enzymes via a thiolate bond between the conserved amino acid and the fifth coordination site of heme iron (Mason et al. 1965; Nebert et al. 1987).

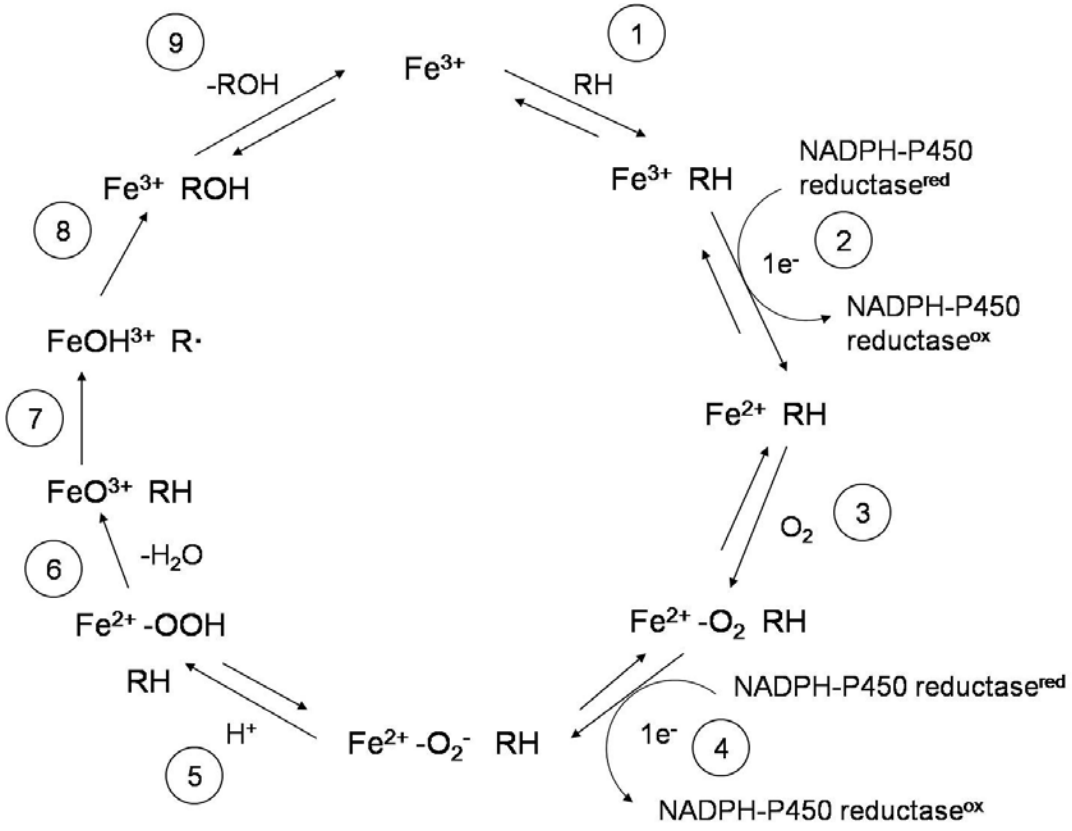

Figure 1.2. Generalized CYP catalytic cycle (adapted from Furge and Guengerich 2006).

The mechanism of catalysis by CYP consists of a cyclic reaction sequence (Figure 1.2). In step 1, the substrate binds to the heme Fe^{3+} of oxidized CYP. Then, one electron is transferred from CPR to CYP, reducing the heme to Fe^{2+} (step 2). Reduced CYP can now bind molecular oxygen, which is then activated by the transfer of the second electron from CPR (steps 3 and 4). The addition of protons (step 5) allows the heterolytic cleavage of the O-O bond to produce a water molecule (step 6). The FeO^{3+} complex then abstracts a hydrogen atom from the substrate to produce a substrate radical (step 7), which then accepts an oxygen or hydroxyl group (step 8). Oxidized substrate leaves CYP, which returns to its initial ferric state (step 9).

Table 1.2. Mechanisms of CYP regulation (adapted from Lewis 2001).

Level of Gene Regulation	CYPs regulated by specific mechanism
gene transcription	1A1, 1A2, 1B1, 2B1, 2B2, 2C7, 2C11, 2C12, 2D9, 2E1, 2H1, 2H2, 3A1, 3A1, 3A6, 4A1, 11A1, 1B1, 17A1, 21A1
mRNA processing	1A2
mRNA stabilization	1A1, 2B1, 2B2, 2C12, 2E1, 2H1, 2H2, 3A1, 3A2, 3A6, 11A1
Protein translation	2E1
Protein stabilization/modification	2E1, 3A1, 3A2, 3A6

Regulation of CYP can be affected by various factors including age, sex, reproductive status, ethnicity, species, and environmental conditions. Of particular interest is the regulation of CYP, both up-regulation (induction) and down-regulation, by both xenobiotics. Increased CYP detoxification activity after treatment with certain PAHs provided the first evidence for CYP induction (Conney et al. 1956). Further research has elucidated multiple mechanisms for controlling CYP expression (Table 1.2). Transcriptional regulation is especially important and various nuclear receptors appear to play an important role in expression of particular subfamilies (Table 1.3). The regulation of the predominant CYP families in xenobiotic biotransformation will be discussed further below.

According to D.R. Nelson's Cytochrome P450 Homepage (http://drnelson.uthsc.edu/cytochromeP450.html), as of August 2009, 120 CYP families have been identified in animals but only around 20 of those are found in vertebrates. With regard to xenobiotic biotransformation, CYP1, CYP2, and CYP3 families are considered particularly important in vertebrates and have been considerably studied in mammals; these enzymes will therefore be discussed further.

Table 1.3. Nuclear receptors associated with induction of various CYPs and other biotransformation enzymes (adapted from Nebert and Dalton 2006).

Nuclear receptor	Regulated Enzymes	Typical inducers
AhR	CYP1, CYP2A*, CYP2C*,CYP2S1, NQO1, ALDH3A1, GSTA1, UGT1A6, UCGT1A7	Dioxins, planar PAHs, planar PCBs, benzoflavones
CAR	CYP2B, FMO, ALDH, GST, SULT, UGT1A6	Phenobarbital, androstanes
FXR	CYP7A1, CYP8B1	Bile acids
HNF-1α	GSTA2, UGT1A1, UGT1A7, UGT2B17	Bile acids
HNF-3α,β,γ	CYP2C, CYP3A, CYP7A1	Lipopolysaccharide, epidermal growth factor
HNF-4α	CYP2D6	Long-chain fatty acyl-CoA thioesters
LXRα,β	CYP7A1	Oxysterols
PPARα	CYP4A1, CYP4A3	Fatty acids, fibrates
PPARγ	CYP4B1	Fatty acids, eicosanoids
PXR	CYP1, CYP2A, CYP2B, CYP2C, CYP3A, CYP4F, MAO, CAT, FMO, GST, UGT, SULTST2A	Rifampicin, lithochloic acid, pregnenolone 16α-carbonitrile
RARα,β,γ	CYP26A1	Retinoic acids
VDR	CYP24A1, CYP27B1	1α,25-dihydroxy-vit D_3

* only one or very few members regulated; AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; FXR, farnesoid X receptor; HNF, hepatocyte nuclear factors; LXR, liver X receptors; PPAR, peroxisome proliferators-activated receptors; PXR, pregnane X receptor; RAR, retinoic acid receptors; VDR, vitamin D_3 receptor

The CYP1 family, which, in mammals, includes CYP1A1, CYP1A2, and the relatively new member CYP1B1, can be induced by many planar PAHs, such as 3-methylcholanthrene (3MC) and their nitrogenous derivatives, as well as other planar environmental contaminants including HOCs (see Table 1.3) (Murray et al. 2001). The CYP1 family is particularly notorious for induction by substrates which are then bioactivated by the induced enzyme, for example benzo(a)pyrene. While 7-ethoxyresorufin-O-deethylase (EROD) activity is considered a classic reaction of CYP1A enzymes (Lewis 2001), all three CYP1 enzymes catalyze O-dealkylation of alkoxyresorufins, such as 7-ethoxyresorufin, although CYP1B1 tends to have lower activity (Murray et al. 2001; Parkinson 2001). In mammals, CYP1A1 basal expression is generally negligible while substantial constitutive expression of CYP1A2 and CYP1B1 have been found in many tissues (Nebert et al. 2004). Historically, CYP1A1 and CYP1A2 were distinguished by differences in catalytic activity towards substrates – CYP1A1 usually catalyzes hydroxylation of hydrophobic HOCs and PAHs, e.g., benzo(a)pyrene, while CYP1A2 generally catalyzes oxidation of more hydrophilic heterocyclic amines and amides, e.g., 2-aminonaphthalene (Kawajiri and Hayashi 1996; Lewis 2001). Relatively little information is available on the more recently discovered CYP1B1 (Sutter et al. 1994). While 4-hydroxylation of estradiol is characteristic of this enzyme (Hayes et al. 1996), CYP1B1 has also been implicated in the activation of hydrophobic HOCs and PAHs (Guengerich 2000; Nebert et al. 2004; Nebert and Dalton 2006). Thus, both CYP1A1 and CYP1B1 could be implicated in the metabolism of coplanar PCBs.

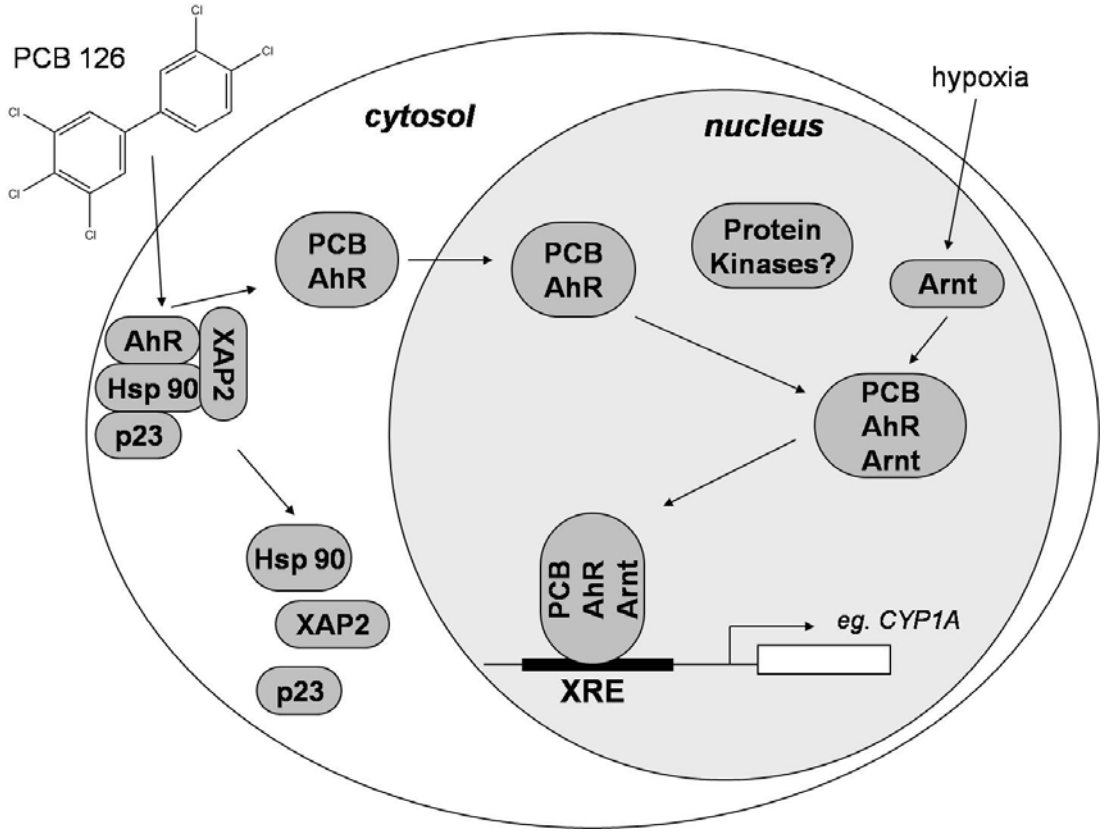

Figure 1.3. Signaling pathway of AhR (adapted from Mandal 2005).

The aryl hydrocarbon receptor (AhR), a ligand-activated basic-helix-loop-helix transcription factor (Hahn 2002), is involved in the induction of all three *CYP1* genes (Denison and Nagy 2003). AhR bind numerous planar aromatic compounds, with the highest affinity for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Poland et al. 1976; Poland et al. 1985). Homologs of this receptor have been found in many vertebrates and its overall function is conserved throughout (Hahn 2002; Hahn et al. 2006).

AhR mediates a complex signaling pathway involving many genes, most of which are normally regulated in species-, cell-, and developmental stage-specific manner. The

signaling pathway is depicted in Figure 1.3. In the absence of ligand, AhR exists as a cytosolic complex with HSP90, co-chaperone p23, and immunophilin-like protein XAP2 (Meyer et al. 1998; Kazlauskas et al. 1999; Meyer and Perdew 1999). The binding of a ligand, such as dioxin, triggers translocation to the nucleus where the liganded AhR replaces its partner molecules with AhR nuclear translocator (ARNT), forming a DNA-binding protein complex that activates gene transcription (Whitlock 1999). The AhR/ARNT-ligand complex binds xenobiotic responsive elements (XREs) upstream of genes coding for phase I and II biotransformation enzymes, including *CYP1* genes, as well as genes involved in cell proliferation, cell cycle regulation, and apoptosis (Mimura and Fujii-Kuriyama 2003).

The CYP2 family is the one of the largest and most diverse families of animal CYPs, consisting of many subfamilies (Nelson et al. 1996). The enzymes of the CYP2 family catalyze greater than 50% of all phase I drug oxidations in humans (Rendic and DiCarlo 1997). While the substrate specificities vary (and overlap) within CYP2, as compared to CYP1 and CYP3, CYP2 substrates tend to have smaller molecular weights (Lewis 1998). Stereotypical substrates and inhibitors of major CYP2 enzymes are shown in Table 1.4. Of particular interest is the ability of CYP2B enzymes to oxidize non-planar PCBs, which can also induce this enzyme in mammals (McFarland and Clarke 1989). Although several CYP2 and CYP3A isoforms are also induced, the CYP2B subfamily is the main hepatic CYP isoform induced by phenobarbital (PB) and other barbiturates in mammals (Lewis 2001). Preferred substrates of CYP2B include barbiturates, aliphatic amines, and non-planar, V-shaped hydrocarbons (David F. V.

Lewis et al. 1999; D. F. V. Lewis et al. 1999). 7-Pentoxyresorufin-O-depentylase activity is characteristic of PB-induced hepatic microsomes (Lewis 2001).

Table 1.4. Major CYP2 subfamilies and their typical substrates (adapted from Lewis 1998)

CYP	Substrate	Inhibitor
2A	coumarin	pilocarpine
2B	phenobarbital	orphenadrine
2C	tolbutamide	sulphaphenazole
2D	debrisoquine	quinidine
2E	*p*-nitrophenol	disulphiram

As shown in Table 1.3, there are several transcription factors associated with regulation of CYP2 enzymes including constitutive androstane receptor (CAR) and pregnane X receptor (PXR) (Nebert and Dalton 2006). PB-induced expression of CYP2B is mediated through the nuclear receptor CAR (Honkakoski et al. 1998). However, unlike TCDD and AhR, CAR shows constitutive basal activity and ligands, such as PB, do not appear to bind directly to CAR (Moore et al. 2000). Phosphorylation pathways and other indirect mechanisms are thought to be involved in xenobiotic induction of CAR by promoting its translocation to the nucleus (Honkakoski et al. 1998; Kawamoto et al. 1999; Sueyoshi et al. 1999; Tirona and Kim 2005). Upon translocation, CAR dimerizes with retinoid X receptor (RXR), which binds 9-*cis*-retinoic acid (Figure

1.4) (Honkakoski et al. 1998). The heterodimer can then bind phenobarbital responsive enhancer units (PBRU) initiating transcription of *CYP2B* (Waxman and Azaroff 1992; Tirona and Kim 2005).

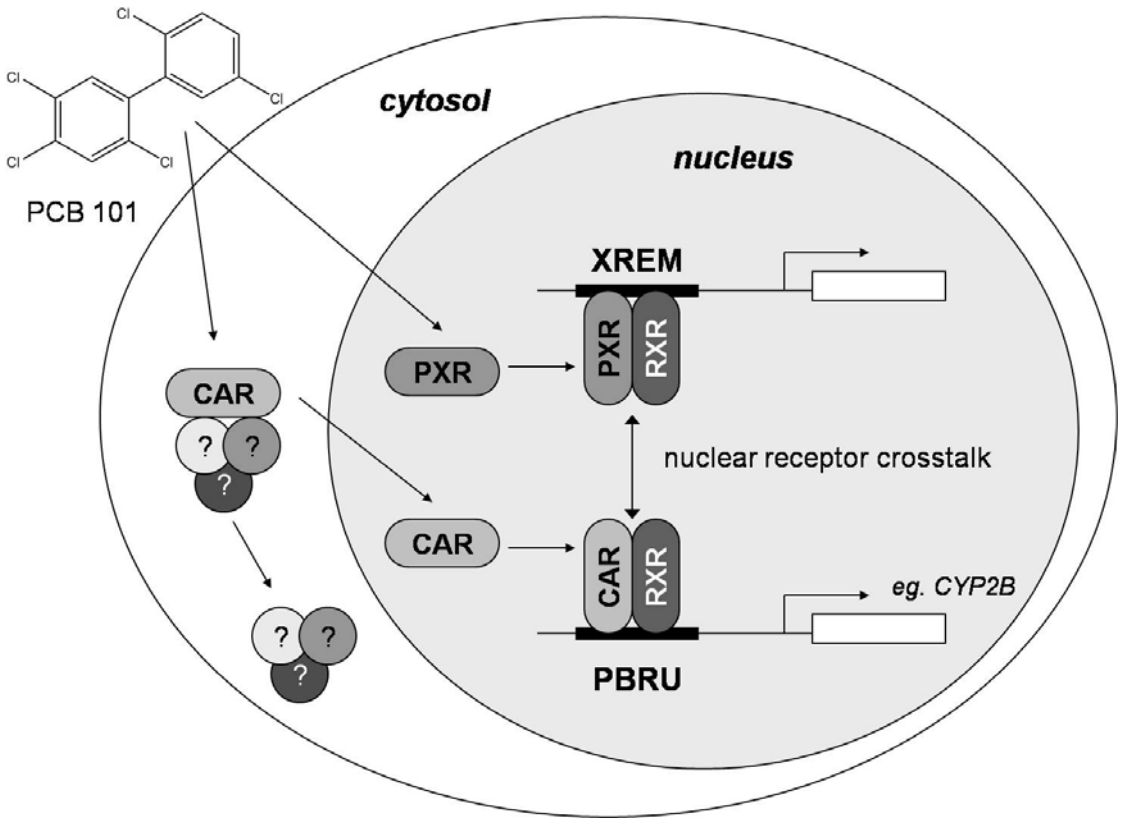

Figure 1.4. Activation of CAR and PXR responsive genes (adapted from Handschin and Meyer 2003).

The enzymes of the CYP3A subfamily are the main contributors to the CYP3 family, although several other subfamilies have been identified in non-mammalian species. Of the CYP3A subfamily, CYP3A4, CYP3A5, and CYP3A7 are the most abundant human hepatic isoforms (Lewis 2001; Parkinson 2001). CYP3A4 appears to be

expressed, with some variability, in all human livers while CYP3A5 and CYP3A7 are highly expressed in fetal livers (Wrighton and Stevens 1992; Parkinson 2001). CYP3A4 alone is responsible for the metabolism of 34% of known drugs in humans (Lewis 2003). Because of its role in the biotransformation of many drugs (Table 1.5), CYP3A4 has serious potential consequences during drug therapy. For example, grapefruit juice is prohibited for many drug therapy patients because of its inhibition of CYP3A4 by flavones and related compounds in the juice (Parkinson 2001).

Table 1.5. A selection of CYP3A4 substrates (adapted from Rendic and DiCarlo 1997; Lewis 2003)

Warfarin (R)	Midazolam	Erythromycin
Cortisol	Testosterone	6-Aminopyrene
Progesterone	Nifedipine	Dexamethasone
Quinidine	Aflatoxin B1	Ethynylestradiol
Budesonide	Phenactin	7-Benzyloxyquinoline
Verapamil	Rifampicin	Nevirapine
7-Benzyloxy-4-trifluoromethylcoumarin		

Many of the clinically significant drug-drug interactions involving the CYP3A subfamily have been linked to induction via PXR (Table 1.3) (Bertilsson et al. 1998; Blumberg et al. 1998b; Kliewer et al. 1998; Lehmann et al. 1998). The nuclear receptor PXR has been cloned from a range of animals including mammals, fish, and birds

(Bertilsson et al. 1998; Blumberg et al. 1998a; Lehmann et al. 1998; Zhang et al. 1999; Handschin et al. 2000; Jones et al. 2000; Savas et al. 2000; Moore et al. 2002). Once activated by ligand, PXR, like CAR, translocates to the nucleus where it heterodimerizes with RXR (Figure 1.4) (Willson and Kliewer 2002). The heterodimer can then bind to xenobiotic responsive enhancer module (XREM) and initiate the transcription of many genes, including *CYP3A* (Handschin and Meyer 2003). Activation of PXR has been demonstrated by a wide range of chemicals with no obvious similarities except for their induction of CYP3A, and even classic inducers of CYP3A via PXR vary by species – rifampicin induces CYP3 in rabbit and human (but not rat and mouse) whereas pregnenolone 16α-carbonitrile induces in rat and mouse (but not rabbit and human) (Xu et al. 2005).

It is important to note that induction of xenobiotic enzymes is a complex process. As shown in Table 1.3, numerous transcription factors have been implicated in the regulation of biotransformation enzymes, and overlapping control of some enzymes have been elucidated. While the interconnectedness of CAR and PXR is evident from the fact that both receptors heterodimerize with RXR, multiple factors obfuscate a clear understanding of biotransformation regulation by transcription factors. Many CAR ligands, such as PB, are also PXR ligands (Willson and Kliewer 2002). CAR- and PXR-mediated induction of various biotransformation enzymes can be modulated by the glucocorticoid receptor signaling, which can also induce the expression of *CAR* and *PXR* (Tirona and Kim 2005). Other nuclear receptors including HNF-4α participate in CAR and PXR induction by acting on distal elements (Tirona and Kim 2005). *In vitro* studies

have shown that CAR and PXR can bind to the same regulatory sequences in *CYP2B* and *CYP3A* promoters, but CAR activates the *CYP2B* promoter, and PXR activates the *CYP3A* promoter (Xie et al. 2000; Goodwin et al. 2001; Smirlis et al. 2001). Furthermore, PXR regulates the expression of not only itself, but also CAR and AhR (Maglich et al. 2002). Thus, there is significant evidence of crosstalk between xenobiotic responsive transcription factors, although the biochemical and toxicological impacts have yet to be elucidated.

Glutathione *S*-Transferase

Of the phase II enzymes, glutathione *S*-transferases (GST) play a critical role in the detoxification of the electrophilic products of phase I biotranformation, as well endogenous electrophiles. GSTs are present in most tissues at levels ranging from 5-100 μM (Hayes and Pulford 1995). While nuclear, microsomal and mitochondrial GSTs have been identified, cytosolic GSTs are most prominent. In fact, GSTs can account for up to 10% of cytosolic proteins (Boyer 1989; Will 1999). In vertebrates, GSTs are distributed throughout most tissues - especially lungs, heart, intestines, and liver – and are expressed in a tissue-specific manner (Vos and Van Bladeren 1990; Awasthi et al. 1994).

In 1961, the first evidence for an enzyme in rat liver catalyzing conjugation reactions with glutathione (GSH) was reported (Booth et al. 1961a). Several years later, GST was found to be the same protein as ligandin (W. H. Habig et al. 1974), which had been isolated (and named) on the basis of its ability to bind a variety of hydrophobic compounds. Because early reports of GSH conjugation demonstrated proteins which

eluted differentially on chromatography columns and demonstrated different catalytic abilities, it was evident that multiple mammalian isoforms of GST existed (Listowsky and Arias 2007). The endogenous function of GST, inactivation of secondary metabolites during oxidative stress, predicates its ubiquity in oxygen-consuming organisms. GST proteins have been found in a wide range of eukaryotes, as well as certain prokaryotes, such as cyanobacteria, which use GSH as their major intracellular thiol (Vuilleumier and Pagni 2002). Multiple GST isoforms occur in any given organism; in humans, 24 GSTs are known (Hayes et al. 2005). Genome analysis in human tissues estimates the occurrence of 40 forms making up 0.2% of the genome (Holt et al. 2002). Across many taxa, including insects, worms, yeast, fish, and mammals, approximately 0.2% of all proteins coded by their genome are GSTs (Holt et al. 2002). Today, 4 major groups of enzymes with GST activity have been identified: bacterial fosfomycin-resistance proteins (fosA), membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG), mitochondrial kappa-class GSTs, and canonical (cytosolic) GSTs (Figure 1.5). Of these four groups, the cytosolic GST enzymes are the most numerous, most extensively studied, and the main contributor to biotransformation by GSTs (Zimniak and Singh 2007). Therefore, canonical GSTs will be discussed in further detail.

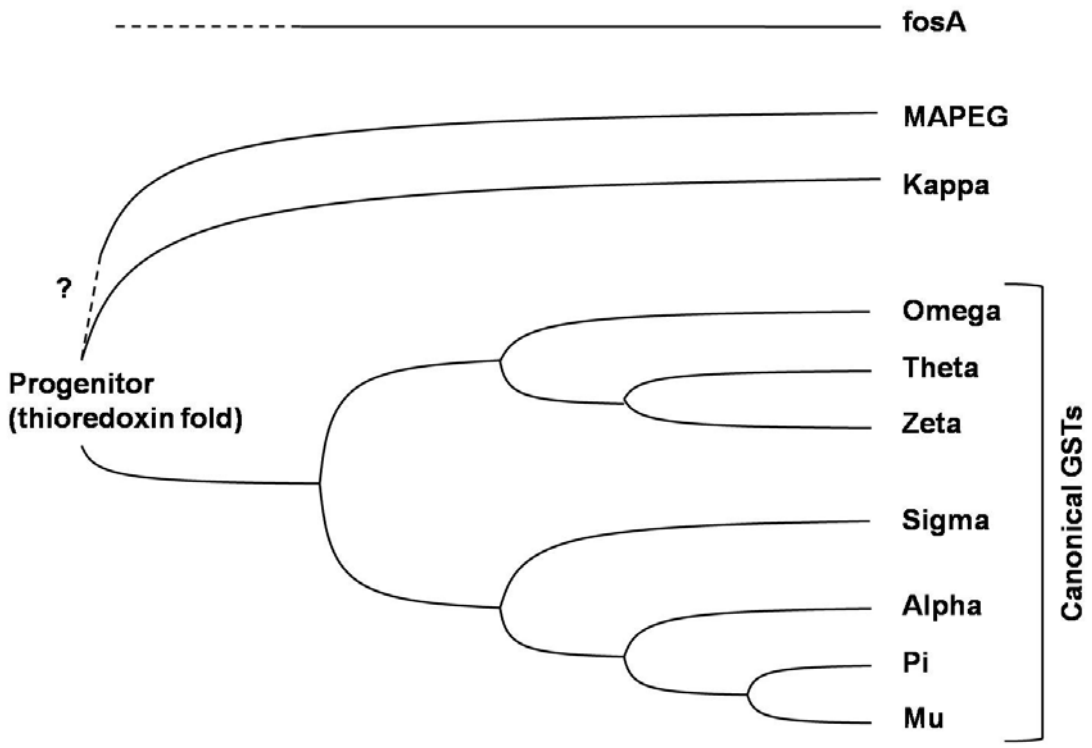

Figure 1.5. Phylogenetic tree of major GST classes with arbitrary branch lengths (adapted from Zimniak and Singh 2007).

As with CYPs, GSTs were initially characterized on the basis of catalytic activity (Boyland and Chassea 1969), e.g., glutathione aryltransferase (Grover and Sims 1964). But in 1973, several GSTs were purified from rat liver which exhibited overlapping substrate specificities (Pabst et al. 1973), indicating the need for a consensus systematic nomenclature. First proposed by Mannervik and colleagues in 1992, a naming system was established for human GSTs which followed conventions for naming other biotransformation enzymes by protein sequence homology (Mannervik et al. 1992). The nomenclature was later adopted for other vertebrate species (Zimniak and Singh 2007), invertebrates (Chelvanayagam et al. 2001), and plants (Edwards et al. 2000). The naming

system abbreviates the protein glutathione *S*-transferase as GST, or *GST* when referring to the gene (and capitalization follows the rules established for the species). Although there is not a rigid threshold, GSTs generally share 40% or greater homology within a class (and between classes, less than 25% sequence identity). The mammalian cytosolic classes include alpha, mu, pi, sigma, theta, zeta, and omega. Additional classes of GSTs, including beta, delta, epsilon, lambda, phi, rho, and tau have been identified in nonmammalian organisms, such as fungi, plants, insects, fish (Blanchette et al. 2007). GST classes are abbreviated using the Roman letter, e.g., alpha-class GST is denoted GSTA. Following the class designation is an Arabic numeral, which is assigned according to the chronological order of reported sequences within a species, e.g., GSTA1 is the first alpha-class GST reported from a given species, GSTA2 is the second, and so on. If sequences are reported in an organism for which no GSTs have been reported, the numbering reflects the chromosomal position of the gene. Canonical GSTs are dimeric proteins; both homodimers and heterodimers can exist within a class, but dimers composed of subunits from different classes are rare or nonexistent *in vivo* (Zimniak and Singh 2007). Thus, dimeric proteins are denoted by two numerals representing the two subunits, e.g., GSTA1-1 or GSTA1-2. Lastly, a prefix is usually appended to the name to indicate species – one letter for common species, e.g., hGSTA1 for human GSTA1, and two letters derived from the taxonomic name for other species, e.g., DrGSTA1 for zebrafish (*Danio rerio*) GSTA1.

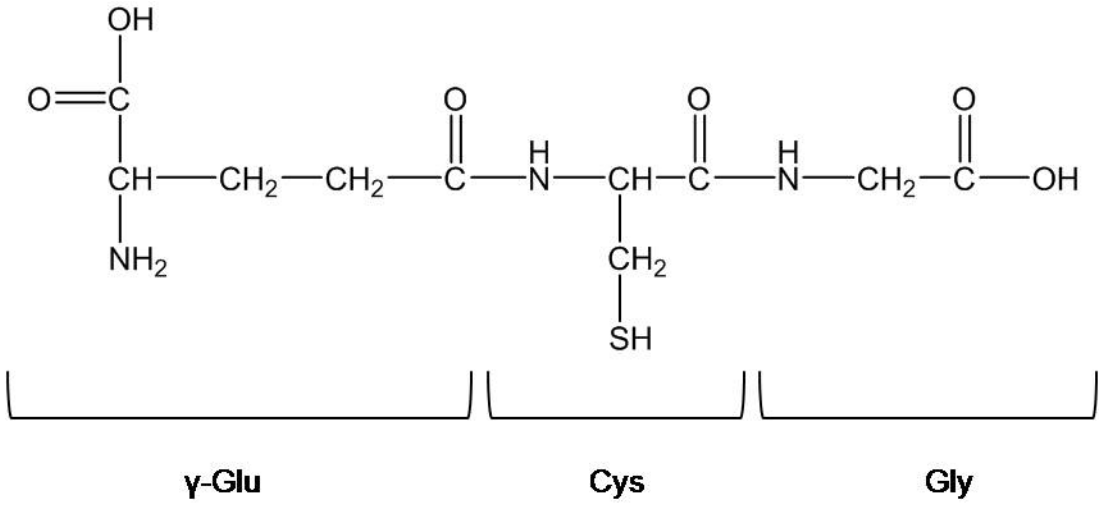

Figure 1.6. Structure of reduced glutathione (GSH).

Since its discovery in the late nineteenth century (De-Rey-Pailhade 1888b, a), the tripeptide GSH (Figure 1.6) has been the focus of much research in the fields of biochemistry, cell biology, and toxicology, with particular focus in its role as antioxidant. GSH has a peptide sequence of γ-glutamine-cysteine-glycine. It is synthesized by two ATP-dependent reactions catalyzed by γ-glutamylcysteine synthetase and glutathione synthetase (Anderson 1998). GSH has 2 unique properties: the γ-glutamyl amide bond which protects it from the hydrolytic degradation by cellular proteases, and the high reduction potential of the thiol group of cysteine (Meister 1983). Within the cell, GSH is present at concentrations of 0.5 to 10 mM and levels are tightly regulated in order to provide essential protection from oxidative stress (Will 1999). Numerous cellular reactions use GSH as a reductant, and in many cases GSH is converted to glutathione disulfide (GSSG), the disulfide formed from two oxidized GSH molecules. Glutathione reductase, a NADPH dependent enzyme, is responsible for the reduction of GSSG to

GSH and therefore is essential to maintenance of intracellular GSH levels (Anderson 1998). While GSH is involved in several cellular functions, its role in xenobiotic biotranformation will be discussed further.

Although the formation of mercapturic acid as a mechanism for excretion of xenobiotics has also been known since the late nineteenth century (Baumann and Preusse 1879), it was not until almost a century later that the enzymatic reaction responsible for the first step in the mercapturic acid pathway was found to be the conjugation of xenobiotics with GSH (Booth et al. 1961b; Boyland et al. 1961; Combes and Stakelum 1961; William H. Habig et al. 1974). The full mercapturic acid pathway is depicted in Figure 1.7. The reaction catalyzed by GST involves the nucleophilic attack by reduced glutathione on a diverse group of hydrophobic compounds which contain an electrophilic carbon, nitrogen, or sulfur atom. The basic stoichiometry of GSH conjugation, which can occur to some degree nonenzymatically, is:

$$GSH + RX \rightarrow R\text{-}SG + XH$$

Most GST catalyzed reactions involve GSH conjugation, but other reactions catalyzed by GST have been identified (Table 1.6). In addition to xenobiotic biotransformation, GST metabolizes a small number of endogenous compounds, including the isomerization of Δ5-3-ketosteroids to Δ4-3-ketosteroids (Benson et al. 1977), maleylacetoacetate to fumarylacetoacetate (Fernandez-Canon and Penalva 1998), 13-*cis*-retionic acid to all-*trans*-retinoic acid (Chen and Juchau 1997, 1998), and prostaglandin H_2 to prostaglandin D_2, E_2, or F_2 (Ujihara et al. 1988).

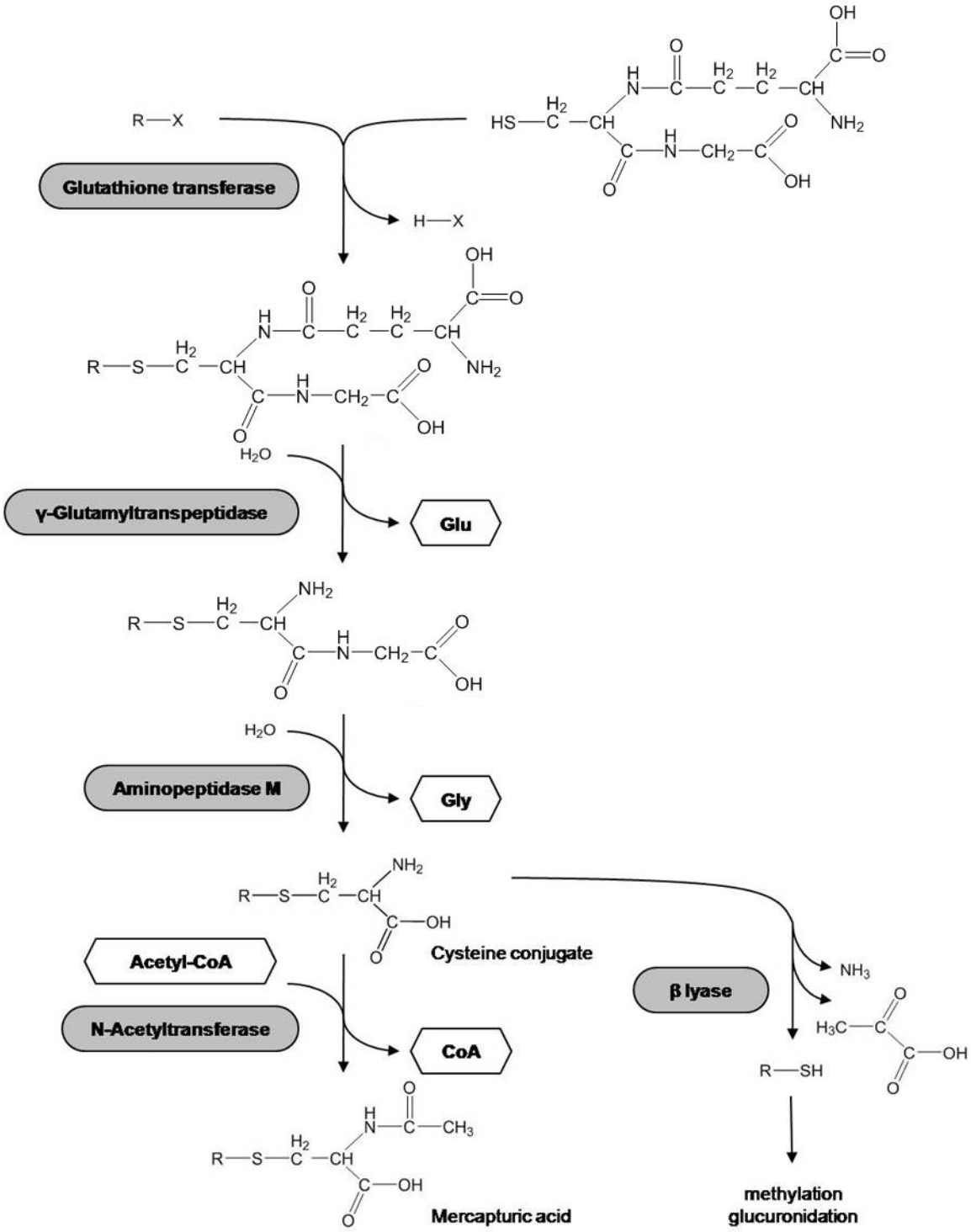

Figure 1.7. The mercapturic acid pathway (adapted from Parkinson 2001).

Table 1.6. GST mediated reactions (adapted from Parkinson 2001; Zimniak 2007).

Heteroatom displacement	Quinone addition
Michael addition	Quinoneimine addition
Arene oxide addition	Organic hydroperoxide reduction
Alkene epoxide addition	Reductive dehalogenation
Isomerization	Heteroatom reduction
Disulfide reduction	Sulfine reduction

Conjugation of GSH with a xenobiotic almost always leads to detoxification. In fact, GST plays a critical role in the detoxification of many reactive intermediates, such as arene oxides, produced during phase I metabolism. For example, GST is a major contributor to the cellular defense mechanism against DNA damage caused by diol epoxides of PAHs (Xiao and Singh 2007). Furthermore, the conjugation of GSH to a hydrophobic xenobiotic aids in its excretion and elimination via the mercapturic acid pathway (see Figure 1.7). The next step in the formation of mercapturates is catalyzed by γ-glutamyl transpeptidase (GGT), which is located in canalicular membranes, luminal membrane of bile ducts, small intestine epithelium, and kidneys (Parkinson 2001). Thus, GSH conjugates (GS-Rs) must be extruded from hepatocytes. Extrahepatically formed GS-R metabolites are thought to be transported through the blood to the liver by carriers known to transport amino acids and small peptides (McGivan and Pastor-Anglada 1994) and are then taken up by hepatocytes. The hepatic cellular efflux of GS-Rs is mediated by multiple transport mechanisms (Muller et al. 1994; Ballatori and Truong 1995),

including several members of the multidrug resistance protein family of ATP-dependent pumps (Cole and Deeley 2006). Once a GS-R enters the bile, GGT catalyzes the removal of glutamic acid and aminopeptidase M catalyzes the removal of glycine to form a cysteine conjugate (Parkinson 2001). The cysteine conjugate can then be reabsorbed into the liver where N-acetyltransferase can catalyze the formation of mercapturic acid, or it passes into the intestine where β-lyase can catalyze the formation of a thiol conjugate (Parkinson 2001). Depending on the molecular weight and physiochemical properties, mercapturate elimination is urinary or biliary (Will 1999).

However, GSTs do participate in the bioactivation of some xenobiotics. Mechanisms of GSH dependent bioactivation include: (1) formation of unstable GS-Rs which rapidly degrade to reactive intermediates, e.g., GSH conjugation to vicinal dihalogenoalkanes leads to the formation of reactive episulfonium groups; (2) formation of reversibly bound electrophile which allows transport of the GS-R and subsequent release of the reactive electrophile, resulting in toxicity by the reactive electrophile and/or intracellular depletion of GSH, e.g., isothiocyanates; and (3) formation of GS-Rs that are bioactivated via further metabolism of GSH or the xenobiotic, e.g., hydroquinone GS conjugates are metabolized via the mercapturic acid pathway to cysteine conjugates which can then undergo redox cycling (Commandeur et al. 1995). These mechanisms of toxicity are highly dependent upon the location in which they occur, the transport to target tissues, and the activities of competing phase II biotransformation enzymes. Moreover, they should be considered the exceptions to the rule, "GSH conjugation is detoxification."

The basic reaction mechanism of canonical GSTs involves two main processes: binding and activation of GSH, and binding and conjugation of the electrophilic substrate. Correspondingly, each subunit of GST has two binding pockets – the N-terminal domain contains the GSH binding site (G-site) while the C-terminal domain contains the hydrophobic electrophile binding site (H-site). Even before any GST structure was resolved, it was suggested that GST evolution involved the fusion of a GSH binding domain with other structural domains which would allow the promiscuity of electrophilic substrates conjugated to GSH (Mannervik 1985). Later, it was determined that the C-terminal H-site domains of the various GSTs classes evolved to bind sets of electrophilic substrates with varying structural and chemical features (Zimniak 2007). Accordingly, C-terminal domains are highly variable between classes, and considerably less variable between individual enzymes within a class (Mannervik et al. 1985; Sinning et al. 1993).

As shown in Figure 1.5, canonical (and kappa-class) GSTs share a common ancestor containing a thioredoxin-like fold (Martin 1995; Ladner et al. 2004). The structural characteristics of the thioredoxin α/β-fold module are present in the N-terminal domain of GSTs and produce the high specificity for GSH binding and activation (Ivarsson and Mannervik 2007). Several amino acids lining the G-site serve to bind the charged tripeptide and orient the molecule for nucleophilic attack. The attack is facilitated by the ionization of the GSH to its thiolate form, taking advantage of a higher reactivity between electrophiles with a thiolate anion than with a sulfhydryl group. The pK_a of the sulfhydryl group in aqueous solution is approximately 9, but when GSH is

bound to GST the pK_a drops to below 7 (Chen et al. 1988). Therefore, the enzyme bound form of GSH is predominately deprotonated at physiological pH and activated for nucleophilic attack on the electrophilic substrate (Graminski et al. 1989). A hydrogen bond between the proton of a hydroxyl group in the enzyme and the sulfur atom of GSH stabilizes the thiolate within the G-site (Parsons and Armstrong 1996; Thorson et al. 1998). In most GSTs, the proton donor is the hydroxyl group of a tyrosine near the N-terminus, but in theta-class and non-vertebrate GSTs the hydroxyl group of serine serves this purpose (Philip et al. 1995). Additionally, the ε nitrogen atom of Arg-15 in alpha-class GSTs interacts with the thiolate to stabilize the deprotonated form of GSH (Bjornestedt et al. 1995). The stabilization of the thiolate form of GSH in the active site of an alpha-class GST is illustrated in Figure 1.8.

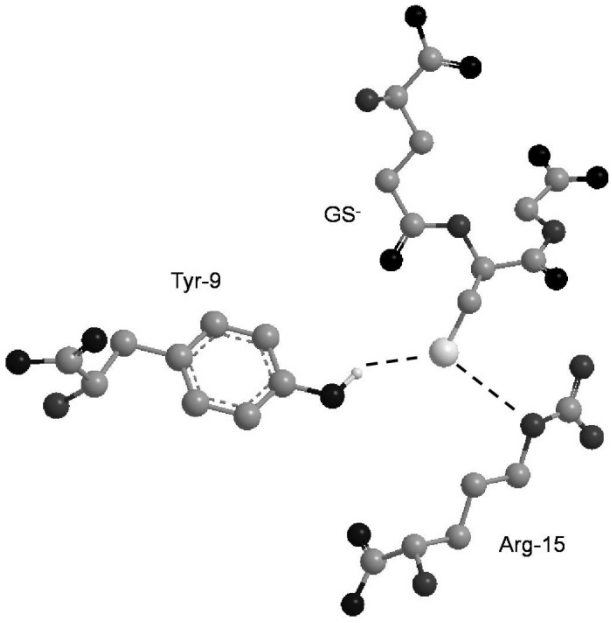

Figure 1.8. Activation of GSH via stabilization of the thiolate anion (adapted from Zimniak 2007).

Regulation of GST can be affected by various factors including age, sex, tissue, ethnicity, strain, species, oxidative stress, and environmental conditions. The regulation of GST provides a mechanism of adaptive response to cellular stress from electrophiles and reactive oxygen species (Hayes and Pulford 1995). Over 100 chemicals have been identified that induce GST genes (Hayes and Pulford 1995). Both GST substrates (Talalay et al. 1988) and GS conjugates (Borroz et al. 1994) are involved in the induction of GST - the former are thought to participate in xenobiotic response regulation while the latter are thought to affect regulation of enzymes that are involved in GSH homeostasis. As with CYPs, various nuclear receptors appear to play an important role in expression of particular subfamilies (Table 1.3). Particularly interesting is the determination of two mechanisms of regulation of GSTs: these enzymes can be regulated by transcription factors which are directly activated by xenobiotics, or regulation can occur through signaling cascades which are activated by cellular stress (Xu et al. 2005). The regulation of GSTs, particularly canonical GSTs will be discussed further below.

While many classes of GSTs have been identified, the cytosolic canonical GSTs have been the most extensively studied, especially with regard to their role in the biotransformation of xenobiotics, and will be the focus of further discussion. Because of structural and chemical variability within the H-site of GSTs, classes can be distinguished through biochemical assays using various substrates. A selection of these substrates is shown in Table 1.7. Alpha-, mu-, and pi-class GSTs are the major mammalian classes and are considered the most highly evolved because of their structural and catalytic adaptation (Caccuri et al. 2001a; Caccuri et al. 2001b). Theta-class GSTs, on the other

hand, are considered to be the closest to ancestral GST (see Figure 1.5) and differ from other classes in that they do not accept 1-chloro-2,4-dinitrobenze (CDNB) as a substrate. In non-mammalian species, theta-class GSTs and other classes of GSTs more closely related to theta- than to alpha-, mu-, and pi-classes have been identified (Zimniak and Singh 2007). Thus, alpha-, mu-, pi-, and theta-class GSTs will be the focus of discussion.

Table 1.7. GST substrate specificity in rat liver (adapted from Hayes and Pulford 1995).

	relative activity of rat GST class (isoenzymes)			
Substrate	alpha (A1 & A2)	mu (M1 & M2)	pi (P1)	theta (T1)
CDNB	++	+++	+	-
ADI	+++	+	?	?
ECA	+	+	+++	?
NBC	+/?	++	?	+++

+ indicates activity towards the substrate, with ++ representing higher relative activity than +; - indicates that activity was below limits of detection; ? signifies that substrate has not been tested with the class/isoenzyme; CDNB, 1-chloro-2,4-dinitrobenzene; ADI, Δ^5-androstene-e,17-dione; ECA, ethacrynic acid; NBC, 4-nitrobenzylchloride

The H-site of theta class GSTs have a rigid structure while those of the more recently evolved alpha-, mu-, pi-classes are flexible in ways which optimize substrate binding and catalytic activity (Caccuri et al. 2001a; Caccuri et al. 2001b). Both alpha- and mu-class GSTs contain relatively hydrophobic substrate binding pockets, providing for overlapping substrate specificities, while the pi-class H site is partly hydrophilic and

partly hydrophobic, indicating a higher affinity for substrates containing polar and nonpolar bonds (Ji et al. 1997). All three classes have been shown to have activity towards diol epoxides of PAHs but vary in their activity and stereoselectivity. For example, rGSTA5 detoxifies aflatoxin B1-8,9-*exo*-epoxide while rGSTM2 detoxifies the *endo*-epoxide.

The alpha-class of GSTs, which, in rats, includes GSTA1, GSTA2, GSTA3, GSTA4, GSTA5, and possibly several other genes, are the major renal and hepatic isoforms (Hayes and Pulford 1995). GSTAs participate in the isomerization of Δ^5-androstene-e,17-dione (Johansson and Mannervik 2001) and conjugation of 4-hydroxynon-2-enal (Zimniak 2007). Six mu-class GSTs, GSTM1-6, have been identified in rats. GSTMs contribute to the synthesis of prostaglandin E_2 from prostaglandin H_2. In rats and humans, only one pi-class GST, GSTP1, has been identified. GSTP1 is considered a major extrahepatic GST. The over expression of GSTP1 in tumor cells has been implicated in tumor drug resistance and makes it a focus of anticancer drugs (Findlay et al. 2007). GSTP1 is associated with the detoxification of products of oxidative damage to nucleic acids while GSTAs and GSTMs are associated with that of lipid peroxidation (Berhane 1994). Theta-class GSTs, including GSTT1-3 in rats, are responsible for the bioactivation of dihaloalkanes (Sherratt et al. 1998), but they also serve to detoxify several carcinogenic sulfate esters of methyl PAHs produced during biotransformation (Hiratsuka et al. 1990).

Generally, in rats treated with xenobiotics, induction of GSTA2 and M1 is the highest, with less dramatic increases in GSTA1 and GSTA3, and only a modest, if any

change in GSTA4 and GSTM2 (Hayes and Pulford 1995). Early studies with β-napthoflavone, an AhR ligand, showed expression of GSTAs in rat and mouse following exposure (Friling et al. 1990; Rushmore et al. 1990). Furthermore, the induction of GSTA1, GSTA2, and GSTM1 by 3-methylcholanthrene was shown to mediated through AhR and XRE (Rushmore and Kong 2002). But in other induction studies, the pathway of regulation was not as evident. For example, treatment with PB, the classic activator of CAR, showed increased expression of GSTA1, GSTA2, GSTA3, and GSTM2 but no PBRU has been found upstream of the coding region (Honkakoski and Negishi 1997; Waxman 1999; Rushmore and Kong 2002). Thus, both non-planar (PB-like) and planar PCBs (dioxin-like) show potential for induction of GSTs. Yet, as mentioned earlier, the regulation of GSTs is complicated by two levels of induction – the first involves the activation of transcription factors by substrates, while the second mechanism involves signaling cascades related to cellular stress.

Several transcription factors are have been implicated in the direct induction of GSTs, for example AhR induces GSTA1, GSTA2, and GSTM (Schrenk 1998; Rushmore and Kong 2002) and PXR induces GSTA1, GSTA2, GSTA4, GSTM1, and GSTM2 (Falkner et al. 2001; Maglich et al. 2002; Rosenfeld et al. 2003). The overlapping induction patterns suggested that the mechanism(s) for induction of phase II enzymes was not clearly understood. The discovery of the antioxidant regulatory element (ARE, also called electrophile response element) upstream of GST genes and other biotransformation genes provided key findings for stress induced regulation of phase II enzymes (Daniel et al. 1988; Daniel et al. 1989; Friling et al. 1990; Paulson et al. 1990; Rushmore and

Pickett 1990; Rushmore et al. 1991). Basic leucine zipper transcription factor nuclear factor-erythoroid 2 p45-related factor 2 (Nrf2) was identified as protein which binds ARE (Hayes et al. 2000; Enomoto et al. 2001; Kwak et al. 2001; McMahon et al. 2001; Ishii et al. 2002; McMahon et al. 2003; Kobayashi et al. 2004; Motohashi and Yamamoto 2004; Kobayashi and Yamamoto 2005). As depicted in Figure 1.9, it is now believed that chemical stress from electrophiles and reactive oxygen species (both of which can result from phase I biotransformation) activate Nrf2 by non-receptor mediated signaling cascades (Xu et al. 2005). Additionally, genes induced by transcription factors such AhR may also participate in the signaling cascade that activates Nrf2. Nrf2 binds ARE upstream of the coding region in many genes causing an increase in enzymes responsible for the detoxification of xenobiotics and/or reactive oxygen species, including GSTAs, GSTMs, and GSTPs (Kwak et al. 2003). Therefore, the induction of GST occurs through direct activation of transcription factors by ligands, and also occurs through non-receptor-mediated pathways in response to cellular stress.

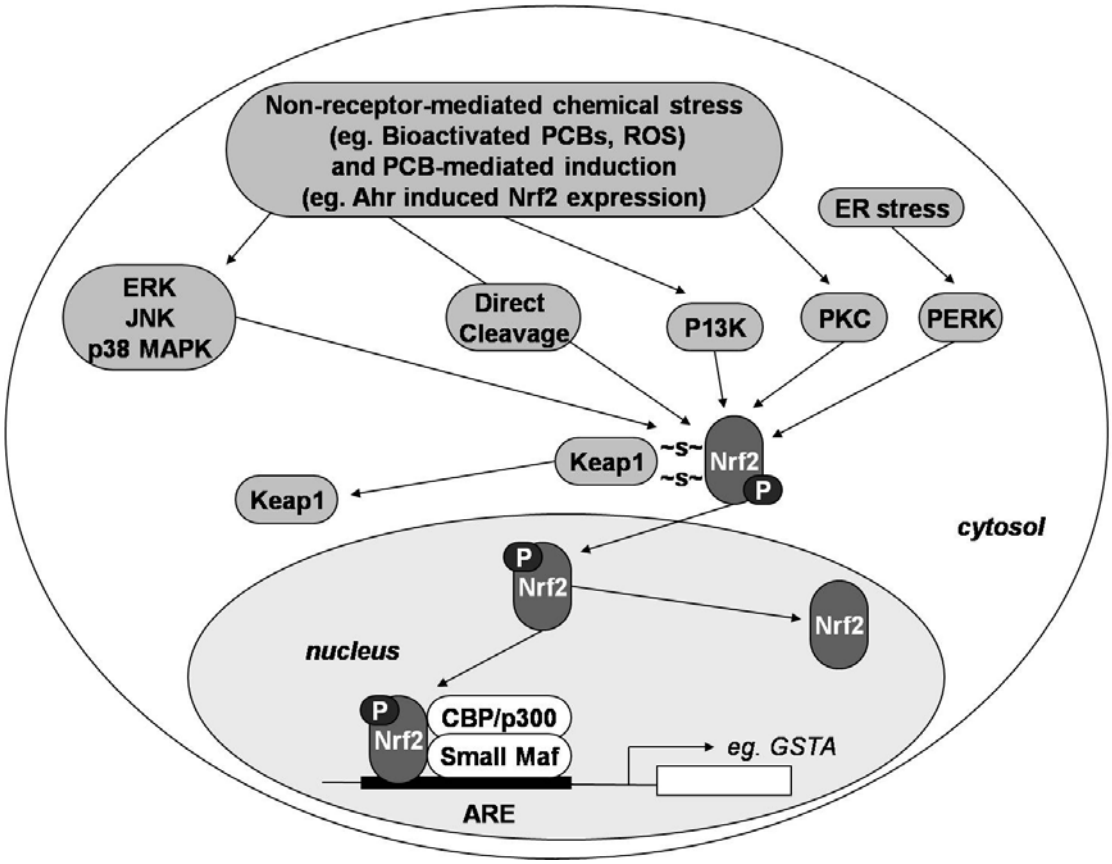

Figure 1.9. Cellular stress from electrophiles and reactive oxygen species activate antioxidant response element-mediated induction of phase II biotransformation enzymes (adapted from Xu et al. 2005).

Biotransformation and PCBs

PCBs are chemically quite stable and some are resistant to biotransformation by both prokaryotes and eukaryotes, yet many undergo some degree of metabolism under normal cellular conditions. The biotransformation of PCBs is a major influence on the toxicokinetics of PCBs, affecting both their biological half lives, and as well as their toxicity (Bakke and Gustafsson 1984; Norstrom and Letcher 1997). The potential pathways of PCB metabolism are shown in Figure 1.10 (James 2001).

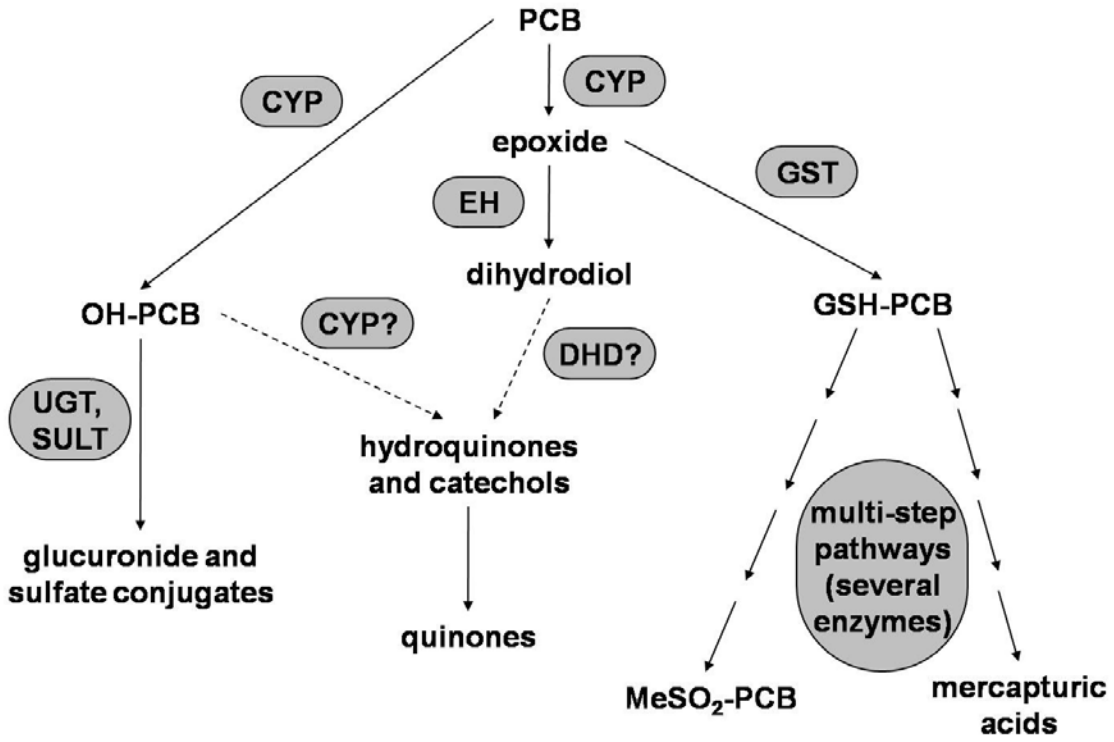

Figure 1.10. Potential biotransformation pathways of PCBs (adapted from James 2001).

In phase I metabolism, oxygen is introduced through the cytochrome P450 (CYP) enzyme system. Addition of oxygen to the aromatic ring can result in the formation of an epoxide or a hydroxylated metabolite, which is the most common metabolic product. The epoxide can spontaneously rearrange into a hydroxylated metabolite (OH-PCB), or it can be further metabolized by epoxide hydrolase into a dihydrodiol, or conjugated with GSH by GST. Phase II metabolism of the hydroxylated metabolite may include conjugation with glucuronide or sulfate in reactions catalyzed by UDP-glucuronosyltranferase (UGT) or PAPS-sulfotransferase (SULT), respectively. Glucuronic acid and sulfate conjugates have been shown to be excreted from laboratory

animals (Bakke et al. 1982; Bergman et al. 1982; Bakke and Gustafsson 1984), however OH-PCBs are relatively poor substrates for glucuronidation or sulfation and therefore these conjugation reactions may not significantly contribute to PCB biotransformation (Koga et al. 1990; James 2001). The hydroxylated metabolite may also be further hydroxylated to dihydrodiols or reactive quinones. Glutathione conjugates may be further metabolized into mercapturic acids or methyl sulfones ($MeSO_2$-PCB). Because OH- and $MeSO_2$-PCBs have been detected in laboratory animals, humans, and wildlife, and have been implicated in the toxicity of PCBs, the formation of these metabolites will be further discussed (Figure 1.11).

CYP mediated biotransformation of PCBs can occur through 2 mechanisms: 1) a hydroxyl group (OH) can be added directly at the *meta* and less frequently *para* position or 2) an arene oxide is formed, which is subject to rearrangement (Letcher et al. 2000b). The formation of *meta-para* arene oxide seems to be the most common mechanism (Preston and Allen 1980; Ishida et al. 1991; Koga et al. 1995), although this varies with species as dog and guinea pig have been shown to form arene oxides at the *ortho-meta* position (Ariyoshi et al. 1997). While it is possible for epoxide hydrolase to hydrolyze an arene oxide into a dihydrodiol PCB, this pathway does not seem common (Norback et al. 1976; Ariyoshi et al. 1994). Arene oxides are electrophilic and unless they open to the hydroxyl metabolite or are detoxified by phase II enzymes, may react with macromolecules.

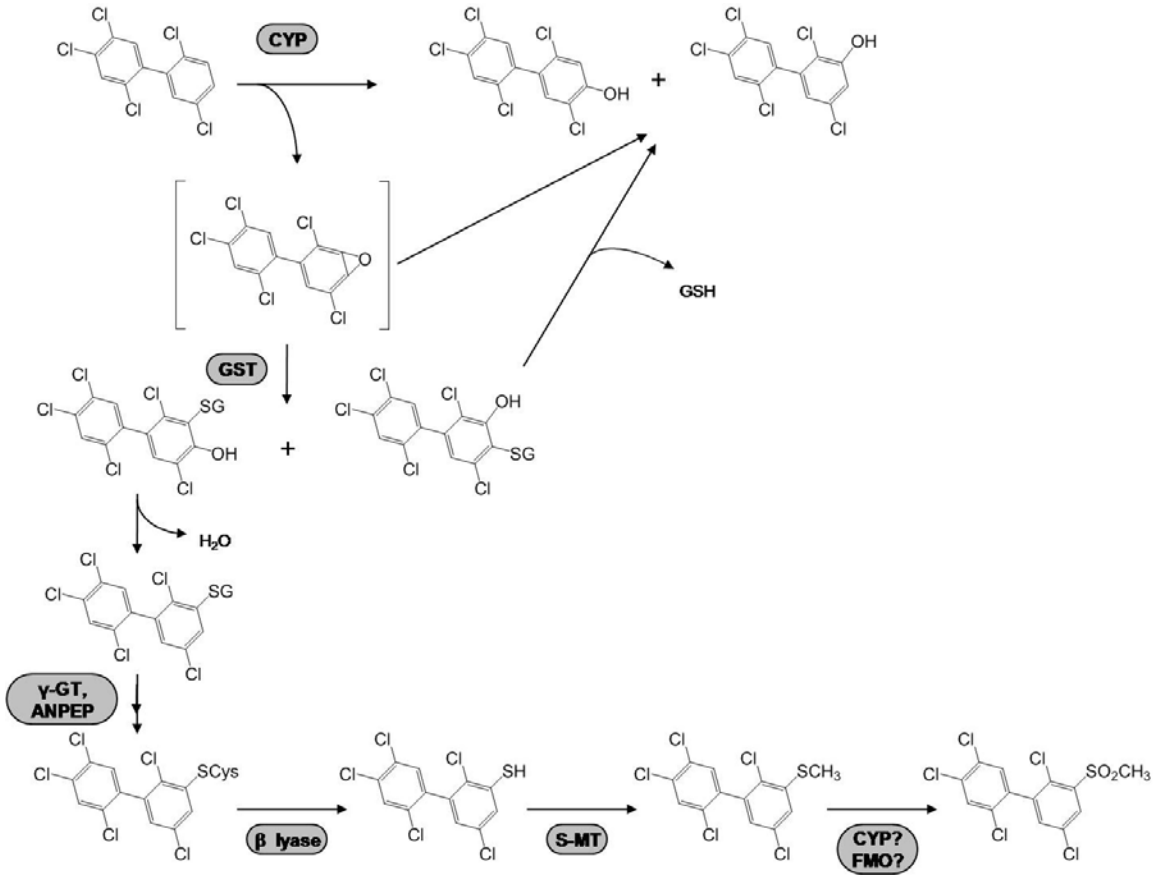

Figure 1.11. Biotransformation pathway of PCBs leading to the formation of hydroxylated and methyl sulfone metabolites (adapted from Letcher et al. 2000b).

PCB arene oxide intermediates may react with GSH, spontaneously and/or via GST mediated conjugation (Bakke 1989; Vermeulen 1996). While arene oxide opening is generally thought to be the mechanism of GSH conjugation, GSTs are capable of catalyzing heteroatom displacement reactions, and therefore may directly substitute GSH for a chlorine atom on PCB, as has been suggested in northern quahog (*Mercenaria mercenaria*) (Blanchette and Singh 2003). After dehydration, in the case of arene oxide addition, glutathione PCB conjugates (GS-PCB) follow the mercapturic acid pathway to

produce cysteine conjugates and mercapturic acids (see Figure 1.7), which can then be excreted through the bile (Bakke et al. 1982; Bakke and Gustafsson 1986). Once in the gastrointestinal tract, β-lyase of the gut microflora produces thiol-PCB (SH-PCB) (Larsen 1985). The SH-PCB can be excreted as a thiol, or as a S-glucuronic acid conjugate (Bakke 1989; Bakke 1990). In the formation of $MeSO_2$-PCBs, microfloral s-methyl transferase catalyzes the formation of methylthiol-PCB (MeS-PCB) (Brandt et al. 1982). In a two step oxidation, CYP or flavin-containing monooxygenase catalyze the formation of a $MeSO_2$-PCB (Bakke 1989).

The biological retention of PCBs is affected by their susceptibility to biotransformation, which is determined by their structure or more specifically, the number and position of chlorine atoms. Historically, the accumulation of PCBs and other HOCs was thought to be a function of the lipid content of an organism: increasing Kow up to ~5-6 resulted in increasing bioconcentration (Hamelink et al. 1971; Shaw and Connell 1986). However, more recent studies have indicated that differences in lipid content between organisms are irrelevant to bioaccumulation in an ecosystem, instead metabolic efficiency is the key factor (Kannan et al. 1995). In most species, PCBs are resistant to biotransformation, at least to some degree, when they contain five or more chlorine atoms. Structure activity relationships further suggest that the biotransformation of PCBs is based on the absence/presence of vicinal protons as these are necessary for CYP activity (Boon et al. 1989; McFarland and Clarke 1989; Kannan et al. 1995). PCBs which lack *ortho-meta* and *meta-para* vicinal hydrogen atoms are the most persistent congeners and are generally considered non-metabolizable.

In rodents, PCB oxidation is mediated by CYP1A and CYP2B, although CYP2C and CYP3A may also contribute to phase I metabolism (McFarland and Clarke 1989; Lewis 1998). The position of the arene oxide is dependent on the CYP isoform catalyzing the reaction. PB-induced CYPs, e.g., CYP2B, insert an oxygen atom at non-sterically hindered sites of globular molecules whereas 3MC- induced CYPs, e.g., CYP1A, catalyze oxygenation at sterically hindered sites on planar molecules (Parke 1985). For PCBs, the formation of an arene oxide at the *meta-para* position is less sterically hindered than at the *ortho-meta* position. Therefore, PCBs containing vicinal *meta-para* protons are metabolized by CYP2B-like enzymes while PCBs with vicinal protons in the *ortho-meta* positions are biotransformed by CYP1A-like enzymes. PCBs that have both *ortho-meta* and *meta-para* vicinal hydrogen atoms can be metabolized by both types of enzymes. Because both PB- and 3MC-induced enzymes can catalyze the formation of an epoxide, both CYP types can bioactivate compounds. However, metabolites from PB-induced enzymes go on to phase II biotransformation (James 1987). The steric hindrance of the oxygen atom provides stability and may inhibit detoxification so 3MC-induced enzymatic activity may present a greater potential for toxicity (McFarland and Clarke 1989).

While the presence of vicinal protons is requisite for the formation of OH-PCBs, the formation of persistent $MeSO_2$-PCBs has been demonstrated for only PCBs that possess *meta-para* vicinal protons (Letcher et al. 2000b). Furthermore, conversion of PCBs to OH-PCB or $MeSO_2$-PCB is determined, at least in part, by chlorination. As chlorination of a PCB increases, the ratio of OH to $MeSO_2$ metabolites increases but the

overall extent of biotransformation decreases (Haraguchi et al. 1997). The exact GST isoforms responsible for GSH conjugation critical to the formation of $MeSO_2$-PCBs have yet to be identified.

PCBs can also influence their own biotransformation through induction of CYPs, GSTs, and other biotransformation enzymes. X-ray crystallography studies have shown that the preferred conformation for all PCBs is nonplanar (McKinney and Singh 1981). However, certain PCBs which contain one or no *ortho* chlorine atoms can assume a planar conformation similar to the 3 x 10 Å planar volume of TCDD (McKinney and Singh 1981; McFarland and Clarke 1989). Coplanar PCBs with chlorine substitution at both *para* positions and at least 2 *meta* positions, which include four non-*ortho* PCBs (77, 81, 126, and 169) and eight mono-*ortho* PCBs (105, 114, 118, 123, 156, 167, 189), are termed "dioxin-like PCBs" because they are agonists of the AhR and cause induction of CYP1A and other biotransformation enzymes which are regulated by XRE promoter regions (Safe 2001). PCB 81 and all of the mono-*ortho* PCBs showed mixed-type induction – both 3MC-like (CYP1A) and PB-like (CYP2B) induction patterns are observed (Safe 2001). Furthermore, several di-*ortho* analogs of the mono-*ortho* PCBs (128, 138, 158, 166, 168, and 170) are also mixed-type inducers in mammals, although less potent than non-*ortho* and mono-*ortho* inducers (Safe et al. 1985). Lastly, many non-planar, globular PCB congeners have been shown, or are predicted based on structure activity rules to cause PB-like induction (McFarland and Clarke 1989). These congeners include: 11, 14, 15, 47, 52, 54, 66, 75, 80, 85, 87, 99, 100, 101, 133, 136, 137, 139, 140, 146, 151, 155, 159, 163, 165, 171, 180, 181, 182, 183, 184, 190, 191, 194, 195,

196, 197, 203, 204, 205, 206, 207, and 209. PCBs can therefore cause induction of enzymes responsible for their metabolism and influence the direction of their biotransformation pathway.

Toxicological Impacts of PCBs

The toxicity of PCBs as individual congeners and commercial mixtures has been the subject of much research. In laboratory animals (including fish, birds, and mammals), commercial mixtures elicit a broad range of toxic responses which vary depending on chlorine content of the mixtures, animal species and strain, age and sex of animal, as well as route and duration of exposure, but the liver appears to be a common target organ (Safe 1994). Responses observed in laboratory animals after exposure to PCB mixtures include: acute lethality, reproductive toxicity, growth inhibition, porphyria, hepatotoxicity, immunotoxicity, endocrine disruption, neurotoxicity, thymus toxicity, dermal toxicity, and carcinogenicity.

Early studies of PCB mixtures showed that many of their toxic effects resembled those of TCDD and structurally related compounds (Alvares 1977; Yoshimura et al. 1979). Structure-activity studies indicated that the coplanar congeners in these mixtures were responsible for these dioxin-like effects (Safe 1994). The chemical carcinogenesis of PCBs is thought to be a combined result of the direct and indirect effects of the AhR mediated signaling pathway. Extensive studies using AhR knockout mice have shown that the toxic effects of TCDD are mediated by AhR (FernandezSalguero et al. 1996; Mimura et al. 1997; Shimizu et al. 2000). Furthermore, the *in vitro* induction mediated

by AhR binding of dioxin-like PCBs shows strong correlation with *in vivo* mammalian toxicity (Sawyer and Safe 1982; Casterline et al. 1983; Sawyer et al. 1983; Sawyer and Safe 1985; Safe 1987). DNA microarray analysis revealed that TCDD exposure up- or down-regulates 310 genes in HepG2 cells, both directly and indirectly, suggesting a complex cellular response resulting in toxicity (Frueh et al. 2001). Most of the toxic responses from PCBs seem to stem from dioxin-like congeners, but the toxicity resulting from exposure to nonplanar PCBs is less clear. Nonplanar PCBs have been implicated in neuroendocrine toxicity (Coburn et al. 2007), hepatoxicity (Goldstein et al. 1976; Kohli et al. 1979; Biocca et al. 1981) and tumor promotion (Buchmann et al. 1986; Buchmann et al. 1991; Laib et al. 1991).

Although the purpose of biotransformation is to detoxify the body by making the compounds more readily excreted, many PCB metabolites persist in tissues. Thus, biotransformation intermediates and products may also be responsible for toxicity associated with PCBs. For example, PCB-induced porphyria is thought to be induced by parent PCBs while the cytotoxic effects result from arene oxide intermediates (Schnellmann et al. 1985). The hydroxylated and methylsulfone metabolites that are produced may be selectively bound to proteins and therefore retained in cells (Lans et al. 1993; Bergman et al. 1994; Letcher et al. 2000b). HO-PCBs are more hydrophilic than $MeSO_2$-PCBs and PCBs, so OH-PCBs are usually detected in blood while lipophilic $MeSO_2$-PCBs and PCBs persist in lipids (Letcher et al. 2000b). The retention of these 2 types of metabolites may lead to some of the toxic effects seen in animals because they have been shown to affect oxidative phosphorylation, inhibit some CYPs, and interfere

with the endocrine system (Letcher et al. 2000b). Furthermore, in environmental exposures, PCBs are often not the sole contaminant, indicating that changes in cellular biochemistry induced by PCBs may affect not only their own toxicity, but also that of other contaminants.

The toxic manifestations of PCBs may be based, at least in part, on oxidative stress (Twaroski et al. 2001a; Twaroski et al. 2001b). Oxidative stress, defined as an imbalance between reduced oxygen species (ROS) and antioxidant capacity, results in damage to cellular nucleophiles such as proteins, lipids, and DNA. Evidence of increased oxidative stress after PCB exposure has been detected *in vitro* (McLean et al. 1996; Oakley et al. 1996; Ludewig et al. 1998; Schlezinger et al. 1999; Slim et al. 1999; Schlezinger et al. 2000; Slim et al. 2000) and *in vivo* (Kamohara et al. 1984; Dogra et al. 1988; Pelissier et al. 1990; Saito 1990). As shown in Figure 1.10, the biotransformation of minimally chlorinated PCBs can give rise to catechols and hydroquinones, which have the potential to generate ROS by redox cycling (McLean et al. 1996; McLean et al. 2000). However, in rats treated with minimally chlorinated PCBs, oxidative stress was not observed (Twaroski et al. 2001a; Shertzer et al. 2004) and thus, this mechanism is not thought to significantly contribute to PCB-induced oxidative stress. Instead, the NADPH-dependent uncoupling of electron transfer and oxygen reduction from monooxygentaion by CYP causes the production of ROS (Schlezinger et al. 1999; Schlezinger et al. 2000). The binding of a substrate to CYP initiates the catalytic cycle (see Figure 1.2), triggering the transfer of an electron to the heme of CYP. But if the substrate is refractory to metabolism, as is the case for the dioxin-like PCBs, the ROS

formed during the catalytic cycle can be released from the enzyme (Lewis 2002). The formation of ROS via CYP uncoupling has been shown for many CYP isoforms, including CYP1A, CYP2B4, CYP2E1, CYP3A (Kuthan and Ullrich 1982; Ekstrom et al. 1986; Ahmed et al. 1995; Schlezinger et al. 1999). Thus, dioxin-like PCBs uncouple CYP1As in vertebrates, which causes oxidative stress (Shertzer et al. 2004). Clearly, multiple pathways contribute to the toxic effects associated with PCBs.

PCBs in Sea Turtles, Reptiles, and Others

Turtles belong to the most ancient order of reptiles, with earliest fossil records dating to the Triassic period (Gaffney 1990). From fossil evidence, it appears that turtles initially developed terrestrially (because this environment is required for oviposition). Approximately 100 million years ago, some turtles adapted to marine life (Pritchard 1997) and became an important component of marine ecosystems. Today, only seven species of sea turtles survive – green (*Chelonia mydas*), olive ridley (*Lepidochelys olivacea*), Kemp's ridley (*L. kempii*), loggerhead (*Caretta caretta*), hawksbill (*Eretmochelys imbricata*), leatherback (*Dermochelys coriacea*), and flatback (*Natator depressus*). Throughout their long life, these endangered turtles face many hazards from human activity, including: direct take (hunting), fisheries impacts (incidental), loss of nesting habitat due to coastal development, global warming, and pollution/pathogens (Mast et al. 2005). Of these hazards, the effects of anthropogenic contaminants and their metabolites is probably the least studied. In fact, only 1.4% of the reptile abstracts listed in ISI Web of Science in 2005 dealt with toxicology (Sparling 2006). While this

discussion will focus on sea turtles and other reptilian species, the paucity of reptilian toxicology data mandates that other wildlife species be included.

Sea turtles are slow growing, late maturing, and long lived organisms, which makes them particularly susceptible to population decline caused by toxicity associated with bioaccumulation (Rowe 2008). Their life history patterns involve terrestrial habitats for oviposition and embryonic development as well as marine habitats, including coastal waters and open ocean, for development and foraging (Bolten 1997). As oviparous organisms, sea turtle exposure to lipophilic contaminants starts in the egg – vitellogenesis, the process of yolk deposition, transfers contaminants along with maternal dietary or stored lipid to the oocyte. Maternal transfer of PCBs, PHAHs, and pesticides has been observed in snapping turtles (*Chelydra serpentina*) (Bishop et al. 1994; Kelly et al. 2008) and alligators (*Alligator mississippiensis*) (Rauschenberger et al. 2004). The transfer of lipophilic contaminants from sandy beaches across the eggshell would not be expected to contribute significantly to the body burden of embryos. Upon hatching, neonates emerge from the nest, crawl towards the shore, and disappear only to reappear as much larger adults. This period of a sea turtle's life was called the "lost years" – however, evidence is building that the first 2-10 years of a sea turtles life are spent in pelagic habitats, often in the *Sargassum* habitat of oceanic convergence zones and gyres (Musick and Limpus 1997). The *Sargassum* mats serve to camouflage the young sea turtles from predators and presumptively, they also provide food in the form of flora and fauna which also shelter in the mats, as well as *Sargassum* itself. After the "lost years," coastal zones, especially estuaries, provide developmental and feeding grounds for sea

turtles (Musick and Limpus 1997). Depending on the species, adult sea turtles occupy coastal or oceanic habitats and are known to travel great distances between foraging areas and mating/nesting areas (Bolten 2003). Post-hatching, ingestion of contaminated food is likely the greatest source of exposure, especially considering that the extremely high nutrient conversion efficiency of reptiles would most likely be associated with high rates of contaminant accumulation (Hopkins 2007). Indeed, environmental contamination has been demonstrated in a wide range of reptiles (Meyers-Schone and Walton 1994; Campbell and Campbell 2000, 2001; Campbell 2003), including sea turtles (Lake et al. 1994; Rybitski et al. 1995; McKenzie et al. 1999; Corsolini et al. 2000; Storelli and Marcotrigiano 2000; Miao et al. 2001; Keller et al. 2004b; Keller et al. 2004c; Storelli et al. 2007). Organohalogen contamination in sea turtles typically ranges from below limits of detection to the low 100's ng/g.

Reptiles possess a functional cytochrome P450 monooxygenase system (Ertl and Winston 1998). Investigation of reptilian phase I biotransformation has focused primarily on CYP1A because it is often used as a biomarker of PCB and other HOC exposure. The presence of CAR has not been investigated in reptiles, but a photoaffinity labeling study of AhR demonstrated the presence of AhR in two out of three painted turtles, but not in the one alligator examined in the study (Hahn et al. 1994). Overall, reptiles show lower CYP content as well as differences in substrate activities, isoform content, and induction patterns relative to mammals (Ertl and Winston 1998; Mitchelmore et al. 2006). Additionally, interesting species variability of CYP1A has been found in reptiles. Snapping turtles collected from the Great Lakes region, notorious

for HOC contamination (Bishop et al. 1998), and painted turtles (*Chrysemys picta picta*) collected from a military chemical waste disposal facility (Rie et al. 2000) showed relatively high induction of CYP1A protein and EROD activity as compared to turtles from lesser contaminated sites, but not as high compared to other vertebrates. Conversely, marsh turtles (*Mauremys caspica rivulata*) from a petrochemical contaminated site failed to demonstrate CYP1A induction (Yawetz et al. 1997) and, in alligators collected from three different sites representing a gradient of contamination, EROD activity was inhibited at the more contaminated sites (Gunderson et al. 2004). Laboratory studies with the classic CYP1 and CYP2 inducers, 3MC and PB respectively, have shown little to no induction of protein levels or CYP activities in snakes, alligators, and turtles (Mitchelmore et al. 2006). *In vivo* injections of Aroclor 1254 to marsh, painted, and red-eared slider (*Trachemys scripta elegans*) turtles showed no effect on total CYP content, but lower CYP1A protein levels in marsh turtles as compared to the other two species (Yawetz et al. 1997). In African brown house snakes (*Lamprophis fuliginosus*), treatment with 2 potent CYP1A inducers, TCDD and PCB 126, resulted in dose-dependent increases in CYP1A activity, while other CYP1A inducers, PCB 77, PCB 81, and PCB 169, caused no induction (Hecker et al. 2006). The partial isolation of four CYP proteins from the liver of a Kemp's ridley sea turtle, which showed low levels of CYP1A activity, represents the only CYP study to date in sea turtles (Goldman and McClellan-Green 2001).

Because of the anoxic and hypoxic conditions often faced by reptiles, the role of GSTs (and associated enzymes and cofactors) in oxidative stress associated with such

conditions has been investigated (Mitchelmore et al. 2006). Two GSTs, one homodimeric and one heterodimeric, have been purified from red-eared slider turtles and activities against a variety of substrates and inhibitors suggested that both enzymes were of the alpha-class (Willmore and Storey 2005). Changes in overall GST activity, as measured by conjugation of CDNB with GSH, have been observed following exposure to hydrogen peroxide, anoxic conditions, and tetrachloroethylene contaminated sites in red-sided garter snakes (*Thamnophis sirtalis parietalis*) (Hermes-Lima and Storey 1993), red-eared slider turtles (Willmore and Storey 1997a, b), and painted turtles (Rie et al. 2000), respectively. These studies suggest that GSTs are responsive to stressors, possibly via the ARE, and that their activity may be altered by exposure to HOCs. Recently, Valdivia and colleagues measured GST activity in green sea turtles, and found levels comparable to those reported in the above studies for red-sided garter snakes and red-eared slider turtles, but no comparisons were made to contaminant body burdens (2007). In fish exposed to PCBs and PAHs, increased GST activity has been found in some species, but many other studies show no alterations in GST (van der Oost et al. 2003). Furthermore, corn snakes (*Elaphe guttata emoryi*) exposed to 3MC and PB showed no changes in GST activities (Bani et al. 1998) and no differences were found in GST activities in alligators collected from three different sites representing a gradient of contamination (Gunderson et al. 2004). Because sea turtles and other reptiles often face oxidative stress, basal activities may be high and elucidation of xenobiotic induction of GSTs may be confounded by measurements of overall GST activity towards CDNB. The use of GST

class-specific substrates may provide a better understanding of the effect of PCBs and other xenobiotics on GSTs.

The metabolism of PCBs has been demonstrated in a variety of organisms and generally shows the following pattern of biotransformation capacity: mammal > bird > fish (Matthews and Dedrick 1984). PCB 3, PCB 15, and Aroclor 1254 administered *in vivo* to leopard frogs (*Rana pipiens*) were biotransformed primarily to hydroxylated metabolites similar to those found in rabbits, however oxidation capacity of the frog was greater than rainbow trout but less than mammals (Safe et al. 1976). In a more recent study, both leopard and green (*Rana clamitans*) frogs again showed elimination rates of PCBs between fish and mammals (Leney et al. 2006a). The frogs showed preferential elimination (and therefore metabolism) of PCBs with *meta-para* vicinal protons over those with *ortho-meta* vicinal protons. Preferential elimination of *meta-para* unsubstituted PCBs has also been found many animals, including kestrels (*Falco sparverius*) (Drouillard et al. 2001), ring doves (*Streptopelia risoria*) (Drouillard and Norstrom 2003), flounder (*Pleuronectes americanus*) (Elskus et al. 1994), as well as loggerhead and Kemp's ridley sea turtles (Keller et al. 2004a). Higher relative abundance of *ortho-meta* to *meta-para* unsubstituted PCBs in worm (*Nereis diversicolor*), fish (*Pleuronectes platessa*), bird (*Haematopus ostralegis*), and harbor seal (*Phoca vitulina*) suggest preferential metabolism of PCBs with *meta-para* vicinal protons as well. However, this pattern is more dramatic in mammals and birds than in fish and invertebrates (Boon et al. 1989), and amphibians appear to fall between birds and fish (Leney et al. 2006b). A study investigating the biotransformation of PCB 77 in hepatic

microsomes of several organisms including painted turtle induced with β-naphthoflavone, a AhR agonist, represents the only report of PCB metabolism in reptiles (Schlezinger et al. 2000). Turtle hepatic microsomes oxidized PCB 77 at rates similar to birds and they had the highest CYP1A protein expression as compared to all other induced species, including rat induced with a commercial Aroclor 1254. However, turtle showed very low EROD activity, which was comparable to levels in non-induced chicken (*Gallus domesticus)*, gull (*Larus argentatus*), and eel (*Anguilla rostrata*) (Schlezinger et al. 2000). The discrepancy between low CYP1 activity, high CYP1A protein content, and PCB 77 oxidation further suggests that reptilian CYP1A may have structural or catalytic differences from CYP1A of other vertebrate groups.

The presence of PCB metabolites in a variety of organisms supports the biotransformation in these organisms as well. OH-PCBs have been detected in fish (Asplund et al. 1999; Stapleton et al. 2001; Campbell et al. 2003; Li et al. 2003), birds and their eggs (Klasson-Wehler et al. 1998; Berger et al. 2004; Fangstrom et al. 2005; Verreault et al. 2005; McKinney et al. 2006a; Jaspers et al. 2008; Kunisue and Tanabe 2009; Jorundsdottir et al. 2010), cetaceans (Hoekstra et al. 2003; Houde et al. 2006; McKinney et al. 2006c; Kunisue et al. 2007; Montie et al. 2009; Nomiyama et al.), pinnipeds (Bergman et al. 1994; Kunisue and Tanabe 2009; Park et al. 2009a), polar bears (Sandala et al. 2004; Gebbink et al. 2008; Verreault et al. 2008), canines (Verreault et al. 2008; Kunisue and Tanabe 2009) and humans (Bergman et al. 1994; Sandau et al. 2000; Guvenius et al. 2002; Hovander et al. 2002; Fernandez et al. 2008; Kunisue and Tanabe 2009; Park et al. 2009b; Nomiyama et al. 2010b). Differences in individual OH-

PCB levels, total OH-PCB levels, and ratios of OH-PCB to total PCBs vary between and within species, highlighting variability of biotransformation and elimination in wild organisms. For example, despite the fact that fish have been shown to be slow metabolizers of PCBs, levels of OH-PCBs in the plasma of benthic and pelagic fish from the highly contaminated Detroit River have been shown in the ppm range and in some fish OH-PCB levels were higher than PCB concentrations (Li et al. 2003; Valters et al. 2005). Additionally, although it is generally believed that mammals preferentially metabolize *meta-para* unsubstituted PCBs, hepatic microsomes from beluga whales have been to preferentially metabolize *ortho-meta* unsubstituted PCBs (McKinney et al. 2006b). $MeSO_2$-PCB have been detected primarily in marine mammals (Jensen and Jansson 1976; Karlson et al. 2000; Letcher et al. 2000c; Gebbink et al. 2008) and humans (Guvenius et al. 2002), but also in the deepwater sculpin (*Myoxocephalus thompson*) (Stapleton et al. 2001) and guillemot (*Uria aalge*) (Jorundsdottir et al. 2006). Persistent *meta*- and *para*-$MeSO_2$-PCBs are preferentially retained in fatty tissues, especially the liver, and represent an abundant class of contaminants in wildlife (Letcher et al. 2000b). In marine mammals, levels of $MeSO_2$-PCBs can exceed that of PCBs, illustrating that although some marine mammals have shown preferential metabolism of *ortho-meta* unsubstituted PCBs (McKinney et al. 2006b), metabolism of *meta-para* unsubstituted PCBs may occur. While the presence of PCB metabolites has not been investigated in reptiles, the presence of metabolites in a variety of wildlife indicates that sea turtles may also metabolize PCBs.

The toxicity of PCBs and their metabolites has been investigated, to some degree, in reptiles and other organisms. In alligators and snapping turtles, maternal transfer of PCBs and other HOCs has been correlated with population level effects including hatching success and hatchling deformities (Bishop et al. 1998; Guillette 2000). In order for sea turtle eggs to develop properly, the nest temperature must be 26 - 32°C and sexual differentiation is determined by temperature – cooler temperatures produce males and warmer temperatures produce females (Miller 2007). However, environmentally relevant PCB mixtures, individual congeners, and hydroxylated metabolites have been shown to alter sex ratios (i.e., feminization) in experimentally dosed eggs of red eared slider turtles (*Trachemys scripta elegans*) (Bergeron et al. 1994; Willingham and Crews 1999). Thus, maternal transfer of PCBs and related HOCs may compromise the reproductive success of sea turtles. In caspian terns (*Sterna caspia*), herring gulls (*Larus argentatus*), and harbor seals immunosupression has been linked with feeding on PCB contaminated food (Grasman et al. 1996; Ross et al. 1996; Grasman and Fox 2001). Recently, Keller and colleagues have found that concentrations of HOCs adversely modulate immunity in loggerheads (Keller et al. 2004c; Keller et al. 2006). Immunosuppression in turtles may make them more susceptible to pathogenic infection, as has been suggested in the case of green turtle fibropapillomas, which are benign, yet often debilitating cutaneous (and less commonly visceral) tumors up to 30 cm in diameter (Cray et al. 2001; Aguirre and Lutz 2004). Additionally, PCB metabolites have been linked to endocrine disruption in laboratory animals. OH-PCBs with the hydroxyl group in *meta* or *para* position and one or more adjacent chlorine atoms show a high potency for displacing thyroxine from

transport proteins such as transthyretin (Lans et al. 1993). OH-PCBs and $MeSO_2$-PCBs show opposing effects on the catabolism of thyroid hormones – some OH-PCBs have been shown to inhibit thyroid hormone sulfotransferase (Schuur et al. 1998a; Schuur et al. 1998b) while some $MeSO_2$-PCBs induce UGT responsible for glucuronidation of thyroxine (Visser et al. 1993; Kato et al. 2000). Thus, endocrine disruption and developmental effects seen in reptiles may result from the products of PCBs metabolism.

Table 1.8. Dietary preferences of adult sea turtles (adapted from Bjorndal 1997)

Species	Common Name	Preferred food
Chelonia mydas	green	sea grass, algae
Dermochelys coriacea	leatherback	gelatinous zooplankton
Natator depressus	Flatback	gelatinous zooplankton, soft benthos
Caretta caretta	loggerhead	benthic invertebrates
Eretmochelys imbricata	hawksbill	Sponges
Lepidochelys olivacea	olive ridley	fish, salps
Lepidochelys kempii	Kemp's ridley	Crabs

Because lipophilic, persistent contaminants, including PCBs bioaccumulate as trophic level increases, the feeding strategy of each species can affect its exposure. The dietary preferences of sea turtles has been extensively studied through stomach content analysis (Bjorndal 1997). Juveniles tend to show opportunistic feeding strategies. As adults, while not completely exclusive, the various species generally exhibit preferred

food sources as shown in Table 1.8. In numerous field studies, reptiles, like other vertebrates, show bioaccumulation reflective of their trophic status (Meyers-Schone and Walton 1994; Campbell and Campbell 2000, 2001; Campbell 2003). Bioaccumulation has been suggested, but not correlated, with residues of PCBs in green, loggerhead and Kemp's ridley sea turtles (Gardner et al. 2003; Milton and Lutz 2003).

Dietary preferences of sea turtles may also influence biotransformation pathways as exogenous compounds can modulate the expression of phase I and phase II enzymes. Thus, feeding at a higher trophic level results in higher potential absorption of contaminants and may result in higher induction of biotransformation enzymes. However, it is important to note the incidental ingestion of marine debris, particularly plastics, that has been found in many juvenile and adult sea turtles, both juveniles and adult (Bjorndal 1997). Adsorption of PCBs to the surface of hydrophobic plastics results in accumulation of PCBs at levels up to 10^6 higher than surrounding sea water (Mato et al. 2001). The concentration of PCBs in the fat of puffins (*Puffinus gravis*) was positively correlated with the mass of plastic ingesta (Ryan et al. 1988). Taken together, these studies suggest that plastic debris concentrate and transport significant levels of PCBs and may serve as a potential source of toxicants to animals which incidentally ingest plastics. This may be especially important for juvenile sea turtles during their "lost years" spent in oceanic gyres, as plastic and other debris accumulates in these gyres (Thompson et al. 2004) and the contribution of plastic-accumulated PCBs may skew the bioaccumulation at various trophic levels and life stages. Additionally, anthropogenic

contaminants in the diet are not the only xenobiotic compounds turtles ingest – the diet itself may contain toxic chemicals.

Marine benthic organisms, such as sponges, produce a wide variety of halogenated secondary metabolites as chemical defense against consumers (Hay 1996). Among these are brominated pyrrole alkaloids, which are conserved natural products from *Agelas* sponges, a genus of demosponge (Pawlik et al. 1995; Chanas et al. 1996). 4,5-dibromopyrrole-2-carboxylic acid (DBPC) has been identified as a primary feeding deterrent in several sponges of the *Agelas* genus (Chanas et al. 1996). However, adaptive processes, such as changes in biotransformation enzyme expression, may allow some organisms to consume such noxious compounds. For example, a species of butterflyfish (*Chaetodon capistratus*) that preferentially feeds on chemically-rich gorgorian corals had higher total CYP content and CYP2 and CYP3 enzymes than a cogeneric species that avoid eating gorgonians (Vrolijk et al. 1994). This may hold true for the hawksbill sea turtle since the primary component of the diet of adults from the Caribbean Sea is marine demosponges (Meylan 1988; Leon and Bjorndal 2002). Therefore, it plausible that sea turtles may be exposed to toxic chemicals in their diet which would result in altered biotransformation pathways.

Hypotheses

While sea turtles and other reptiles show biotransformation capacity, little is known about the biotransformation enzymes or pathways of PCBs or other HOCs in these endangered animals. Thus, the purpose of this study is to understand the accumulation and metabolism of halogenated organic compounds in several species of sea turtles. The following hypotheses will be addressed:

a. PCBs will be detected in liver samples from several species of sea turtles and expression of biotransformation enzymes will reflect contaminant exposure in these turtles.

b. Liver samples from various species of sea turtles possess glutathione *S*-transferase (GST) biotransformation potential and catalytic activities will be reflective of their evolutionary adaptations to diverse feeding strategies.

c. Liver samples from various sea turtle species will exhibit both phase I and phase II biotransformation of PCBs and catalytic activity will reflect trophic level &/or biotransformation potential of each species.

d. Liver samples from hawksbill turtles will demonstrate an enhanced capacity to biotransform 4,5-dibromopyrrole-2-carboxylic acid (DBPC), a natural sponge product representative of the organohalogens found in sponges.

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Chapter 2: Polychlorinated Biphenyls and Biotransformation Enzymes in Three Species of Sea Turtles from the Baja California Peninsula of Mexico

Abstract

Concentrations of polychlorinated biphenyls (PCBs) as well as the expression patterns of cytochrome P450 (CYP) enzymes and glutathione *S*-transferase (GST) activities were measured in livers of loggerhead (*Caretta caretta*), green (*Chelonia mydas*), and olive ridley (*Lepidocheyls olivacea*) sea turtles from the Baja California peninsula of Mexico. The mean concentrations of total PCBs were 18.1, 10.5, and 15.2 ng/g wet weight (wet wt), respectively for the three species and PCB 153 was the dominant congener in all samples. Total PCB concentrations were dominated by penta- and hexa-chlorinated biphenyls. The mean estimated TEQs were 42.8, 22.9, and 10.4 pg/g wet wt for loggerhead, green, and olive ridley, respectively, and more than 70% was accounted for by non-*ortho* PCBs. Western blots revealed the presence of hepatic microsomal proteins which cross-reacted with anti-CYP2K1 and anti-CYP3A27 antibodies, but not with anti-CYP1A antibody. There were no significant differences in GST activities between species. Grouping congeners based on structure-activity relationships for CYP isoenzymes suggested limited activity of CYP1A contribution to PCB biotransformation in sea turtles. These results suggest potential accumulation of PCBs that are CYP1A substrates and provide evidence for biotransformation capacity, which differs from known animal models, highlighting the need for further studies in reptiles, particularly those threatened with extinction.

Introduction

The coast of the Baja California (BC) peninsula of Mexico serves as a critical feeding and developmental area for sea turtles (Clifton et al. 1982). Five of the world's seven extant sea turtle species can be found along BC: leatherback (*Dermochelys coriacea*), hawksbill (*Eretmochelys imbricata*), olive ridley (*Lepidochelys olivacea*), green (*Chelonia mydas*), and loggerhead (*Caretta caretta*). All of the world's seven species of sea turtles are globally considered critically endangered, endangered or vulnerable (IUCN 2008) and throughout their life span sea turtles face many hazards from human activity, including pollution and pathogenic infections (Mast et al. 2005). Organochlorine compounds, such as polychlorinated biphenyls (PCB) have ecotoxicological significance as environmental contaminants because of their persistent nature, accumulation, and biomagnification through the food web (James 2001). Yet, compared to other taxa such as fish or mammals, relatively few studies have focused on levels of environmental contaminants such as PCBs in sea turtles worldwide (Lake et al. 1994; Rybitski et al. 1995; McKenzie et al. 1999; Corsolini et al. 2000; Storelli and Marcotrigiano 2000; Miao et al. 2001; Keller et al. 2004b; Keller et al. 2004c; Storelli et al. 2007) and only one other study has been conducted in the BC region (Gardner et al. 2003). No data exists on threshold levels for toxicity of these compounds in sea turtles or reptiles. With life history traits optimized for reproductive success throughout their long life span, turtle populations may be particularly susceptible to the accumulation of persistent environmental contaminants (Rowe 2008).

Within the food chain, PCBs are subject to biotransformation by xenobiotic metabolizing enzymes to different degrees. This highly conserved transformation process generally occurs in the liver and/or intestines of vertebrates. Initial metabolism reactions typically involve the introduction of a functional group such as oxygenation by the cytochrome P450 (CYP) mono-oxygenases, typically resulting in an increase in hydrophilicity. The CYP enzyme system is critical to the metabolism of both exogenous and endogenous chemicals, with CYP families 1, 2, and 3 considered the most important for xenobiotic metabolism in vertebrates. Additional transformation reactions include conjugation of the xenobiotic with various polar endogenous moieties, such as glutathione (GSH) by glutathione *S*-transferases (GST). Conjugation results in a large increase in hydrophilicity of the metabolite, thereby aiding its elimination. Exposure to coplanar or dioxin-like PCBs enhance CYP1 family expression and exposure to globular or non-planar PCBs has been shown to increase expression of CYP2 and 3 families particularly in mammals (Safe 1994). In addition to modifying expression, PCBs are substrates for CYP1A or CYP2B subfamilies in mammals based on the position of vicinal protons within the congener (Parke 1985; Boon et al. 1989; McFarland and Clarke 1989). Xenobiotic accumulation can be alleviated by enhanced biotransformation which can occur by exposure to enzyme inducing agents. PCBs provide a unique situation where specific enzyme substrates may also induce the same enzyme and potentially their own biotransformation.

Polychlorinated biphenyl congeners can be separated into four metabolic groups based on structure-activity relationships described by Kannan et al.(Kannan et al. 1995). Group 1, considered nonmetabolizable, consists of congeners which lack *meta-para* (*m-p*) and *ortho-meta* (o-*m*) vicinal protons. Group 2, metabolized by CYP2B in mammals, consists of congeners which have only *m-p* vicinal protons. Group 3, metabolized by CYP1A in mammals, consists of congeners which have only *o-m* vicinal protons. Group 4, considered readily metabolizable as both of the above enzyme families can oxidize these congeners, consists of congeners which have both *m-p* and *o-m* vicinal protons. Through comparison of congener profiles with expression patterns of biotransformation enzymes, a better understanding of PCB metabolism and accumulation can be attained.

The present study measured the concentrations of PCBs in the livers of three species of sea turtles – loggerhead, green, and olive ridley from the Baja California peninsula of Mexico and evaluated species-specific variation in PCB accumulation. Congeners that bind the aryl hydrocarbon receptor (AhR) were grouped into toxic equivalency units (Van den Berg et al. 1998). Finally, profiles of CYP isoform and GST activities were compared to PCB congeners to explore biotransformation and accumulation differences among the three species of sea turtles.

Materials and Methods

Chemicals

Optima grade acetone and hexane, as well as ACS plus grade sulfuric acid were purchased from Fisher Scientific (Pittsburgh, PA, USA). The PCB standards were

purchased from AccuStandard Inc. (New Haven, CT, USA). GSH and CDNB were purchased from Sigma Chemical (St. Louis, Mo, USA). Various buffers, salts, and cofactors were purchased from Fisher Scientific or Sigma Chemical.

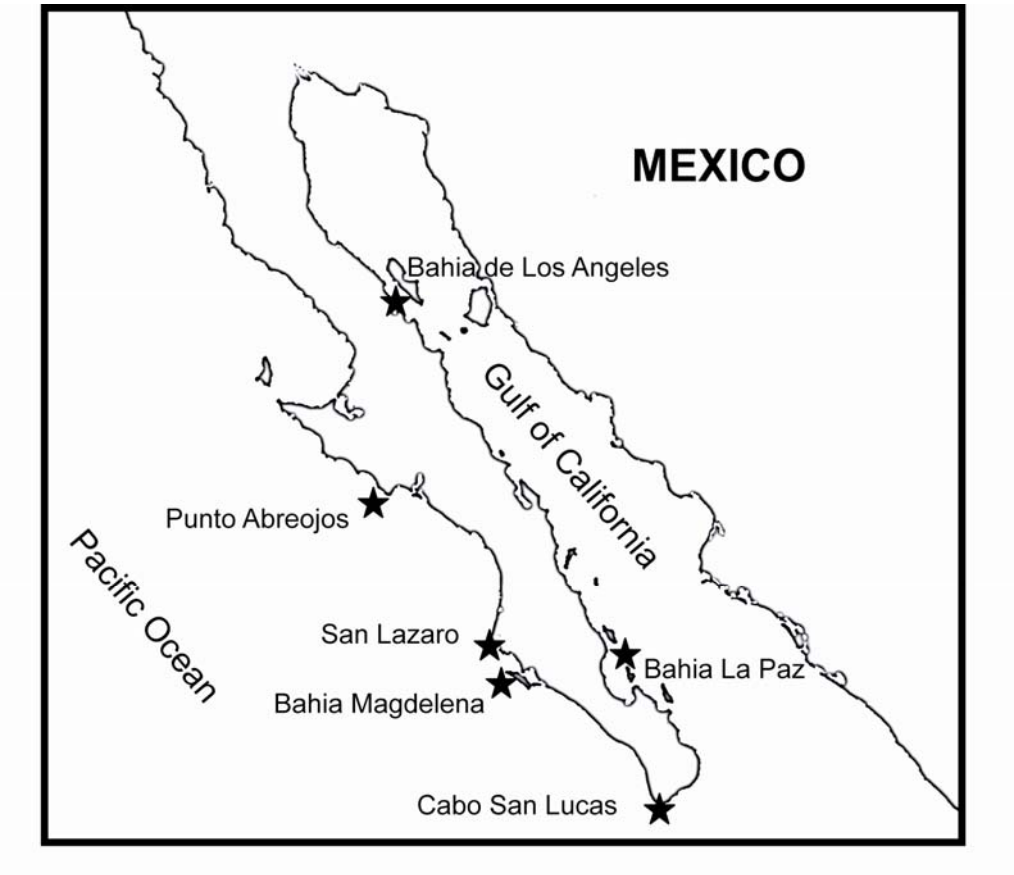

Figure 2.1. A Map of the Baja California peninsula. Stars indicate sampling locations

Sample Collection

Between 2001 and 2003, samples of liver tissue were collected from stranded wild-born loggerhead, green, and olive ridley sea turtles from the waters surrounding the Mexican states of Baja California and Baja California Sur (see Figure 2.1). Tissue

samples were obtained as a result of natural mortality, incidental fisheries or unknown causes of death and were only collected from animals for which time of death could be approximated within 24 hours. Turtles were necropsied and a sample of liver tissue was placed in aluminum foil and stored on ice for transport, then stored at -80°C until analysis. Table 2.1 provides specific features of the sampled individuals.

Table 2.1. Dietary preferences of adult sea turtles and information on individual specimens used in this study (adapted from Chapter 3).

			Specimen Information		
Species	Common Name	Preferred food	ID	Carapace Length (cm)	Sex
Chelonia mydas	green	sea grass, algae	CM-1	52.5	Female
			CM-2	58.5	Unknown
			CM-3	50	Unknown
			CM-4	55	Male
Caretta caretta	loggerhead	benthic invertebrates	CC-1	55	Male
			CC-2	66.5	Unknown
			CC-3	62	Female
			CC-4	57	Unknown
Lepidochelys olivacea	olive ridley	fish, salps	LO-1	61	Unknown
			LO-2	57	Unknown
			LO-3	61.5	Unknown
			LO-4	53	Unknown

Chemical Analysis

Approximately 3 g of liver tissue from 4 loggerhead, 3 green, and 4 olive ridley turtles was spiked with PCB 65 surrogate standard prior to homogenization with anhydrous sodium sulfate followed by extraction with hexane using an ultrasonic disruptor (550 Sonic Dismembrator, Fisher Scientific) according to EPA method 3550B. The extract was evaporated to 2 mL and 250 µL were removed for gravimetric lipid determination. The remaining extract was treated by vigorous vortexing with 2 mL of hexane-cleaned concentrated sulfuric acid. The acid layer was then back-extracted 2 times with hexane and the hexane fractions were combined and evaporated to 0.25 mL for GC-ECD analysis.

Sample extracts were amended with the internal standard PCB 30 prior to analysis using a Hewlett Packard HP-6890 gas chromatograph equipped with a ^{63}Ni electron capture detector (Wilmington, DE, USA) with hydrogen and nitrogen used as carrier and make-up gases, respectively. The column, a 60 m x 0.25 mm i.d. DB-5MS fused silica (J&W, Agilent Technologies, Santa Clara, CA, USA), was programmed from 50 to 200 °C at 30°C/min, held at this temperature for 1 min, raised to 220°C at 3°C/min, held at this temperature for 1 min, raised to 255 °C at 1.5°C/min, then raised to 290°C at 2°C/min and held at this temperature for 5 min. The chemical identity was verified using a 60 m x 0.25 mm i.d. DB-35 fused silica column (J&W) programmed from 50 to 190 °C at 1.5°C/min then raised to 320°C at 3.5°C/min. The inlet and detector temperatures were 250°C and 310°C, respectively

Quantification of individual PCB congeners (IUPAC PCB 8,18, 28, 44, 52, 66, 77, 81, 101, 105, 114, 118, 123, 126, 128, 138, 153, 156, 157, 167, 169, 170, 180, 187, 189, 195, 206, and 209) was performed using the relative response factors generated from the calibration curve of standards (Mullin et al. 1984). The total PCB (tPCB) concentration in samples was defined as the sum of these congeners.

Analytical performance was monitored using blanks, spiked samples, and surrogate recovery in all samples. PCB recoveries ranged from 70-95% and 71-103% for spiked samples and surrogates, respectively. The method detection limit ranged from 0.02 to 0.25ng/g.

Polychlorinated biphenyl congeners are present in the environment as mixtures, but the concentration of each congener in various compartments is controlled by physiochemical as well as biological processes. PCB 153, which is ubiquitous in the environment, lacks vicinal protons and therefore is generally considered recalcitrant to biotransformation (Safe 1984). In order to examine the metabolic influence on PCB profiles, metabolic ratios for each congener were calculated as molar PCB *X* / PCB153 (Kannan et al. 1995).

Toxic Equivalency Calculations

2,3,7,8-Tetrechlorodibenxo-p-dioxin (TCDD) equivalents (TEQs) were estimated for non-ortho and mono-ortho dioxin-like PCBs using bird toxic equivalency factors (TEFs) reported in 1998 by the World Health Organization (Van den Berg et al. 1998). Because TEFs have not been established for reptiles (but are available for mammals, fish,

and birds), bird TEFs were selected because of the three taxa; birds are most similar to reptiles.

Isolation of Subcellular Fractions

Liver tissue (1-2g/sample) from each sea turtle sample, as well as liver from laboratory-reared rainbow trout (*Oncorhynchus mykiss*) for use as controls, was homogenized, at 4°C in buffer (1:4 w/v) containing 0.1 M Tris HCl, 0.15 M KCl, 1 mM EDTA, and 0.1 mM phenylmethanesulphonylfluoride (PMSF), pH 7.4, with a Wheaton tissue grinder with a Teflon pestle (Wheaton Scientific Products, Millville, NJ, USA). The subcellular fractions were isolated by sequential centrifugation at 20,000*g* for 30 min and then at 100,000*g* for 90 min, both performed at 4°C to prevent protein degradation. After the final spin, the supernatant (cytosolic fraction) was aliquoted to clean tubes, and the pellet (microsomal fraction) was resuspended in buffer containing 0.1M potassium phosphate buffer, pH 7.4, with 20% glycerol and aliquoted. All subcellular fractions were then stored at -80°C until analysis. Protein concentrations of the fractions were determined using the microassay Bradford method (Brogdon and Dickinson 1983), stained with Coomassie Plus and Bovine Serum Albumin as a standard (both Pierce Biotechnology Inc, Rockford, IL, USA).

Anti-CYP Western Blotting

Due to limited biomass, profiles of CYP isoforms in sea turtles were evaluated by Western blotting using CYP anti-serum raised against fish CYP isoforms since antibodies

for sea turtle CYP isoforms are not available. Microsomal proteins (50 μg per lane), along with molecular weight markers (SeeBlue Plus2, Invitrogen, Carlsbad, CA, USA) were resolved using polyacrylamide gels (sodium dodecyl (SDS)-PAGE, 10% gradient). Four loggerhead, six green, and six olive ridley samples were used for these analyses. Rainbow trout microsomes from animals pre-treated with β-naphthoflavone (25 mg/kg twice over 4 days) were used as positive controls and were run on each gel for densitometry normalization. Resolved proteins were transferred to 0.45 μm nitrocellulose membranes using semi-dry transfer. Membranes were blocked with 5% non-fat milk in Tris buffered saline (TBS, 20 mM Tris, 150 mM NaCl, pH 7.5) for at least 30 min. Primary antibodies consisted of mouse anti-fish monoclonal CYP1A (Biosense, Bergen, Norway) and rabbit anti-rainbow trout polyclonal CYP2K1 and CYP3A27 antibodies (provided by Dr. D.R. Buhler, Oregon State University). Goat anti-mouse (CYP1A) and anti-rabbit (CYP2K1, CYP3A27) IgG alkaline phosphatase was used as the secondary antibody (BioRad, Hercules, CA, USA). Bands were visualized using a commercial alkaline phosphatase substrate conjugation kit (BioRad). Blots were scanned using a Biorad GelDoc. After measuring the density of each band with Quantity One software (BioRad), bands were normalized to the rainbow trout control run on each gel and expressed as relative optical density.

GST Activities

Cytosolic GST activities toward 1-chloro-2,4-dinitrobenzene (CDNB), was determined spectrophotometrically using the assays of Habig and Jakoby (Habig and

Jakoby 1981). Four loggerhead, four green, and four olive ridley samples were used for these analyses. Catalytic activity assays were conducted at 25°C, at pH 7.2 and were corrected for nonenzymatic activity. CDNB was dissolved in ethanol and prepared so that the final concentration of ethanol was less than 0.01% in the reaction. The reaction mixture (1 mL final volume) contained 30 μg of cytosolic protein, along with 1mM CDNB, 1mM GSH, and assay buffer [0.1M phosphate (pH 7.2)]. The reaction was started, after a 7-minute preincubation of GSH and protein in buffer, by adding CDNB, and the linear portion of change of absorbance was monitored over time. Duration and optimization of conditions is described elsewhere (Chapter 3).

Statistical Procedures

Statistical analyses (ANOVA and linear regression) were carried out using Minitab for Windows software (Minitab Inc., State College, PA, USA) and Prism 5 for Windows software (Graphpad Software Inc., La Jolla, CA, USA). PCB concentrations on a wet-weight basis were analyzed. For individual PCB congeners below detection limits, a random number between the detection limit and one-half of the detection limit was used for statistical analysis.

The GST activities were analyzed once corrected for nonenzymatic activity. For regression analysis, only data points for which both endpoints were available were included and outliers were removed when appropriate. If an overall significance was detected by ANOVA, Tukey's tests were performed in order to determine differences

among species or groups. Results are presented as mean ± standard deviation. A p-value of less than 0.05 was considered significant.

Results

PCB Concentrations

PCB concentrations and lipid content for loggerhead, green, and olive ridley turtles are given in Table 2.2 and the pattern of PCB congeners in all three species are given in Figure 2.2. Lipid content varied among individual samples (0.787 – 10.7%) but did not significantly differ across species ($p=0.96$). Furthermore, regression analysis showed no relationship between lipid content and total PCB (tPCB) wet weight concentrations (data not shown). Thus, wet weight concentrations were used for further analysis.

Table 2.2. Mean concentrations of PCBs (ng/g wet wt) and 2,3,7,8-TCDD equivalents (TEQs pg/g wet wt)

		loggerhead			green			olive ridley		
PCB	TEF[a]	Mean (SD[b])	*n* > DL[c]	TEQ (SD)	Mean (SD)	*n* > DL	TEQ (SD)	Mean (SD)	*n* > DL	TEQ (SD)
8		0.241 (0.481)	1		BDL[d]	0		1.22 (2.16)	2	
18		0.792 (0.919)	2		0.415 (0.718)	1		1.43 (1.67)	2	
28		BDL	0		BDL	0		BDL	0	
44		1.53 (2.34)	2		0.389 (0.338)	2		0.907 (0.230)	4	
52		BDL	0		BDL	0		0.343 (0.686)	1	
66		1.46 (1.35)	3		0.884 (1.53)	1		0.0748 (0.150)	1	
77	0.05	0.184 (0.367)	1	9.18 (1.84)	0.507 (0.639)	2	22.0 (26.3)	BDL	0	>0.05
81	0.1	BDL	0	>0.1	BDL	0	>0.1	BDL	0	>0.1
101		0.106 (0.213)	1		0.561 (0.971)	1		1.14 (2.27)	1	
105	0.0001	1.08 (0.631)	4	0.106 (0.0659)	0.525 (0.126)	3	0.0496 (0.0172)	0.775 (0.246)	4	0.0746 (0.0194)
114	0.0001	0.202 (0.234)	2	0.0113 (0.0200)	0.0773 (0.134)	1	0.000770 (0.00133)	0.382 (0.165)	4	0.0382 (0.0165)
118	0.00001	0.856 (0.762)	3	0.00855 (0.00760)	BDL	0	>0.00001	0.835 (0.951)	3	0.00835 (0.00951)
123	0.00001	1.81 (1.28)	4	0.0176 (0.0124)	0.304 (0.372)	2	0.00304 (0.00372)	0.970 (0.541)	4	0.00969 (0.00539)
126	0.1	0.338 (0.676)	1	32.6 (65.262)	BDL	0	>0.1	0.0982 (0.196)	1	9.82 (19.6)
128		0.687 (0.684)	3		0.424 (0.165)	3		0.198 (0.231)	2	
138		2.43 (0.427)	4		1.147 (0.288)	3		1.88 (0.757)	4	
153		4.17 (1.09)	4		2.86 (0.992)	3		3.49 (1.03)	4	
156	0.0001	0.159 (0.184)	2	0.0159 (0.06585)	0.593 (0.593)	2	0.0532 (0.05038)	0.334 (0.259)	3	0.0334 (0.0259)
157	0.0001	BDL	0	>0.0001	0.127 (0.221)	1	0.0127 (0.0221)	0.0667 (0.0770)	2	0.0334 (0.00642)
167	0.00001	0.448 (0.564)	3	0.00442 (0.00567)	BDL	0	>0.00001	0.0296 (0.0591)	1	0.000296 (0.000591)

		loggerhead			green			olive ridley		
PCB	TEF[a]	Mean (SD[b])	*n* > DL[c]	TEQ (SD)	Mean (SD)	*n* > DL	TEQ (SD)	Mean (SD)	*n* > DL	TEQ (SD)
169	0.001	1.257 (1.13)	3	0.878 (1.00)	0.839 (0.900)	3	0.823 (0.910)	0.400 (0.390)	3	0.368 (0.423)
170		0.670 (0.695)	3		0.763 (0.164)	3		0.295 (0.380)	2	
180		0.201 (0.289)	2		0.116 (0.201)	1		0.561 (0.183)	4	
187		0.693 (0.879)	2		0.125 (0.217)	1		BDL	0	
189	0.00001	BDL	0	>0.00001	0.306 (0.278)	2	0.00306 (0.00278)	0.304 (0.377)	2	0.00129 (0.00210)
195		BDL	0		BDL	0		BDL	0	
206		BDL	0		BDL	0		0.249 (0.498)	1	
209		BDL	0		BDL	0		BDL	0	
tPCB[e]		19.3 (5.80)			10.5 (4.92)			15.2 (6.17)		
tDioxin-like[f]		5.76 (2.10)			3.04 (2.05)			3.93 (0.849)		
tTEQ[g]				42.8 (62.2)			22.9 (27.2)			10.4 (19.4)
percent lipid		5.05 (4.83)			4.44 (2.67)			5.24 (3.26)		

[a] bird Toxic Equivalent Factors (Van den Berg et al. 1998); [b] SD = standard deviation; [c] n>DL number of samples above detection limit; [d] BDL = below detection limit; [e] tPCB = total PCB concentration; [f] tDioxin-like = total dioxin-like concentration; [g] tTEQ = total TEQ concentration

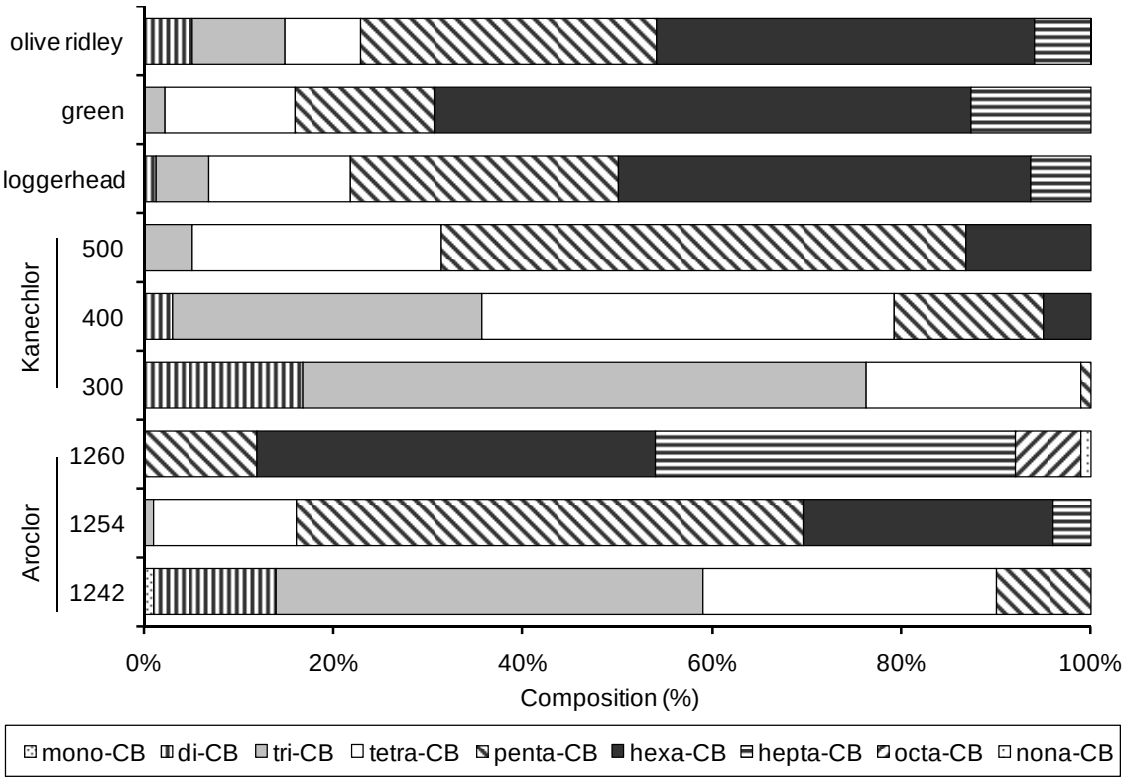

Figure 2.2. Percent composition of PCB congeners in loggerhead, green, and olive ridley turtles. Also presented are the compositions of two commercial PCB mixtures.

In each sample the number of congeners detected ranged from 7 to 17. Several PCB congeners (28, 81, 195, and 209) were not detected in any sample and were removed from further analysis. Three congeners, which were detected in all samples, 105, 138, and 153, constituted 33 - 62% of the tPCB concentration in each sample, with 153 as the most abundant congener (Table 2.2). The tPCB concentration was determined as the sum of the concentrations of each congener detected in any particular sample. Total PCB concentrations ranged considerably, from 5.23 ng/g in one green turtle to 24.9 ng/g in one

loggerhead turtle. Mean tPCB concentrations were 18.1, 10.5, and 15.2 for loggerhead, green, and olive ridley turtles, respectively. However, no significant differences were found in the tPCB concentrations between species ($p=0.451$). In the green turtles, a negative trend was found between carapace length (CL) and tPCB concentration ($r^2=0.99$, $p=0.06$), yet CL was not associated with tPCB concentrations in loggerhead or olive ridley turtles (data not shown).

Homologue Composition

The PCB profiles of all three species showed somewhat similar patterns. All three were dominated by higher chlorinated homologues. Hexachlorinated biphenyls were the predominant homologues, accounting for 36 - 74% of the tPCB concentration (Figure 2.2). Pentachlorinated biphenyls also contributed considerably to tPCBs, accounting for an additional 12-44%. Di-, tri-, tetra-, and octachlorinated biphenyls also contributed to tPCBs in all three species, but to a lesser degree.

Dioxin-like PCBs and TEQs

Co-planar or dioxin-like PCB congeners (77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169, and 189) accounted for 18-46% of the tPCB concentrations in each sample (Table 2.2). With the exception of one loggerhead and one olive ridley turtle in which none were detected, non-*ortho*-PCBs contributed more than 70% of the total TEQ. Mean total TEQ (tTEQ) were 42.8, 22.2, and 10.4 pg/g in loggerhead, green, and olive ridley turtles, respectively. However, no significant differences were found in the tPCB

concentrations between species (p=0.326). In the loggerhead turtles, a positive trend was found between CL and tTEQ concentration (data not shown, r^2=0.90, p=0.06). However, no relationship was observed in green or olive ridley turtles (data not shown).

Metabolic group profiles

Polychlorinated biphenyl load normalized to congener 153 (sum molar X/153 ratios) and sorted by the various metabolic groups based on vicinal proton availability are shown in Figure 2.3. For each species, group 2 and group 4 were significantly lower than both group 1 and group 3 ($p<0.05$). No significant differences were found between species.

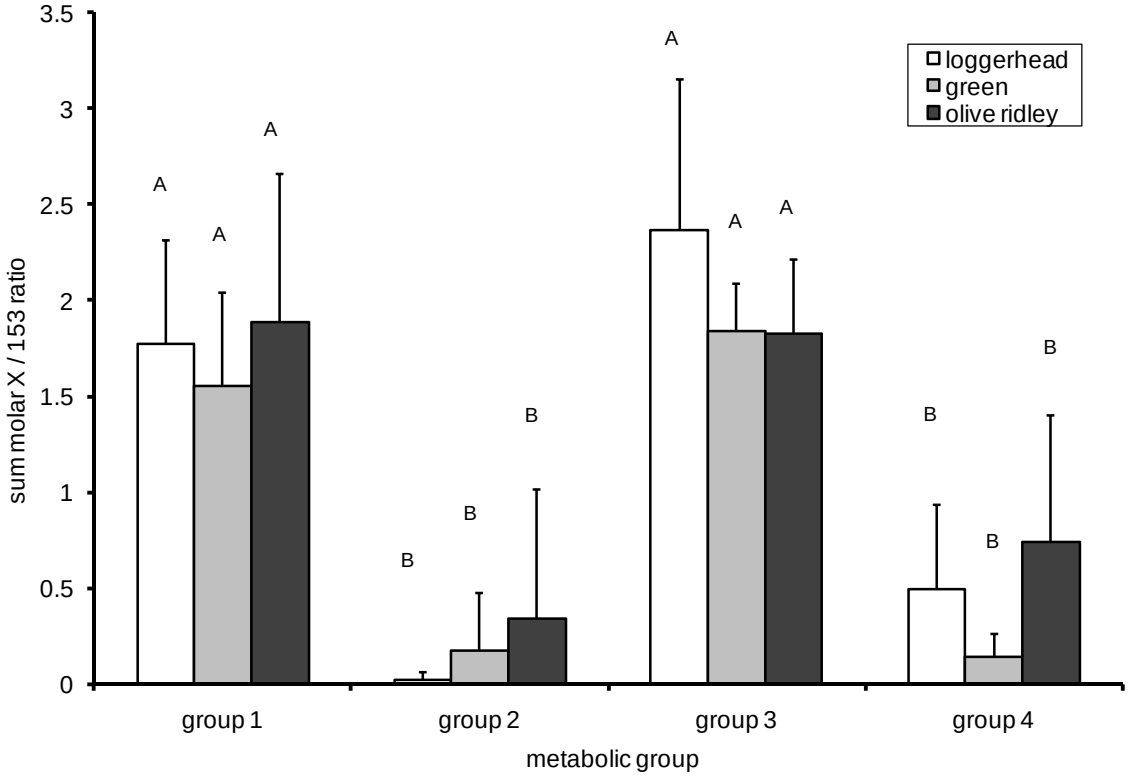

Figure 2.3. Sum contribution of PCBs (molar X / PCB 153) metabolic groups for loggerhead (white bars), green (gray bars), and olive ridley (black bars) turtles. Error bars indicate standard deviation. Different letters indicate significant difference within species ($p<0.05$)

CYP Western Blotting

Hepatic microsomal protein showed no cross-reactivity with the anti-fish CYP1A antibody, yet did cross-react with the anti-trout CYP2K1 and anti-trout CYP3A27 antibodies (Figure 2.4). There was a high degree of variability in the patterns of bands between individuals – some turtles exhibited a singlet band while others displayed doublets. No correlations were observed when comparing CYP expression with tPCB or PCB metabolic groups.

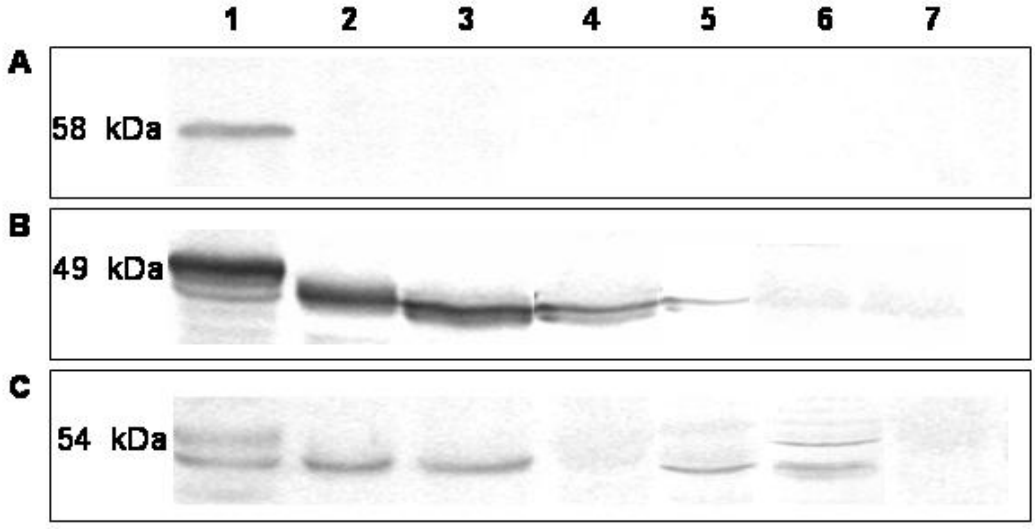

Figure 2.4. Representative Western blots of microsomal A) CYP1A, B) CYP2K1, and C) CYP3A27 proteins for rainbow trout positive control (lane 1), green (lanes 2 & 3), loggerhead (lanes 4 & 5), and olive ridley turtles (lanes 6 & 7)

GST activities toward reference substrates

No significant differences in cytosolic GST activity were observed between species. CDNB activities (± SD) in olive ridley were 877 ± 300 nmol/min/mg, and 1103 ± 267 nmol/min/mg in green sea turtles. In loggerhead turtles, the CDNB activity was 1047 ± 436 nmol/min/mg. GST activities showed no significant correlation with tPCB, or metabolic groups of PCBs.

Discussion

Few studies have investigated the levels of PCB contamination in sea turtles worldwide, and data for sea turtles from the Eastern Pacific are particularly sparse. The total PCB concentration of the sea turtle livers analyzed in the current study fall within the range of reported concentrations (< 3.0 – 58.1 ng/g) from the one study on sea turtles from the Baja California (BC) region of Mexico (Gardner et al. 2003). Yet, when compared to reported PCB levels in the livers of sea turtles worldwide (< 1.0 – 1950 ng/g), BC turtles contain relatively low levels (Lake et al. 1994; Rybitski et al. 1995; McKenzie et al. 1999; Corsolini et al. 2000; Storelli and Marcotrigiano 2000; Miao et al. 2001; Storelli et al. 2007). The locality of sample collection may explain these differences, as BC is a relatively unindustrialized region of the world and would therefore be expected to have lower pollution levels than more industrialized regions. This is supported by studies of other marine inhabitants; for example, tPCB levels in California sea lions (*Zalophus californianus californianus*) from BC were found to be more than one order of magnitude less than tPCB in sea lions from various locations throughout California, USA (Toro et al. 2006).

The contributions of individual PCB congeners to tPCBs in loggerhead, green, and olive ridley turtles presented a similar overall pattern. The two predominant congeners, 153 and 138, showed similar relative contributions in all three species. Congener 153 is typically the most prevalent congener reported in terrestrial and marine organisms due to the lack of vicinal protons necessary for biotransformation (Safe 1984). Furthermore, the predominance of 153 and 138 and hexachlorinated congeners is

consistent with patterns reported for sea turtles worldwide, including loggerhead turtles in the Mediterranean area (Corsolini et al. 2000; Storelli and Marcotrigiano 2000; Storelli et al. 2007), green turtles in the Pacific (Miao et al. 2001), loggerhead and Kemps' ridley (*Lepidochelys kempii*) turtles in the Atlantic (Rybitski et al. 1995; Keller et al. 2004a).

On the basis of trophic level, species differences in PCB accumulation were previously predicted in a qualitative examination of one carnivorous olive ridley and one carnivorous loggerhead as compared to seven herbivorous green turtles (Gardner et al. 2003). In contrast, the current data set, consisting of 4 loggerhead, 3 green, and 4 olive ridley turtles failed to show significant differences in mean tPCB levels. However, species differences were observed for liver concentrations of non-ortho congeners.

Four non-*ortho* PCBs (77, 81, 126, and 169) and eight mono-*ortho* PCBs (105, 114, 118, 123, 156, 167, 189) are stereoisomers of the highly toxic 2,3,7,8-tetrechlorodibenxo-p-dioxin (TCDD) and have been shown to act through the same mechanism of action: binding to the aryl hydrocarbon receptor (AhR). The relative potency of dioxin-like PCBs can thus be calculated as TCDD toxic equivalents (TEQs) using the congener concentrations and Toxicity Equivalency Factors (TEFs), which are based on taxa-specific relative potency at the AhR relative to TCDD (Safe 1992, 2001). Because reptile TEFs have not been determined, TEFs for birds, which range from 0.00001 to 0.1, were used in the present study (Van den Berg et al. 1998). For all three species, non-*ortho* congeners, accounted for more than 70% of the TEQ, similar to TEQs calculated previously for Mediterranean loggerhead turtles (Storelli et al. 2007). Congener 126 was observed in livers from one carnivorous loggerhead and one

carnivorous olive ridley species, but was not observed in any herbivorous green sea turtle. Congener 126 represents less than 0.01% of commonly used Aroclors (Frame 2001) and thus detection in these turtles may indicate bioaccumulation. The more abundant congener 77 was observed in two green and one loggerhead, but not any olive ridley turtle. The occurrence of both congeners in loggerheads suggests carnivorous species may be more prone to accumulation of non-ortho PCBs. The variability between turtles may result from a number of life-history factors. As oviparous organisms, sea turtle exposure to lipophilic contaminants starts in the egg and the turtles in this study may have emerged from the shell with differing levels of various PCBs depending on maternal transfer. Maternal transfer of PCBs, PHAHs, and pesticides has been observed in snapping turtles (*Chelydra serpentina*) (Bishop et al. 1994; Kelly et al. 2008) and alligators (*Alligator mississippiensis*) (Rauschenberger et al. 2004). Additionally, whereas the diet of adult turtles (see Table 2.1) has been extensively studied through stomach content analysis, juveniles tend to show opportunistic feeding strategies (Bjorndal 1997) which could influence the PCB profiles of individual turtles.; for example, a juvenile green turtle may prey upon organisms of a higher trophic level (and therefore higher predicted accumulated PCBs) than it would as an adult, and therefore could exhibit growth-dilution of accumulated congeners. Additionally, it is important to note the incidental ingestion of marine debris, particularly plastics, that has been found in many sea turtles, both juveniles and adult (Bjorndal 1997). Adsorption of PCBs to the surface of hydrophobic plastics results in accumulation of PCBs at levels up to 10^6 higher

than surrounding sea water (Mato et al. 2001); thus, ingestion of debris may add variability to the concentrations of PCBs in the sea turtles.

The TEQs reported in the present study are higher than those reported for livers of loggerhead turtles in the Mediterranean Sea (Corsolini et al. 2000; Storelli et al. 2007), but lower than those reported in the eggs of snapping turtles from the Great Lakes (Dabrowska et al. 2006) and lower than the proposed TEQ LOEL (210 pg/g) for bald eagle chicks (Elliott et al. 1996). The discrepancy between TEQs for sea turtles may be based on the greater number of congeners detected in BC sea turtles. In loggerhead sea turtles, dioxin-like congeners and TEQs correlated with increased heterophil-lymphocyte ratios (Keller et al. 2004c), a general indicator for disease in sea turtles (Aguirre et al. 1995; Cray et al. 2001; Work et al. 2001), suggesting the possibility of dioxin-like PCB induced immune modulation. In Canadian snapping turtle (*Chelydra serpentina*) populations, TEQs were found to be only weakly correlated with abnormal egg and hatchling development (Bishop et al. 1991; Bishop et al. 1998), suggesting that the toxicological significance of dioxin-like compounds in reptiles is not fully understood.

Biotransformation plays a critical role in the accumulation of xenobiotics. CYP can catalyze the direct insertion of a hydroxyl group on a precursor PCB congener or arene oxide formation (Brouwer et al. 1986; Letcher et al. 2000). Arene oxide intermediates can be conjugated with GSH, catalyzed by GST, and the conjugate can then follow the mercapturic acid pathway to eventually produce methylsulfone PCB metabolites (Bakke 1989; Vermeulen 1996; Letcher et al. 2000). GST (CDNB) activity in loggerhead, green, and olive ridley sea turtles in the present study were within the

range reported for green turtles collected in BC (Valdivia et al. 2007), similar to those reported for red-eared slider turtles (*Trachemys scripta elegans*) (Willmore and Storey 1997a, b), but much higher than those reported for painted turtles (*C. picta*) (Rie et al. 2000). As CDNB is a generic substrate for GST, class-specific reactions known to conjugate arene oxides (i.e. GSTP) may indicate more specific interactions.

Sea turtle microsomal proteins cross-reacted with anti-CYP2K1 and anti-CYP3A27 antibodies, but not with anti-CYP1A antibody. In alligators (*Alligator mississippiensis*), immunoblotting with anti-CYP2K also revealed proteins, which showed induction with phenobarbital and 3-methylcholanthrene and slight induction when treated with Aroclor 1254 or the nonplanar PCB 47 (Ertl et al. 1999). When stained with anti-CYP3A27 antibody, the alligator hepatic microsomes showed very low constitutive expression. However, expression was induced by phenobarbital and 3-methylcholanthrene, but not by Aroclor 1254 or PCB 47 (Ertl et al. 1999). The lack of correlation of sea turtle PCBs to CYP2K1 and CYP3A27 bands and the induction patterns seen in alligators suggests that differences exist in CYP isofrom expression as compared to mammals.

The CYP1 enzymes are induced in vertebrates by planar aromatic hydrocarbons via the AhR (Safe 2001). Despite the presence of planar PCBs, immunoblotting with an antibody, which recognizes a conserved peptide sequence of vertebrate CYP1A failed to indicate the presence of CYP1A in sea turtles. This antibody was developed to recognize amino acids 277-294, which is a conserved region in all vertebrates (Rice et al., 1998). Studies in our lab with human and rat liver microsomes have shown that the antibody

recognized CYP1A (data not shown). Thus, it is unlikely the antibody failed to recognize the homologous turtle protein. Unfortunately, due to biomass limitations CYP1A catalytic activities were not performed. These results support a growing body of evidence for unique regulation and species variability of CYP1A in reptiles. In Kemp's ridley sea turtles, low P450 1A activity was observed in four partially purified P450 enzymes from the liver of wild Kemp's ridley turtles (Goldman and McClellan-Green 2001). Treatment with 400 mg/kg of Aroclor 1254 caused a 4-fold to 5-fold induction of CYP1A in the freshwater turtles *Chrysemys picta picta* and *C. picta elegans*, but much lower induction in another species of freshwater turtle, *Mauremys caspica rivulata* (Yawetz et al. 1997). Furthermore, tissues of *M. caspica rivulata* from sewage oxidation ponds showed no CYP1A induction, although induction was observed in other species, suggesting variability in responsiveness to CYP1A inducers (Yawetz et al. 1997). Reduced responsiveness to mammalian CYP1A inducers was demonstrated in African brown house snake (*Lamprophis fuliginosus*) primary hepatocytes, in which only treatment with 2 potent CYP1A inducers, TCDD and PCB 126, resulted in dose-dependent increased CYP1A activity, whereas other CYP1A inducers, PCB 77, PCB 81, and PCB 169, caused no induction (Hecker et al. 2006). The mean EC_{50} values calculated for *in vitro* induction of CYP1A activity in these snakes were 51.52 ng/L (0.16 nM) and 2.67 µg/L (8.3 nM) for TCDD and PCB 126, respectively (Hecker et al. 2006). Although mean concentrations of PCB 126 in BC sea turtles (below detection limit (BDL) – 0.338 ng/g) were well below the PCB126 values, the mean calculated TEQs in BC sea turtles are just slightly lower than the TCDD EC_{50}, suggesting that levels of dioxin-like

PCB congeners in BC sea turtles may have been adequate to elicit a response in other reptiles. However, concentration response studies would be necessary to confirm this.

Structure-activity relationships suggest that PCBs can be grouped on the basis of vicinal proton position (Parke 1985; Boon et al. 1989; McFarland and Clarke 1989). In all three species of sea turtles examined in the current study, molar ratios of group 3 congeners, which possess only *o-m* vicinal protons and are substrates for CYP1A in mammals, were significantly higher than that of group 2 and 4 congeners, which contain *m-p* vicinal protons and are CYP2B substrates. In fact, group 3 congeners showed no difference from the poorly-metabolized group 1 congeners, which lack vicinal protons. The same pattern (higher relative levels of CYP1A substrates/congeners and low levels of CYP2B substrates/congeners) was observed in loggerhead and Kemp's ridley turtles in the Atlantic (Keller et al. 2004a). Thus, the specific accumulation of CYP1A substrates in sea turtles may indicate poor biotransformation which is consistent with limited CYP1A expression in these species.

In conclusion, PCB levels in loggerhead, green, and olive ridley turtles from the BC region of Mexico are generally lower than those reported for turtles from more polluted regions of the globe. The presence of dioxin-like congeners and relatively high TEQs along with a lack of CYP1A expression suggest a potential mechanism of accumulation of group 1 congeners. Future studies may clarify the contributions of CYP and GST isoforms toward the biotransformation and accumulation of PCBs in sea turtles.

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Chapter 3: The Characterization of Cytosolic Glutathione Transferase from Four Species of Sea Turtles – Loggerhead (*Caretta caretta*), Green (*Chelonia mydas*), Olive Ridley (*Lepidochelys olivacea*), and Hawksbill (*Eretmochelys imbricata*)

Abstract

Glutathione *S*-transferases (GST) play a critical role in the detoxification of exogenous and endogenous electrophiles, as well as the products of oxidative stress. As compared to mammals, GST activity has not been extensively characterized in reptiles. Throughout the globe, most sea turtle populations face the risk of extinction. Of the natural and anthropogenic threats to sea turtles, the effects of environmental chemicals and related biochemical mechanisms, such as GST catalyzed detoxification, are probably the least understood. In the present study, GST activity was characterized in four species of sea turtles with varied life histories and feeding strategies: loggerhead (*Caretta caretta*), green (*Chelonia mydas*), olive ridley (*Lepidochelys olivacea*), and hawksbill (*Eretmochelys imbricata*). Although similar GST kinetics were observed between species, rates of catalytic activities using class-specific substrates show inter- and intra-species variation. GST from the spongivorous hawksbill sea turtle shows 3-4.5 fold higher activity with the substrate 4-nitrobenzylchloride than the other 3 species. GST from the herbivorous green sea turtle shows 3 fold higher activity with the substrate ethacrynic acid than the carnivorous olive ridley sea turtle. The results of this study may provide insight into differences in biotransformation potential in the four species of sea turtles and the possible health impacts of contaminant biotransformation by sea turtles.

Introduction

Glutathione *S*-transferases (GSTs, EC 2.5.1.18) are a multigene family of enzymes that play a critical role in the detoxification of the exogenous and endogenous electrophiles, as well as the products of oxidative stress. GST proteins have been found in a wide range of eukaryotes, as well as certain prokaryotes, such as cyanobacteria, which use glutathione (GSH) as their major intracellular thiol (Vuilleumier and Pagni 2002). GSTs have been assigned to various classes based on sequence homology. Generally, GSTs share 40% or greater homology within a class (and between classes, less than 25% sequence identity) (Hayes and Pulford 1995). The major mammalian classes include alpha (GSTA), mu (GSTM), pi (GSTP), sigma (GSTS), theta (GSTT), zeta (GSTZ), and omega (GSTO) while additional classes of GSTs, including beta (GSTB), delta (GSTD), epsilon (GSTE), lambda (GSTL), phi (GSTF), rho (GSTR), and tau (GSTU) have been identified in nonmammalian organisms, such as fungi, plants, insects, and fish (Blanchette et al. 2007).

The primary reaction catalyzed by GSTs is the nucleophilic attack by reduced GSH on a diverse group of hydrophobic compounds which contain an electrophilic carbon, nitrogen, or sulfur atom. GSTs are present in most tissues at levels ranging from 5-100 μM (Hayes and Pulford 1995). While nuclear, microsomal and mitochondrial GSTs have been identified, cytosolic GSTs are the most prominent. In fact, GSTs can account for up to 10% of cytosolic proteins (Will 1999). In vertebrates, GSTs are distributed throughout most tissues - especially lungs, heart, intestines, and liver – and are

expressed in a tissue-specific manner through constitutive and responsive mechanisms (Vos and Van Bladeren 1990; Awasthi et al. 1994).

Environmental toxicants detoxified by GSTs include polyaromatic hydrocarbons, pesticides, and reactive intermediates produced by phase I biotransformation and other biochemical reactions, and thus GST expression is of importance when considering susceptibility to toxicity by environmental chemicals. For example, GST is a major contributor to the cellular defense mechanism against DNA damage caused by diol epoxides of PAHs (Xiao and Singh 2007). Furthermore, exposure to environmental toxicants can affect the biochemical responses of exposed organisms. Particularly interesting is the determination of two mechanisms of GST induction by xenobiotics: these enzymes can be regulated by transcription factors which are directly activated by xenobiotics, or regulation can occur through signaling cascades which are activated by cellular stress (Xu et al. 2005). Thus, GST expression and activity contributes to an adaptive response to toxic stress within an organism (Hayes and Pulford 1995).

GSTs also contribute to the detoxification of natural products. In humans, several GSTs contribute to the detoxification of carcinogenic heterocyclic amines produced by cooking protein-rich food (Coles et al. 2001). Furthermore, the ability of insects to tolerate dietary phytotoxins has been linked to constitutively high levels of GST activity (Li et al. 2007). GST expression and activity may help explain the evolution of dietary preferences, and the ability of certain animals to exploit chemically-defended prey.

Little is known about GST expression or activity in sea turtles – in fact, only one paper reports GST activity in the green sea turtle, *Chelonia mydas* (Valdivia et al. 2007). Most sea turtle populations throughout the world are considered threatened or endangered (IUCN 2008), facing both anthropogenic and natural stressors throughout their life. Sea turtles are slow growing, late maturing, and long lived organisms, which makes them particularly susceptible to population decline caused by toxicity associated with bioaccumulation (Rowe 2008). In sea turtles, harmful effects from anthropogenic stressors include chronic stress, compromised physiology, impaired immune function, and increase in susceptibility to disease (Aguirre and Lutz 2004). Because of their role in detoxification of endogenous and exogenous chemicals, GSTs may play a critical role in mitigating the potential toxic insult from anthropogenic stressors in sea turtles.

The aims of the present study were to characterize and compare cytosolic GST activity and kinetics in four species of sea turtles with varied life histories and feeding strategies found in the coastal regions of Mexico – green (*C. mydas*), loggerhead (*Caretta caretta*), olive ridley (*Lepidochelys olivacea*), and hawksbill (*Eretmochelys imbricata*) sea turtles in order to obtain a better understanding of the potential role GSTs may play in the physiological response of sea turtles to xenobiotic exposure.

Materials and Methods

Chemicals

Reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid (ECA), and 4-nitrobenzyl chloride (NBC) were purchased from Sigma Chemical (St. Louis, MO, USA). Δ5-androstene-3,17-dione (ADI) was purchased from Steraloids (Wilton, NH, USA). Various buffers, salts, and cofactors used at UCR were purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma Chemical, while those used at Cinvestav were purchased from JT Baker (Phillipsburg, NJ, USA), Productos Químicos Monterrey (Mexico City, DF, MX), Invitrogen (Carlsbad, CA, USA), or Sigma Chemical.

Sample Collection

Four species of sea turtles, loggerhead, olive ridley, green, and hawksbill, inhabitating the coastal regions of Mexico were examined in this study. The four species utilized in this study demonstrate different life histories. The dietary preference of adult sea turtles has been extensively studied through stomach content analysis (Bjorndal 1997), and the various species generally exhibit preferred food sources as shown in Table 3.1.

Table 3.1. Dietary preferences of adult sea turtles (adapted from Bjorndal 1997) and information on individual specimens used in this study.

			Specimen Information		
Species	Common Name	Preferred food	ID	Carapace Length (cm)	Sex
Chelonia mydas	green	sea grass, algae	CM-1	52.5	Female
			CM-2	58.5	Unknown
			CM-3	50	Unknown
			CM-4	55	Male
Caretta caretta	loggerhead	benthic invertebrates	CC-1	55	Male
			CC-2	66.5	Unknown
			CC-3	62	Female
			CC-4	57	Unknown
Eretmochelys imbricata	hawksbill	sponges	EI-1	Immature	
			EI-2	Immature	
			EI-3	Immature	
Lepidochelys olivacea	olive ridley	fish, salps	LO-1	61	Unknown
			LO-2	57	Unknown
			LO-3	61.5	Unknown
			LO-4	53	Unknown

Tissue samples were obtained as a result of natural mortality, incidental fisheries or unknown causes of death and were only collected from animals for which time of death could be approximated. Turtles were necropsied, the liver was removed, and placed on dry ice for subsequent subcellular fractionation. Liver samples from 4 loggerhead, 4 green, 4 olive ridley, and 3 hawksbill samples were used for these analyses,

and information on each individual specimen is included in table 3.1. Methods for the collection of liver tissue from wild loggerhead, green, and olive ridley turtles was described elsewhere (Chapter 2). In July 2007, samples of liver tissue from wild hatchling hawksbill sea turtles were collected from nesting beaches near Celestun, Yucatan, Mexico from animals for which time of death could be approximated within 6 hours.

Isolation of Subcellular Fractions

Liver subcellular fractions were isolated from liver samples using methods similar to those described elsewhere (Chapter 2). Briefly, each liver sample (approximately 0.5 g) was homogenized then subjected to sequential centrifugation at 4°C to isolate microsomal and cytosolic fractions. The cytosolic fraction was aliqouted, transported on dry ice, and stored at -80°C until analysis. Protein concentrations of the fractions were determined using the microassay Bradford method (Brogdon and Dickinson 1983), stained with Coomassie Plus and Bovine Serum Albumin as a standard (both Pierce Biotechnology Inc, Rockford, IL, USA).

GST kinetics studies

GST enzyme kinetics for CDNB were examined spectrophotometrically by varying concentrations of GSH and CDNB using the assays of Habig and Jakoby (1981). All assay incubations were conducted at 25°C. CDNB was dissolved in ethanol, with the final reaction concentration less than 0.01%, and GSH was dissolved in buffer. For all

assays, the reaction mixture (1 mL final volume) contained 30 μg of cytosolic protein, along with substrate, GSH, and assay buffer - 0.1M phosphate buffer for CDNB (pH 7.2). The approximate pH optima for freshwater turtle GSTs was reported as 7.2 (Willmore and Storey, 2005), so this pH was selected for the general substrate CDNB. The reaction was started, after 7-minute preincubation of GSH and protein in buffer, by adding the appropriate substrate, and the linear portion of change of absorbance, as determined through preliminary studies, was monitored over time using a Shimadzu 1601 UV/Visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The apparent enzyme substrate affinity (K_m) and maximum velocity (V_{max}) values for GSH were determined using a GSH range of 0.0625 to 1 mM and a fixed CDNB concentration of 1 mM. The apparent K_m and V_{max} for CDNB were determined using a CDNB range of 0.0625 to 1 mM and a fixed GSH concentration of 1 mM. The kinetic parameters were determined using non-linear regression in Graphpad Prism (Graphpad Software, San Diego).

GST activities

*C*ytosolic GST activities toward 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid (ECA), 4-nitrobenzyl chloride (NBC), and Δ5-androstene-3,17-dione (ADI) were determined spectrophotometrically using the assays of Habig and Jakoby (1981) in mixtures similar to those above except where noted. CDNB, NBC, and ECA were initially dissolved in ethanol, while ADI was dissolved in methanol and all were prepared so that the final concentration of alcohol was less than 0.01% in the reaction. Substrate and GSH concentrations were: 1mM and 1mM for CDNB assays; 0.25mM and 0.5mM

for NBC assays; 0.25mM and 1mM for ECA assays; and 0.05mM and 0.1mM for ADI assays, respectively. Assay buffers were 0.1M phosphate buffer for CDNB (pH 7.2), NBC (pH 6.5), and ECA(pH 6.5); 25 mM Tris-HCl for ADI (pH 8.3). For ECA, preliminary studies using buffers prepared over a range of pH values were conducted in order to approximate the optimal pH value of 6.5. All GST catalytic activity assays were corrected for nonenzymatic activity.

Statistical analysis

Statistical analyses (ANOVA) were carried out using Minitab® for Windows software (Minitab Inc., State College, PA, USA) and Prism 5 for Windows software (Graphpad Software Inc., La Jolla, CA, USA). GST activities were analyzed once corrected for nonenzymatic activity. If an overall significance was detected by ANOVA, Tukey's tests were performed in order to determine differences among species or groups. Results are presented as means ± standard deviation. A p value of less than 0.05 was considered significant.

Results

All four species of sea turtles demonstrated classic Michaelis-Menton enzyme saturation curves for both varying concentrations of CDNB and GSH (Figure 3.1). Apparent Km and Vmax values derived from these plots, as well as catalytic efficiency (Vmax/Km) are shown in Table 3.2. Species comparisons with the other GST substrates indicated hawksbill turtles showed significantly higher activity towards NBC than the other three species ($p<0.05$, Figure 3.2a). A trend of lower hepatic ECA activity was observed in hawksbill and olive ridley relative to green turtles (Figure 3.2b). However, only the olive ridley ECA activity was significantly lower than that of green turtles ($p<0.05$). No significant differences in ADI activity were found between the four species (Figure 3.2c). It is important to note that, with the exception of high NBC activity, hawksbill turtles showed a general trend of lower GST apparent kinetic constants and activities towards the other substrates.

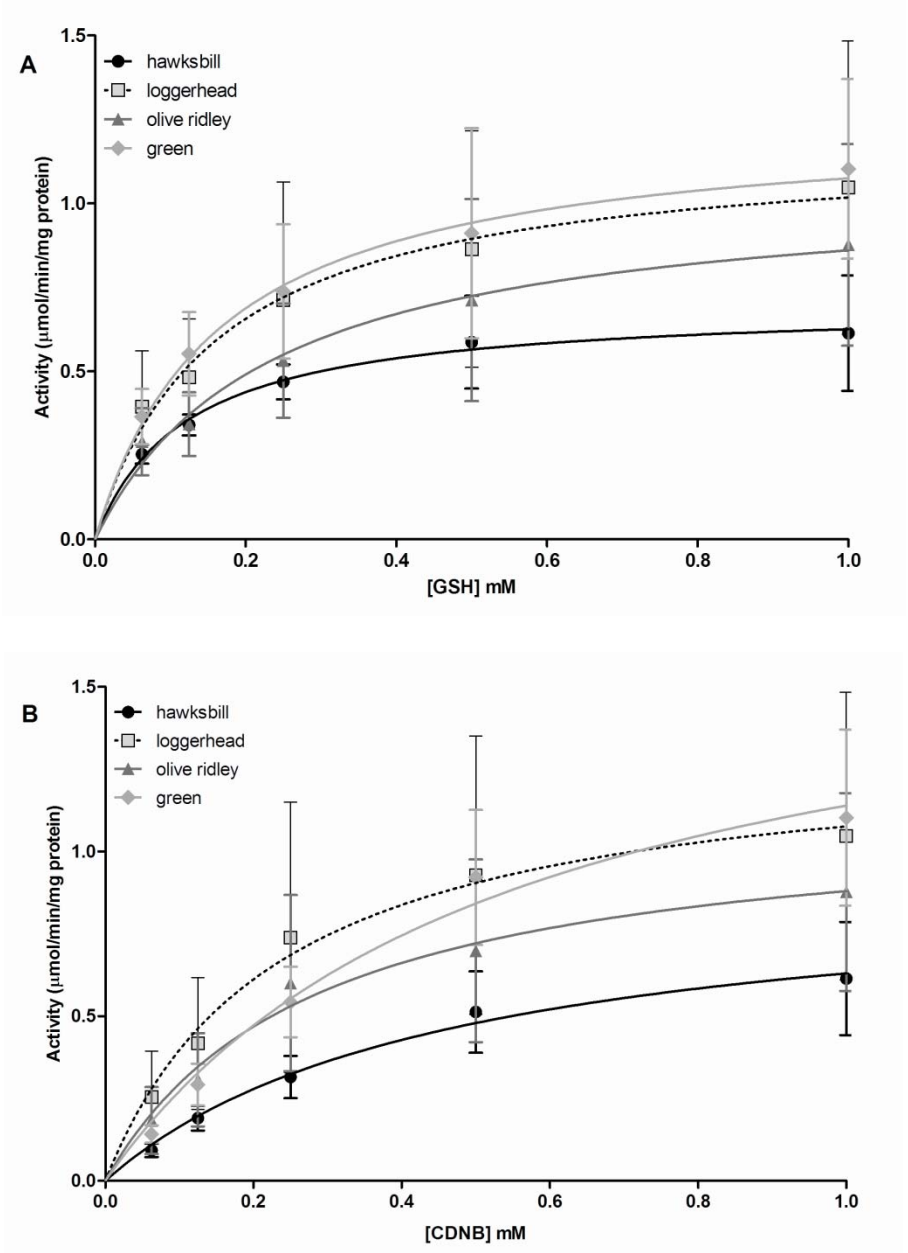

Figure 3.1. Kinetic analysis of the effects of varying reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) concentrations on the initial rates of cytosolic glutathione transferase (GST) CDNB conjugation in hawksbill, loggerhead, olive ridley, and green sea turtles. **A**. Varying concentrations of the nucleophile GSH from 0.0625 to 1 mM with a fixed electrophile (CDNB) concentration of 1 mM. **B**. Varying concentrations of the electrophile CDNB from 0.0625 to 1 mM with a fixed nucleophile (GSH) concentration of 1 mM. Data is expressed as mean of n=4 for all species except hawksbill where n=3. Error bars indicate standard deviation.

Table 3.2. GST kinetic parameters. The maximum velocity (Vmax) is expressed as nmol/min/mg protein, the apparent Michaelis constant (Km) as μM, and the catalytic efficiency (Vmax/Km) as nmol/min/mg protein/ μM. GSH paramters were analyzed by varying concentrations of the nucleophile GSH from 0.0625 to 1 mM with a fixed electrophile (CDNB) concentration of 1 mM, while CDNB paramters were analyzed by varying concentrations of the electrophile CDNB from 0.0625 to 1 mM with a fixed nucleophile (GSH) concentration of 1 mM. Data is expressed as mean (standard deviation) of n=4 for all species except hawksbill where n=3.

	GSH			CDNB		
Species	Vmax	Km	Catalytic Efficiency	Vmax	Km	Catalytic Efficiency
hawksbill	712 (208)	122 (51.1)	6.16 (1.16)	929 (307)	455 (118)	2.02 (0.292)
loggerhead	1201 (512)	172 (53.1)	7.52 (3.89)	134 (502)	269 (87.0)	5.72 (3.42)
olive ridley	1059 (420)	225 (50.5)	4.68 (1.26)	1201 (355)	373 (249)	4.16 (2.12)
Green	1254 (362)	163 (29.3)	7.70 (1.89)	1777 (505)	547 (102)	3.24 (0.652)

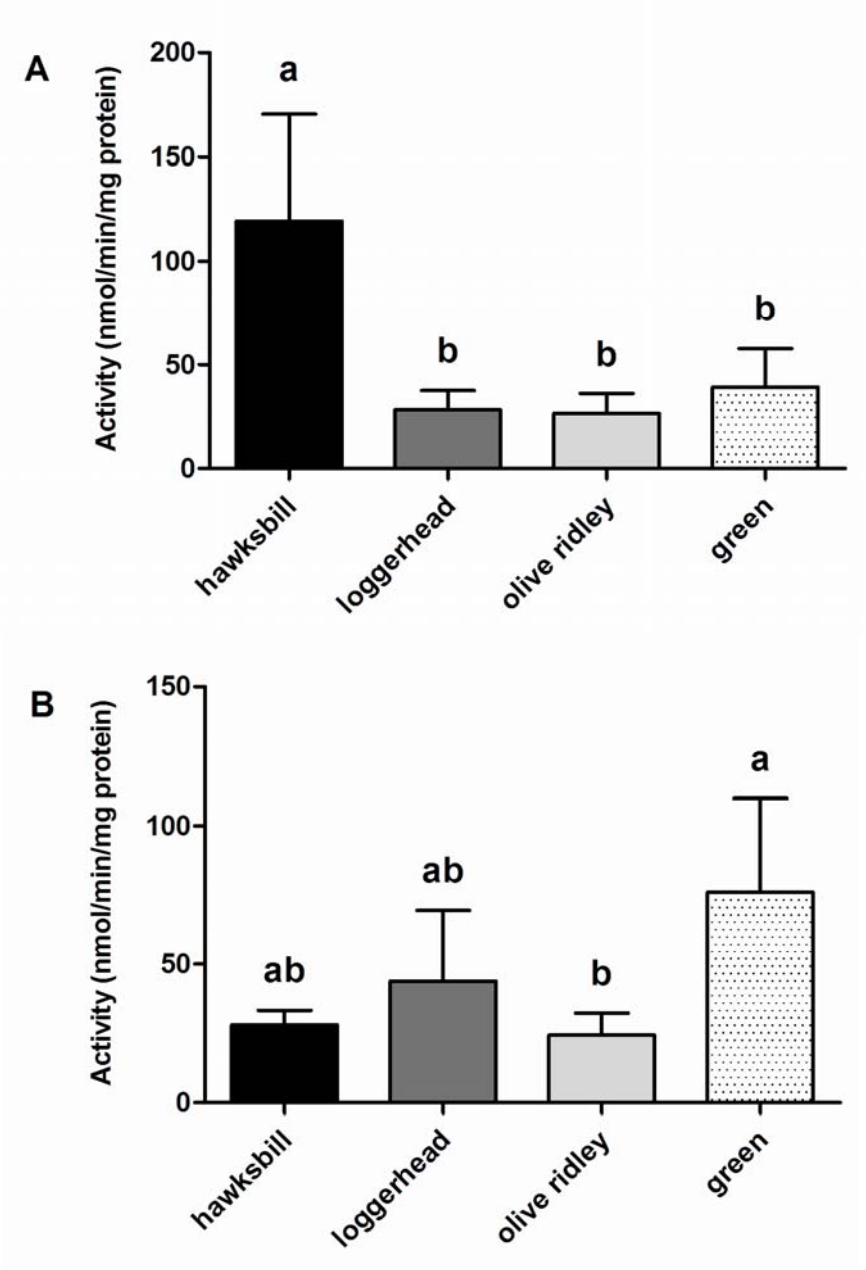

Figure 3.2. Initial rates of hawksbill, loggerhead, olive ridley, and green sea turtle cytosolic glutathione transferase (GST) activities towards various reference substrates. **A**. GST activity towards 4-nitrobenzyl chloride (NBC). **B**. GST activity towards ethacrynic acid (ECA). **C**. GST activity towards Δ5-androstene-3,17-dione (ADI). Different lowercase letters indicate significant difference ($p<0.05$). Data is expressed as mean of n=4 for all species except hawksbill where n=3. Error bars indicate standard deviation.

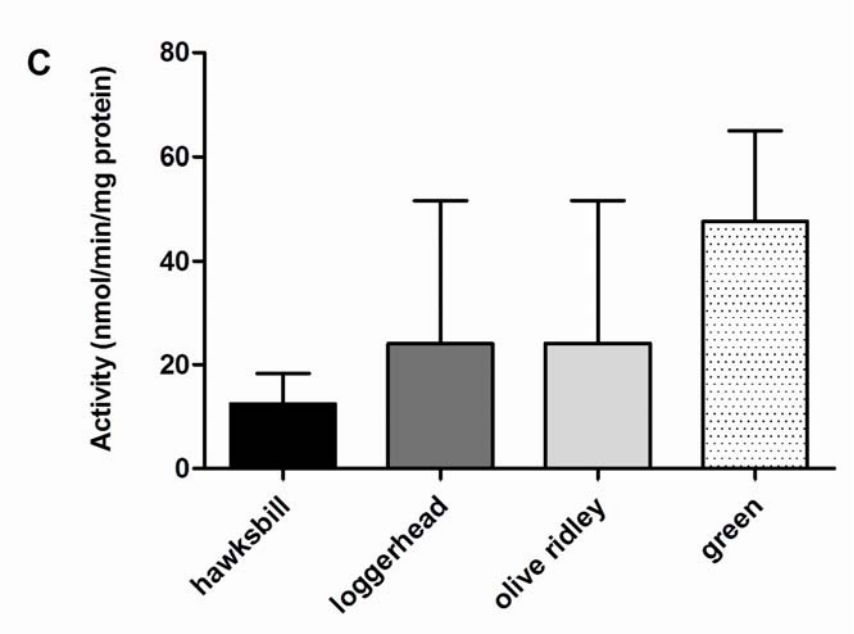

Figure 3.2 Continued. Initial rates of hawksbill, loggerhead, olive ridley, and green sea turtle cytosolic glutathione transferase (GST) activities towards various reference substrates. **A**. GST activity towards 4-nitrobenzyl chloride (NBC). **B**. GST activity towards ethacrynic acid (ECA). **C**. GST activity towards Δ5-androstene-3,17-dione (ADI). Different lowercase letters indicate significant difference ($p<0.05$). Data is expressed as mean of n=4 for all species except hawksbill where n=3. Error bars indicate standard deviation.

Discussion

Most sea turtle populations throughout the world are faced with the threat of extinction. Of the anthropogenic threats, environmental chemicals and mechanisms by which sea turtles deal with their potential toxic insult is probably the least understood. Within the marine environment, sea turtles are exposed to a variety of anthropogenic as well as natural chemicals. In other organisms, GST activity contributes to detoxification of many environmental chemicals, including polyaromatic hydrocarbon epoxides, pesticides, and natural products (Hayes and Pulford 1995), yet there exists a paucity of data on the role of GSTs in sea turtles. In fact, to our knowledge, the present study demonstrates GST activity in hawksbill, loggerhead, and olive ridley sea turtles for the first time.

The calculated catalytic efficiencies for CDNB (Vmax/Km) in the four species of sea turtles were not statistically different, indicating similar turnover of CDNB. The Km values for GSH and CDNB from 2 GST isoforms purified from the livers of red eared slider turtles (*Trachemys scripta elegans*) were 1.5-5 fold and 2-6 fold higher then sea turtle kinetic parameters (Willmore and Storey 2005). GST activities are typically assayed using CDNB as a relatively non-specific GST reference substrate. The use of this substrate integrates almost all GST activity as all isoforms of GST except those of the theta-class can conjugate GSH to CDNB. The initial rates of cytosolic hepatic GST activities towards CDNB for all four species of sea turtle were not significantly different between species. Additionally, they were similar to CDNB activity reported for green turtles also collected from the Baja California region of Mexico (Valdivia et al. 2007),

indicating similar CDNB activity in all sea turtle species. Furthermore, alligators (*Alligator mississippiensis*) and red-sided garter snakes (*Thamnophis sirtalis parietalis*) demonstrated hepatic CDNB activities which were similar to those reported for sea turtles (Hermes-Lima and Storey 1993; Gunderson et al. 2004). However, CDNB activities for the freshwater turtles red eared sliders (*Trachemys scripta elegans*) and painted turtles (*Chrysemys picta*) were approximately 2-7 fold higher than those for sea turtles (Willmore and Storey 1997; Rie et al. 2000). The differences in CDNB activities among reptiles may be due to differences in genetics, life history, diet, environmental factors, or experimental protocol. Differences between freshwater and saltwater turtles include osmoregulation capabilities, thermoregulation strategies, age to maturation, and home range.

Changes in overall GST activity, as measured by conjugation of CDNB with GSH, have been observed following exposure to hydrogen peroxide, anoxic conditions, and to tetrachloroethylene contaminated sites in red-sided garter snakes (*Thamnophis sirtalis parietalis*) (Hermes-Lima and Storey 1993), red-eared slider turtles (Willmore and Storey 1997), and painted turtles (Rie et al. 2000), respectively. These studies suggest that GSTs are responsive to stressors and that their activity may be altered by exposure to environmental chemicals. However, Richardson et al. (2010) found no differences or correlations between hepatic polychlorinated biphenyl (PCB) levels and CDNB activities in green, loggerhead, and olive ridley sea turtles (Chapter 2). Furthermore, corn snakes (*Elaphe guttata emoryi*) exposed to 3-methylcholanthrene and phenobarbital, which induce changes in mammalian GST expression, showed no changes

in GST activities (Bani et al. 1998) and alligators collected from three sites of varying degrees of contamination showed no difference in CDNB activity (Gunderson et al. 2004) suggesting that GST expression in reptiles may be recalcitrant to induction by anthropogenic environmental chemicals.

In addition to CDNB, class specific activities were also evaluated including NBC (relatively specific for GSTT and GSTM), ECA (relatively specific for GSTP, with less specificity for GSTA), and ADI (relatively specific for GSTA) (Mannervik and Danielson 1988; Hayes and Pulford 1995). Unlike red eared slider GST, which showed no activity towards ECA (Willmore and Storey 2005), all four species of sea turtles demonstrated ECA activity. GST-ECA activity is specific for rat pi-class GSTs, and to a lesser extent, mu-class GSTs (Hayes and Pulford 1995). GSTP and GSTM have been implicated in the metabolism of many environmental chemicals, including poly-aromatic hydrocarbons and pesticides, indicating that these GST iso-enzymes may play an important role in detoxification in the livers of sea turtles as well. ECA activity was highest in the green sea turtles - this was only significantly different from the activity in olive ridley sea turtles.

One potential explanation for higher ECA activity in green sea turtles may be their exposure to natural products which may occur in their diet. Adult green sea turtles are primarily herbivorous (Bjorndal 1997). Exposure to natural products would therefore require the means to biotransform and eliminate such compounds. Phase I biotransformation enzymes were higher in herbivorous fish species compared to species which demonstrated different feeding strategies (Stegeman et al. 1997). GST-mediated

detoxification of isothiocyanate natural products found in the cruciferous diet of certain insects has been postulated to explain plant-food adaptations (Wadleigh and Yu 1988). Furthermore, in a study of frog PCB elimination rates, the authors suggested that because highest elimination rates were observed during metamorphosis, biotransformation activity may naturally increase during this period in order to eliminate phytotoxins accumulated by herbivorous tadpoles (Leney et al. 2006). The somewhat higher levels of ECA activity in green sea turtles as compared to the other three species may provide a mechanism for green turtles to better eliminate both natural and potentially anthropogenic environmental chemicals, although levels of PCBs were found to be relatively low (Chapter 2).

All four species of sea turtles demonstrated similar ADI activities. The isomerization of Δ5-3 ketosteroids to Δ4-3 steroids is necessary for the metabolic conversion of cholesterol to steroid hormones such as testosterone and progesterone. The reference substrate for this isomerase activity is ADI. In rats, GSTAs catalyze this isomerization (Hayes and Pulford 1995). The physiological significance of this activity in liver (an organ not responsible for steroid hormone biosynthesis) is unclear; however, it is possible that the activity in sea turtles may result from an isoform of GST with other physiological functions.

Hawksbill sea turtles showed approximately 3-4.5 fold higher activity for the GSTT substrate NBC than the other three species. The hawksbill NBC activity was similar to that of two GST enzymes purified from the liver of red eared sliders (Willmore and Storey 2005), suggesting this activity is common to several types of turtles. One

explanation for higher NBC activity in hawksbill sea turtles may be related to the fact that hawksbill liver samples were collected from newly hatched animals, whereas samples from the other three species were collected from subadult and adult turtles. Regulation of biotransformation enzymes can be influenced by the age of the organism as has been observed for GST expression in mammals (Gregus et al. 1985), invertebrates (Grant et al. 1991; Kostaropoulos et al. 1996; Papadopoulos et al. 2004) and birds (Coulet et al. 1996). However, hepatic expression profiling in smolting and adult coho salmon (*Onchorhynchus kisutch*) indicated that the major isoform of GST may not be subject to developmental expression (Gallagher et al. 2008). And, similar isoforms of GST were expressed in African clawed frogs (*Xenopus laevis*) embryos and adults while distinct isoforms were expressed in common toad (*Bufo bufo*) (Angelucci et al. 2002). The authors suggested the differential expression seen in the latter amphibian may be attributable to changing from an aquatic to terrestrial environment (and thus higher oxygen concentration) and not ontogenic changes as the former maintains an aquatic habitat throughout its life history. Thus, in some animals, age or growth-stage dependent differences in GST expression are seen, but for others additional life history traits may exhibit a stronger influence on GST regulation. Future research examining the ontogeny of biotransformation enzyme expression in reptiles may help to elucidate the influence of age on GST expression.

Alternatively, diet may also influence isoform expression. The primary component of the diet of adult hawksbill turtles from the Caribbean Sea is marine demosponges (Meylan 1988; Leon and Bjorndal 2002). Marine benthic organisms, such

as sponges, produce a wide variety of halogenated secondary metabolites as chemical defense against consumers (for review see Hay 1996). However, adaptive processes, such as changes in biotransformation enzyme expression, may allow some organisms to consume such noxious compounds. For example, various species of butterflyfish that preferentially feed on chemically-rich corals had higher total CYP2 expression than species that avoid eating corals (DeBusk et al. 2008). GST expression in the marine gastropod *Cympoma gibbosum* varies in response to feeding on different chemically-defended corals (Vrolijk and Targett 1992). Proteomic methods were later used to identify a theta-like GST as one of the GSTs expressed in this gastropod (Whalen et al. 2008). Additionally, theta-class GSTs are considered more primitive GSTs (Zimniak and Singh 2007), so it is plausible that hawksbill sea turtles may have evolved specialized GSTs in order to cope with the myriad of potentially toxic natural products present in their diet, as has been suggested for theta-like GST in the marine gastropod (Whalen et al. 2008). The presence of possible GSTT in hawksbill sea turtles may also influence their susceptibility to anthropogenic as well as natural chemicals. GSTT1-3 are responsible for the biotransformation of dihaloalkanes (Sherratt et al. 1998) and carcinogenic sulfate esters of methyl PAHs (Hiratsuka et al. 1990). Furthermore, theta-class GSTs do not show activity towards CDNB, which may explain relatively high NBC activity but somewhat low CDNB activity, and highlights the necessity of assaying several reference substrates in order to better understand the activity of GSTs within a species.

In summary, the results of the present study indicate that hawksbill, loggerhead, olive ridley, and green sea turtles possess functional GST enzymes with similar kinetic parameters. Activity towards various reference substrates elucidated differences in GST conjugation activity among species which were not evident using the broad specificity substrate, CDNB. The higher GST activities seen in hawksbill and green turtles towards NBC and ECA, respectively, should be further examined in order to elucidate the ecological and physiological significance of these activities. It is plausible that sea turtle GST catalytic activities are reflective of their evolutionary adaptations to diverse feeding strategies. Furthermore, different GST catalytic activities in various sea turtles may alter susceptibility to the potential health impacts of environmental chemicals by various sea turtle species.

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Chapter 4: Biotransformation of 2,2'5,5'-Tetrachlorinated Biphenyl (PCB 52) and 3,3'4,4'-Tetrachlorinated Biphenyl (PCB 77) by Liver Microsomes from Four Species of Sea Turtles

Abstract

The rates of oxidative metabolism of two tetrachlorinated biphenyl congeners were determined in hepatic microsomes from four species of sea turtles, green (*Chelonia mydas*), olive ridley (*Lepidochelys olivacea*), loggerhead (*Caretta caretta*), and hawksbill (*Eretmochelys imbricata*). Hydroxylation of 3,3',4,4'-tetrachlorinated biphenyl (PCB 77), an *ortho-meta* unsubstituted rodent CYP1A substrate PCB, was not observed in sea turtle microsomes. Sea turtle microsomes hydroxylated 2,2',5,5'-tetrachlorinated biphenyl (PCB 52), a *meta-para* unsubstituted rodent CYP2 substrate PCB, at rates ranging from less than 0.5 to 53 pmol/min/mg protein. The cytochrome P450 (CYP) inhibitor ketoconazole inhibited hydroxylation of PCB 52, supporting the role of CYP catalysis. Sea turtle PCB 52 hydroxlyation rates strongly correlated with immunodetected CYP2-like and less so with CYP3-like hepatic proteins. Testosterone 6β-, 16α-, 16β-hydroxylase activities were also significantly correlated with the expression of these isoforms, indicating that CYP2 or CYP3 proteins are responsible for PCB hydroxylation in sea turtles. This study indicated species-specific PCB biotransformation in sea turtles and preferential elimination of *meta-para* unsubstituted PCB congeners over *ortho-meta* unsubstituted PCB congeners consistent with PCB accumulation patterns observed in tissues of sea turtles.

Introduction

Throughout the globe, sea turtle populations are in danger of extinction (IUCN 2008). Adapted to marine life approximately 100 million years ago (Pritchard 1997), sea turtles are now primarily threatened by anthropogenic influences such as fisheries activities, coastal development, directed take, climate change, and effects from pollution, including higher susceptibility to pathogenic infections (Mast et al. 2005). Of these hazards, the effects of anthropogenic contaminants and their metabolites is the least studied (Sparling 2006). As long-lived reptiles with delayed sexual maturity (Heppell et al. 2002), sea turtles may be particularly susceptible to persistent contaminants (Rowe 2008). Halogenated organic compounds (HOCs), such as polychlorinated biphenyls (PCBs), are persistent, bioaccumulative, toxic contaminants (James 2001).

Xenobiotic biotransformation, evolved as a defense mechanism to protect organisms from toxic insult by transforming lipophilic molecules into more polar compounds in order to increase hydrophilicity and subsequent elimination (Parkinson 2001). Biotransformation capacity, exposure, and physicochemical properties of individual congeners can influence PCB toxicokinetics (Norstrom and Letcher 1997). Phase I metabolism of PCB involves the introduction of oxygen to one of the aromatic rings by cytochrome P450 (CYP), which may directly insert a hydroxyl group (OH) or result in the formation of an arene oxide which may undergo rearrangement to produce OH-PCB (Forgue et al. 1979). Oxidation by CYP is the critical step in the formation of OH-PCBs. In rodents, PCB oxidation is primarily mediated by CYP1A and CYP2B, with limited contribution from CYP2C and CYP3A (McFarland and Clarke 1989;

Letcher et al. 2000a). PCB biotransformation is dependent on the number and position of chlorine atoms in each congener (see Figure 1.1). In most species, PCBs are resistant to biotransformation when they contain five or more chlorine atoms. Structure activity relationships further suggest that the biotransformation of PCBs is based on the presence of vicinal protons, as these are necessary for CYP activity (Boon et al. 1989; McFarland and Clarke 1989). Highly chlorinated PCBs which lack vicinal protons are the most persistent congeners and generally do not undergo biotransformation. For PCBs with vicinal protons, the position of the inserted oxygen is dependent on the CYP isoform catalyzing the reaction. Phenobarbital (PB)-induced CYPs, e.g., CYP2B1, insert an oxygen atom at the *meta-para* positions while 3-methylcholanthrene (3MC) -induced CYPs, e.g., CYP1A1, catalyze oxygenation at *ortho-meta* positions of PCBs (Parke 1985; Kannan et al. 1995). PCBs with both *ortho-meta* and *meta-para* protons can be metabolized by either enzyme group, and thus are the most readily metabolized PCBs. Because both PB- and 3MC- induced enzymes can catalyze oxygenation, both CYP families can bioactivate PCBs to hydoxylated PCBs. For oxidative metabolites of PCBs with *ortho-meta* protons, the steric hindrance of the oxygen molecule provides stability and may inhibit detoxification so 3MC-induced enzymatic activity may present a greater potential for toxicity (McFarland and Clarke 1989).

Hydroxylated metabolites may be selectively bound to proteins and therefore retained in cells (Bergman et al. 1994). OH-PCBs are more hydrophilic than the parent compound, so OH-PCBs are usually detected in blood while parent PCBs persist in lipids (Letcher et al. 2000a). OH-PCBs also affect oxidative phosphorylation, inhibit the

catalytic CYPs, and interfere with the endocrine system (Safe 1994). Additionally, PCBs provide a unique situation where specific enzyme substrates may also induce the same enzyme and potentially their own biotransformation because exposure to coplanar or dioxin-like PCBs (non- or mono-*ortho* substituted) produces CYP1A induction, and exposure to globular or non-planar PCBs produces CYP2B induction (Safe 1994). Furthermore, in environmental exposures, PCBs are often not the sole contaminant, indicating that changes in cellular biochemistry induced by PCBs may affect not only their own toxicity, but also that of other contaminants.

While data is sparse compared to mammals, reptiles have been shown to possess CYP enzymes, but *in vitro* and *in vivo* studies with classic inducers have shown induction patterns do not always mimic those of mammals (Mitchelmore et al. 2006). For example, reduced responsiveness to dioxin-like PCBs and other mammalian CYP1A inducers was demonstrated in primary hepatocytes of the African brown house snake (*Lamprophis fuliginosus*), in which only treatment with 2 potent CYP1A inducers, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 3,3',4,4',5-pentachlorinated biphenyl (PCB 126), resulted in dose-dependent increased ethoxyresorufin-O-deethylase (EROD) activity (CYP1A catalytic activity) while other CYP1A inducers, 3,3',4,4'-tetrachlorinated biphenyl (PCB 77), 3,4,4',5-tetrachlorinated biphenyl (PCB 81), and 3,3',4,4',5,5'-hexachlorinated biphenyl (PCB 169), failed to cause induction (Hecker et al. 2006). Few studies have focused on biotransformation capacity in sea turtles. In loggerhead (*Caretta carretta*), green (*Chelonia mydas*), and olive ridley (*Lepidochelys olivacea*) sea turtle hepatic tissue, CYP2K-like and, CYP3A-like proteins, but not

CYP1A-like proteins were observed (Chapter 2), and in one Kemp's ridley sea turtle (*Lepidochelys kempii*) liver, four partially purified CYP proteins demonstrated low levels of EROD activity (Goldman and McClellan-Green 2001). Based on congener-specific PCB accumulation patterns, lower PCB biotransfomation by CYP1A relative to CYP2B has been suggested in green, loggerhead, olive ridley, and Kemp's ridley turtles (Chapter 2; Keller et al. 2004). The only report of reptilian PCB biotransformation is a comparative study of the biotransformation of PCB 77 in hepatic microsomes of several organisms including painted turtles (*Chrysemys picta)* induced with β-naphthoflavone (BNF), a 3MC-like inducer (Schlezinger et al. 2000). Turtle hepatic microsomes hydroxylated PCB 77 at a rate higher than CYP1A-induced fish, within the range of uninduced and induced birds, but lower than CYP1A-induced rat (Schlezinger et al. 2000).

The present study investigated the potential for PCB biotransformation in the livers of four species of sea turtles – loggerhead, green, olive ridley, and hawksbill (*Eretmochelys imbricata*) turtles. Representative PCBs, 2,2'5,5'-tetrachlorinated biphenyl (PCB 52) and PCB 77, were chosen based on the structure-biotransformation patterns seen in other organisms and were incubated with hepatic microsome fractions in order to determine the *in vitro* PCB biotransformation kinetics. The use of a CYP inhibitor, as well as relationships between biotransformation rates, other CYP catalytic activities, and protein expression of biotransformation enzymes provides insight into the potential PCB biotransformation pathways in sea turtles.

Materials and Methods

Reagents

PCB 52, PCB 77, 4-hydroxy-2',4',6'-trichlorinated biphenyl (4-OH-PCB 30), 4-hydroxy-2,2',4,6'-tetrachlorinated biphenyl (4-OH-PCB 50) and 2-hydroxy 2',3',5',6'-tetrachlorinated biphenyl (2-OH-PCB 65) were purchased from Accustandard (New Haven, CT, USA). ^{14}C-testosterone (48 mCi/mmol) was purchased from Perkin-Elmer (Waltham, MA, USA). 6β-hydroxytestosterone, 16α-hydroxytestosterone, and 16β-testosterone were purchased from Steraloids (Newport, RI, USA). Nicotinamide adenine dinucloetide phosphate (NADPH) and ketoconazole were purchased from Sigma-Aldrich (St. Louis, MO, USA). For HPLC analysis, Fisher (Pittsburgh, PA, USA) HPLC grade acetonitrile and water from E-PURE (Barnstead, Thermo Scientific, Waltham, MA, USA) filtration device were filtered with 0.2 μm nylon membranes (Millipore, Billerica, MA, USA) prior to use. All other chemicals were of high analytical grade and were purchased from Fisher or Sigma.

Sea turtle samples

Four species of sea turtles, loggerhead, olive ridley, green, and hawksbill, inhabiting the coastal regions of Mexico were examined in this study. Methods for the collection of liver tissue from wild green, loggerhead, and olive ridley turtles, and for hawksbill turtles, were described previously (Chapters 2 and 3). Liver samples from 5 green, 3 loggerhead, 3 olive ridley, and 3 hawksbill turtles were used for the present analyses.

Isolation of subcelluar fractions

Liver subcellular fractions were isolated from as reported previously (Chapters 2 and 3). Briefly, each liver sample (approximately 0.5 g) was homogenized then subjected to sequential centrifugation at 4°C to isolate microsomal and cytosolic fractions, and were stored at −80°C to preserve enzyme integrity. Protein concentrations of the fractions were determined using the microassay Bradford method (Brogdon and Dickinson, 1983), stained with Coomassie Plus and bovine serum albumin as a standard (both Pierce Biotechnology Inc, Rockford, IL, USA). Protein denaturation was not observed in any sample.

Anti-CYP Western Blotting

CYP isoform expression in the livers of hawksbill microsomes was evaluated by Western blotting using CYP anti-serum raised against rainbow trout (*Oncorhynchus mykiss*) CYP isoforms as described previously for the other 3 species of sea turtles (Chapter 2). Briefly, microsomal proteins (50 µg per lane) from turtle and rainbow trout positive control, along with molecular weight markers (SeeBlue Plus2, Invitrogen, Carlsbad, CA, USA) were resolved using SDS-PAGE and then transferred to nitrocellulose membranes. Immunoblotting was performed with primary antibodies, anti-fish monoclonal CYP1A (Biosense, Bergen, Norway) and anti-rainbow trout polyclonal CYP2K1 antibodies (provided by Dr. D.R. Buhler, Oregon State University), and secondary antibodies linked to IgG alkaline phosphatase (BioRad, Hercules, CA, USA), which allowed band visualization through the use of a commercial alkaline phosphatase

substrate conjugation kit (BioRad). After measuring the density of each band with Quantity One software (BioRad), bands were normalized to the rainbow trout control run on each gel and expressed as relative optical density.

PCB in vitro assays

PCB hydroxylation was determined in hepatic microsomes from the four species of sea turtles. Additionally, PCB hydroxylation was measured in commercially available (Xenotech, Lenexa, KS, USA) microsomes prepared from rats treated with phenobarbital (PB) and β-naphthflavone (BNF), which served as positive controls for PCB 52 and PCB 77 hydroxylation, respectively. In order to optimize incubation conditions for turtles, a range of PCB 52 concentrations (50-1000 μM), microsomal protein content (0.5 to 5 mg/mL), temperature (20-35°C), and incubation times (10 minutes to 1 hour) were employed. These studies were carried out in green sea turtles because ample quantities of tissue were available. Based on optimization studies (see below), 100 μM of PCB and 1 mg/mL protein concentrations were employed. Sea turtle and rat hepatic microsomal fractions (1 mg/mL) were incubated with 100 μM PCB 52 or PCB 77, which was added to the reaction tube in iso-octane and evaporated under nitrogen before addition of other constituents. NADPH (600 μM) was added in 2 aliquots – one to start the reaction, and the second, at 30 minutes, to maintain reduced NADPH concentration. Tris-HCl buffer (100 mM) containing 10 mM $MgCl_2$, pH 7.4 was used to provide a total volume of 0.1 mL. The reaction, incubated at 25 °C or 37 °C for sea turtle and rat, respectively, was initiated by the addition of NADPH, and then stopped at 60 min by the addition of 0.5

mL of ice cold ethyl acetate and transferred to ice. Each sample was amended with 0.7 μM (final concentration) 4-OH-PCB 30 as a recovery standard, vortexed vigorously, sonicated for 5 minutes, and then centrifuged for 5 min at 13,000 *g*. The organic layer was then removed and the extraction process was repeated twice (without the addition of internal standard) and organic layers were combined. The sample was then evaporated to dryness with nitrogen and re-suspended in 50 μL of mobile phase A (see below). Also included were negative controls with conditions similar to those above, but contained either boiled microsomal proteins, or buffer instead of NADPH. Additional incubations with conditions similar to those above employed the use of ketoconazole (100 μM, delivered in 5 μL ethanol; negative controls had 5 μL ethanol added), a potent inhibitor of mammalian and piscine CYP enzymes (Miranda et al. 1998; Correia and Ortiz de Montellano 2005).

The organic fractions isolated from *in vitro* incubations were analyzed by HPLC/UV/MS using a 45 μL injection. The HPLC/UV/MS system consisted of a Dionex (Sunnyvale, CA) AS autosampler, Dionex GS50 gradient pump, Dionex AD25 UV/Vis detector, Dionex Ulitmate 3100 auxillary pump, and a Surveyor MSQ single quadropole MS (Thermo Scientific). The HPLC column was a Hypersil ODS C18 column (5 um, 250 x 4.6 mm; Thermo Scientific), protected by a Phenomenex (Torrance, CA USA) Security Guard™ cartridge system equipped with a C18 4x3 mm cartridge. The mobile phase consisted of A, 60% acetonitrile in water and B, 100% acetonitrile. The HPLC system was operated at a flow rate of 1.0 mL/min with a linear gradient of 100% A to 100% B over 50 minutes, then held at 100% B for 5 minutes before returning

to 100% A over 5 minutes, and then held at 100% A to stabilize the column prior to subsequent injection. The UV/Vis detector used absorption maxima of 236 nm for PCB 52 experiments and 212 nm for PCB 77 experiments. The MS was equipped with an auxiliary isocratic pump, pumping 70% acetonitrile in water at a flow rate of 0.3 mL/min. A switching valve between the UV and MS detectors allowed matrix diversion of LC flow to waste when PCB metabolites were not eluting. The MS was operated in electrospray ionization, negative ionization mode. Because the major metabolites produced by vertebrates from PCB 52 or PCB 77 are not commercially available, MS parameters were optimized using 2 homologues of the known metabolites, 4-OH-PCB 50 and 2-OH-PCB 65. Selective ion monitoring of the three largest peaks in the isotopic cluster (resulting from isotopic variations in chlorine) of hydroxylated tetrachlorinated biphenyl metabolites, *m*/*z* 305, 307, and 309, representing $[M-H]^-$, $[M-H+2]^-$, $[M-H+4]^-$, respectively, were used to identify the metabolite, and m/z 307, the largest peak, was used for quantification. Selective ion monitoring of the three largest peaks for 4-OH PCB 30, the internal standard, *m*/*z* 271, 273, and 275, representing $[M-H]^-$, $[M-H+2]^-$, $[M-H+4]^-$ respectively, were used to identify the standard, and m/z 271, the largest peak, was used for quantification. During the analysis, the ESI probe was held at 550 °C and ultra high purity nitrogen was used as the nebulizing gas (80 psi). The cone voltage was -40V for hydroxylated tetrachlorinated biphenyls and -75V for the internal standard, and full scan data for m/z 30-350 were also collected at these cone voltages. Chromeleon version 6.6 (Dionex, Sunnyvale, CA) was used to control the instrumentation and to quantify the metabolites. Chromatographic peaks of metabolites were quantified and

identified with response factors for a commercially available hydroxylated tetrachlorinated biphenyl, 4-OH PCB 50. The use of 4-OH PCB 50 as a standard for quantification was further validated by the detection of hydroxylation rates in positive control samples within the range of those reported in the literature (see results). All samples were analyzed in 2-3 replicates. The detection limit of each metabolite, based on 4-OH PCB 50, in those conditions was 0.54 pmol/min/mg protein.

Testosterone in vitro assays

Due to tissue limitations, testosterone hydroxylation activities were measured in 2 turtles from each species as described in Martin-Skilton et al. (2006). Briefly, 36 μM total testosterone (68 μCi [^{14}C]testosterone) and 300 μM NADPH were incubated with 1 mg/mL hepatic microsomal protein in a final volume of 50 μL 100 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.4. Samples were incubated for 1 h at 25°C and the incubations were stopped by adding 25 μL of acetonitrile. After centrifugation (10,000 g; 10 min), 45 μL of supernatant was injected onto the HPLC system. HPLC analyses were performed on a SCL-10AVP Shimadzu HPLC system equipped with the same column and precolumn described for LC/MS analyses. The mobile phase consisted of A, 75% water and 25% acetonitrile and B, 45% water and 55% acetonitrile. The HPLC system was operated at a flow rate of 1.0 mL/min with a linear gradient of 100% A to 100% B over 40 minutes, then held at 100% B for 5 minutes before returning to 100% A over 5 minutes, and then held at 100% A to stabilize the column prior to subsequent injection. Chromatographic peaks were monitored by on-line radioactivity detection with a radio-detector β-ram Model 3 (INUS Systems Inc., Tampa, FL) using In-Flow 2:1 (INUS Systems Inc.) as the

scintillation cocktail. Metabolites (6β-, 16α- and 16β-hydroxytestosterone) were identified by co-chromatography with authentic standard compounds and quantified by integrating the area under the radioactive peaks. The detection limit was 0.02 pmol/min/mg protein.

Data analysis

For PCB hydroxylation studies, the recovery of the internal standard for was ≥ 75% for all samples, and hydroxylation rates were corrected for recovery. Statistical analyses [analysis of variance (ANOVA), two-tailed T-test, linear regression, and correlation analyses] were carried out using Prism 5 for Windows software (Graphpad Software Inc., La Jolla, CA, USA). For PCB and testosterone hydroxylation rates which were below detection limits, a random number between the detection limit and one-half of the detection limit was used for statistical analysis. If an overall significance was detected by ANOVA, Tukey's tests were performed in order to determine differences among species or groups. Results are presented as mean ± standard error. A p-value less than 0.05 was considered significant. Kinetic parameters of enzymatic activities were calculated using the same software package.

Results

PCB incubations were optimized as follows: highest activity was seen when incubated at 25°C, the hydroxylation rate was linear up a microsomal protein concentration of 1.2 mg/mL, and for at least one hour of incubation. The retention of hydroxylated tetrachlorinated biphenyl metabolites from both turtle and rat were similar to that of 4-OH PCB 50 (Figure 4.1) and produced identical mass spectra (Figure 4.2). The kinetics of PCB 52 metabolism were characterized in hepatic microsomes from green sea turtles to optimize the assay for comparative studies. NADPH-dependent OH-PCB 52 production was linear until approximately 200 μM PCB 52, showed an apparent K_m of 45.6 (± 6.18) μM, and a V_{max} of 84.3 (± 2.30) pmol/min/mg (Figure 4.3).

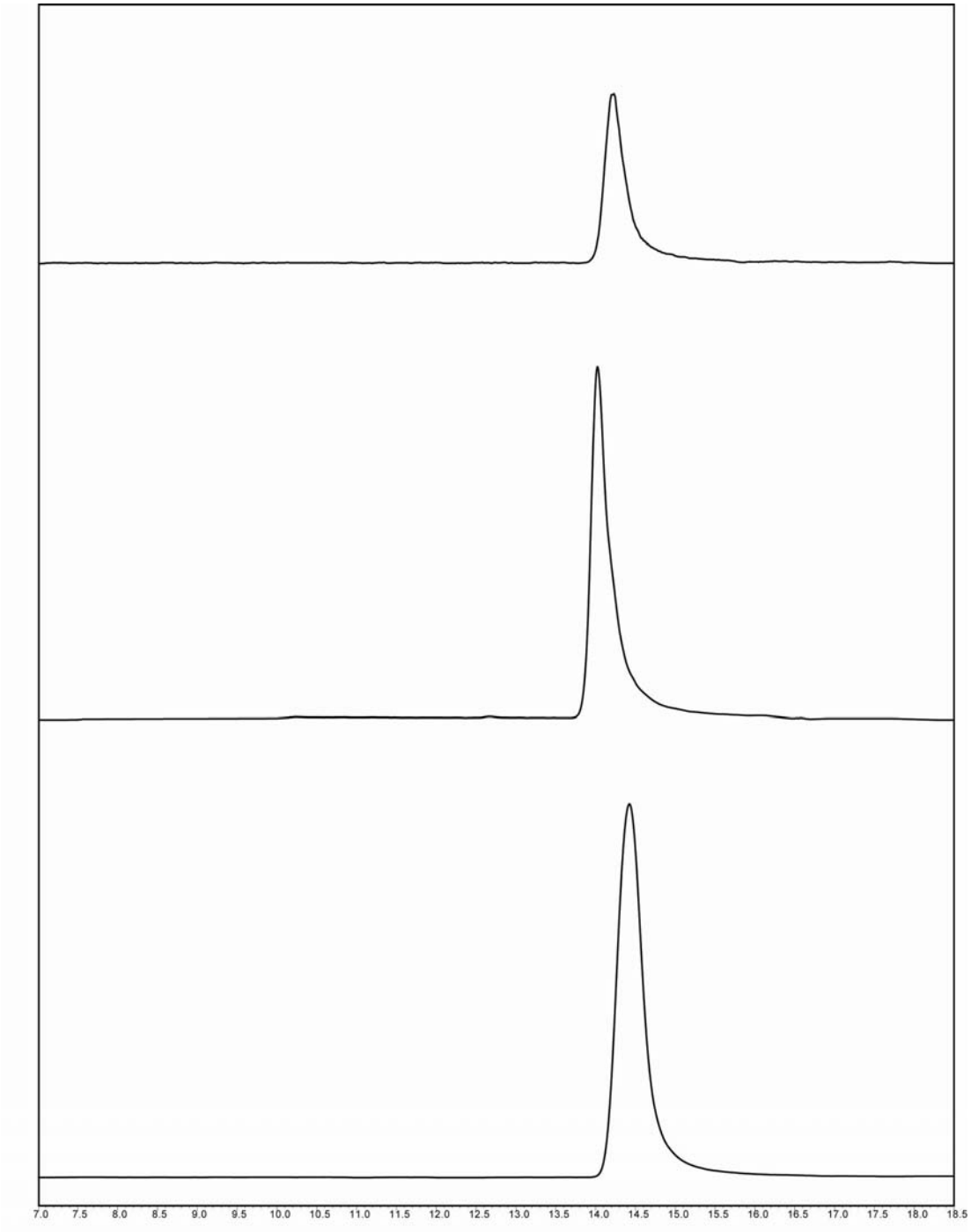

Figure 4.1. Chromatograms showing the relative retention times of a) hydroxylated metabolite from turtle, b) hydroxylated metabolite from rat, and c) 4-OH PCB 50. Retention times are relative to that of the internal standard in each sample.

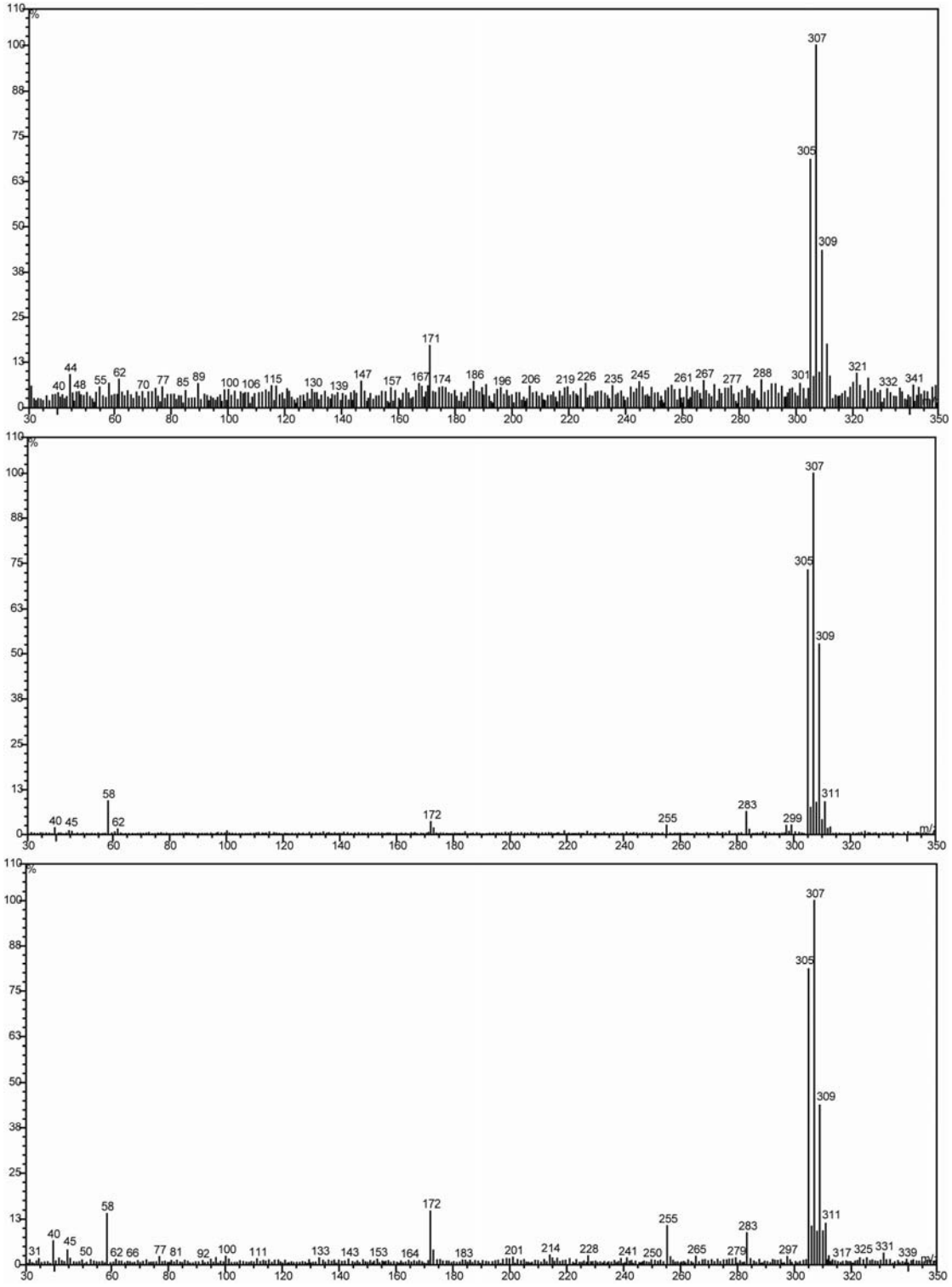

Figure 4.2. Mass spectra of a) hydroxylated metabolite from turtle, b) hydroxylated metabolite from rat, and c) 4-OH PCB 50.

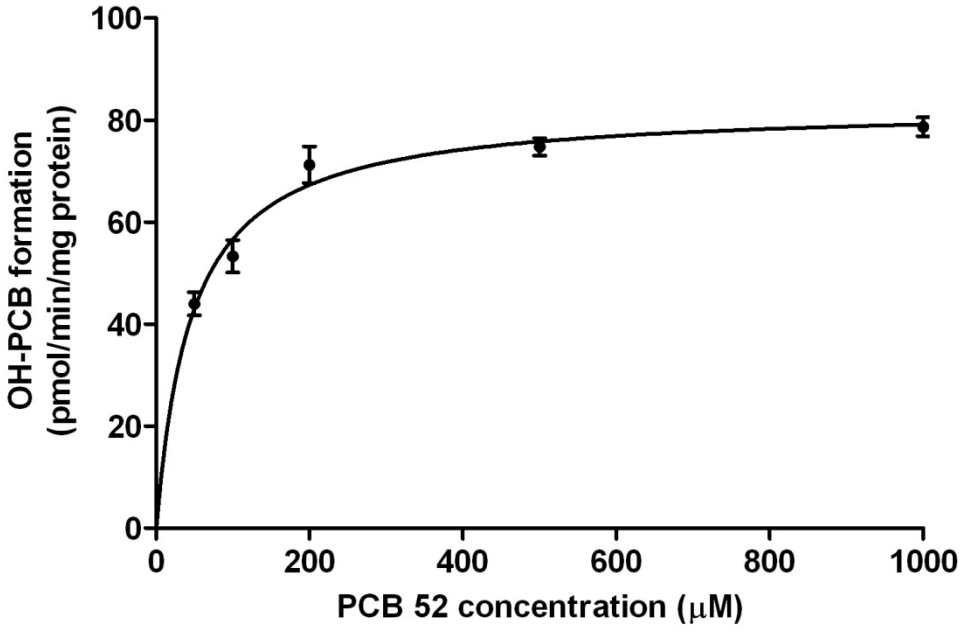

Figure 4.3. Kinetic analysis of the effect of varying PCB 52 concentration on the rate of OH-PCB formation by green sea turtle hepatic microsomes. Data points represent mean (±SE).

PCB 52 metabolism in the four species of sea turtles ranged from below detection limits (BDL, < 0.54 pmol/min/mg protein) to 52.8 pmol/min/mg, and all species showed rates of metabolism lower than PB-treated rat microsomes (107 pmol/min/mg) (Figure 4.4). Green turtle showed high variability (BDL-52.8 pmol/min/mg). Hawksbills showed approximately 10-fold higher hydroxylation rates than loggerheads and olive ridleys (ANOVA, $p<0.001$). PCB 52 hydroxylation was not detected in any incubation containing ketoconazole (Figure 4.5). Within individuals, several statistically significant correlations were found between CYP expression, testosterone hydroxylase activities, and PCB 52 hydroxylase activities were found (Tables 4.1-4.3). PCB 52 hydroxylation

rates showed a stronger correlation (r^2 = 0.8644, Pearson's r = 0.9297, p = 0.001, Figure 4.6) with CYP2-like protein expression as measured by immunoblotting than with CYP3-like expression (r^2 = 0.7227, Pearson's r = 0.854, p = 0.007) (Chapter 2) (Tables 4.1 and 4.2). Of the testosterone hydroxylase activities measured, PCB 52 hydroxylation showed the strongest correlation with 16α-hydroxytestosterone formation (r^2 = 0.7911, Pearson's r = 0.8895, p = 0.0031, Table 4.3), which was most highly correlated with CYP2-like expression (Table 4.1). Hydroxylation of PCB 77 was not observed in any of the sea turtles, but was observed in BNF-treated rat microsomes (21.8 ± 2.41 pmol/min/mg) (Figure 4.7).

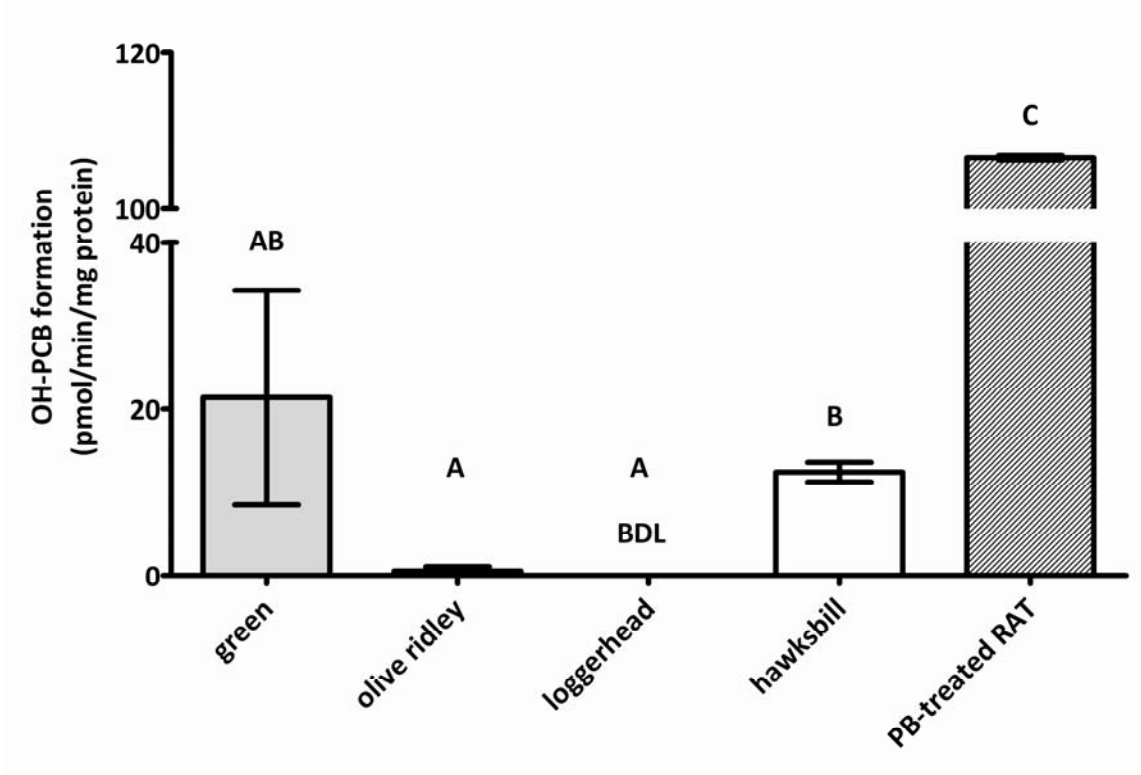

Figure 4.4. Mean (±SE) PCB 52 hydroxylation rates (pmol/min/mg protein) in green, olive ridley, loggerhead, and hawksbill sea turtle hepatic microsomes and PB-treated rat hepatic microsomes. BDL = below detection limit of 0.54 pmol/min/mg protein. Different letters represent statistically significant difference (ANOVA, $p<0.001$).

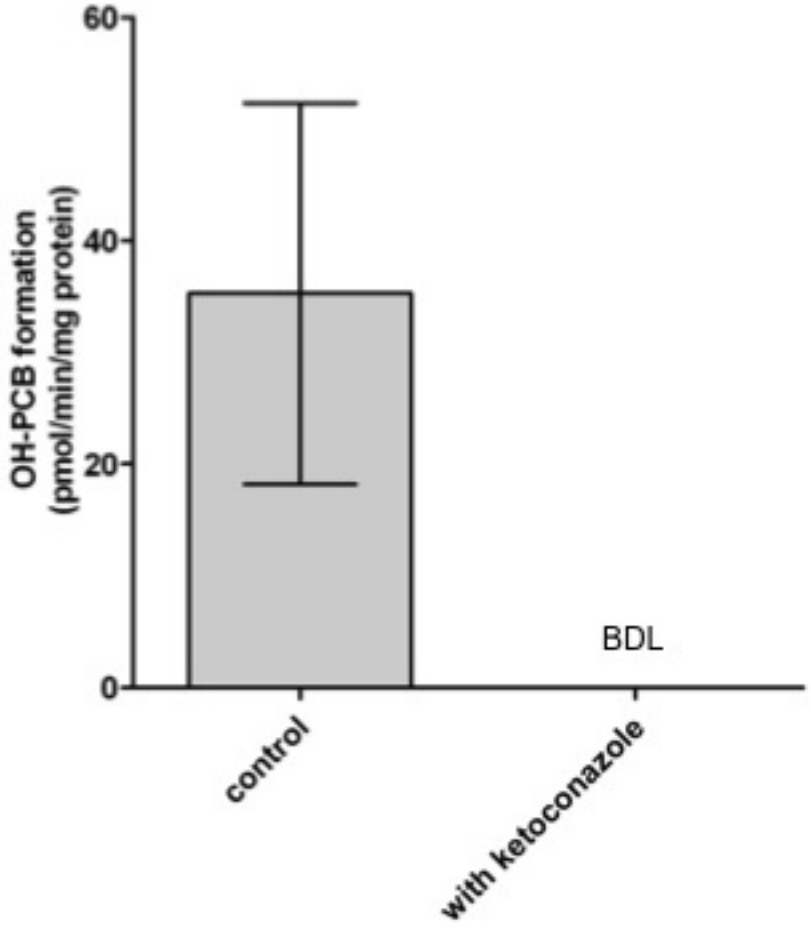

Figure 4.5. The effect of the CYP inhibitor ketoconazole on *in vitro* PCB 52 hydroxylation in green sea turtle hepatic microsomes. Control incubations had 5 µL ethanol added to reaction mixture, whereas, incubations with the inhibitor contained 100 µM ketoconazole added in a 5 µL aliquot of ethanol. BDL = below detection limit of 0.54 pmol/min/mg protein.

Table 4.1. Correlation statistics for CYP2-like expression with testosterone hydroxylase activities and PCB 52 hydroxylase activities.

CYP2-like correlations with:	6β testosterone	16α testosterone	16β testosterone	OH-PCB 52
Slope ± SE	14.7 ± 4.67	9.86 ± 2.85	10.8 ± 4.05	78.0 ± 12.3
R squared	0.625	0.666	0.545	0.869
Pearson r	0.790	0.816	0.738	0.932
P value (two-tailed)	0.020	0.014	0.037	0.001

Table 4.2. Correlation statistics for CYP3-like expression with testosterone hydroxylase activities and PCB 52 hydroxylase activities.

CYP3-like correlations with:	6β testosterone	16α testosterone	16β testosterone	OH-PCB 52
Slope ± SE	3.95 ± 1.30	2.36 ± 0.938	2.61 ± 1.24	19.5 ± 4.84
R squared	0.6039	0.5139	0.4265	0.7293
Pearson r	0.7771	0.7169	0.6531	0.854
P value (two-tailed)	0.0233	0.0454	0.0791	0.007

Table 4.3. Correlation statistics for PCB 52 hydroxylase activity with testosterone hydroxylase activities.

OH-PCB 52 correlations with:	6β testosterone	16α testosterone	16β testosterone
Slope ± SE	0.192 ± 0.0463	0.128 ± 0.0269	0.149 ± 0.0377
R squared	0.7413	0.7911	0.7227
Pearson r	0.861	0.8895	0.8501
P value (two-tailed)	0.006	0.0031	0.0075

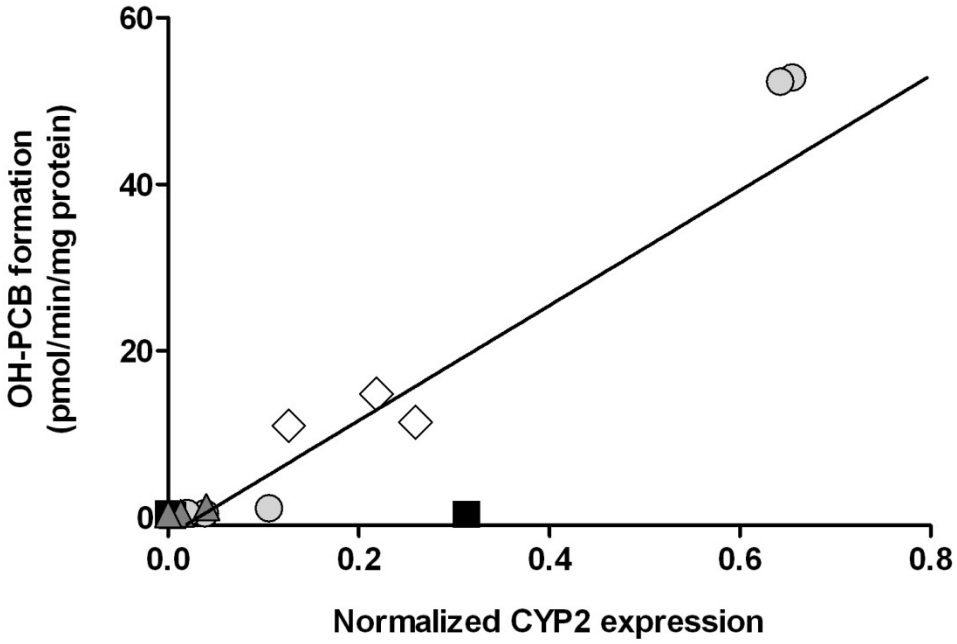

Figure 4.6. Correlation between mean sea turtle PCB 52 hydroxylation rates and normalized CYP2-like expression as measured by immunoblotting (CYP 2 data from Chapter 2 and present study). Each circle represents an individual green sea turtle; diamonds, hawksbills; squares, loggerhead; and triangles, olive ridleys.

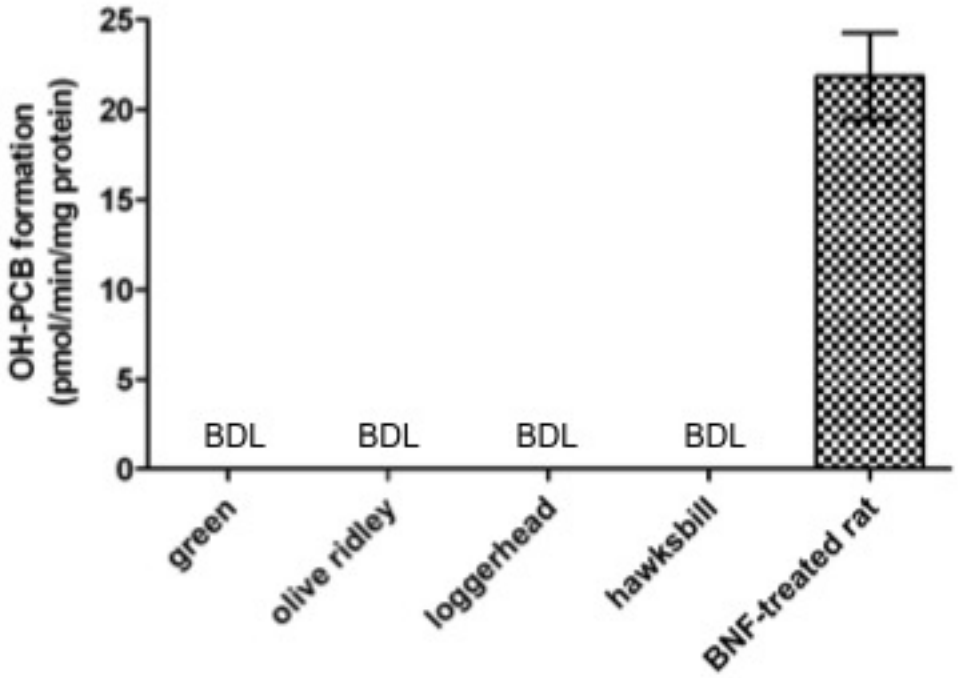

Figure 4.7. Mean (±SE) PCB 77 hydroxylation rates (pmol/min/mg protein) in green, olive ridley, loggerhead, and hawksbill sea turtle hepatic microsomes and BNF-treated rat hepatic microsomes. BDL = below detection limit of 0.54 pmol/min/mg protein.

Discussion

PCB biotransformation in reptiles has been investigated in only one species of freshwater turtle (Schlezinger et al. 2000). In the present *in vitro* metabolism studies in sea turtles, the PCB congeners were selected to represent congeners from biotransformation classifications based on structure-activity relationships found in mammals and aquatic ecosystems (Parke 1985; Boon et al. 1989; McFarland and Clarke 1989; Kannan et al. 1995). PCB 52, a rodent CYP2B substrate congener with *meta-para* vicinal protons, was biotransformed by the microsomes of some sea turtles to a hydroxylated metabolite, while *ortho-meta* unsubstituted PCB 77, rodent CYP1A substrate congener, was not.

PCB 52 hydroxylation varied among sea turtles species. The rates of hydroxylation in the hepatic microsomes of loggerheads, olive ridleys and some green sea turtles, which ranged from below detection limits (BDL) to 1.6 pmol/min/mg, were comparable to those observed in untreated mammalian hepatic microsomes (BDL – 10 pmol/min/mg) (Ghiasuddin et al. 1976; Mills et al. 1985; Ishida et al. 1991; Koga et al. 1995). The rates of metabolism in hawksbill and some green metabolizing sea turtles ranged from 11.0 to 52.8 pmol/min/mg and were greater than rates for untreated, but less than those for PB-treated mammalian microsomes. In this study, PB-treated rat microsomes hydroxylated PCB 52 at a rate of 107 pmol/min/mg, which is within the reported range of 66-1450 pmol/min/mg (Mills et al. 1985; Ishida et al. 1991; Koga et al. 1995). In all species of sea turtles, as well as PB-treated rat, only one hydroxylated metabolite was detected (Figure 4.1). The hydroxylated metabolite in rat and turtles

showed a similar retention time as that of 4-OH PCB 50. The rat metabolite is most likely 3-OH PCB 52 as Preston and Allen showed that this metabolite represented 90% of *in vitro* biotransformation products in PB-treated rat microsomes (1980). And, in turtles the metabolite may also be the 3-OH derivative of PCB 52 because this is also the major metabolite in rainbow trout (*Oncorhynchus mykiss*) (Nichols et al. 2001). The lack of detectable metabolites when incubations contained ketoconazole, a inhibitor of rainbow trout and mammalian CYPs (Miranda et al. 1998; Correia and Ortiz de Montellano 2005), supported the hypotheses that CYP enzymes are responsible for the NADPH-dependent hydroxylation of PCB 52 in the liver microsomes of sea turtles, as is the case for animals ranging from mammals (Letcher et al. 2000a) to nematodes (Schafer et al. 2009).

Because various CYP isoforms demonstrate regio- and stereo-selective metabolism of testosterone, the catalytic activity towards testosterone is often used to characterize CYPs. In rats, the hydroxylation of testosterone in the 6β, 16α, and 16β positions is characteristic of the respective catalytic activities of CYP3A, CYP2C, and CYP2B (Lewis 2001), but these enzymes do show overlapping specificities (e.g., CYP2B1 can hydroxylate testosterone in both the 16α and 16β positions). In rainbow trout, CYP3A27 is primarily responsible for the formation of 6β-hydroxytestosterone, but both CYP2K1 and CYP3A27 contribute to the formation of 16α- and 16β-hydroxytestosterone, although the rates of 16β-testosterone formation are somewhat higher for CYP3A27 (Miranda et al. 1989). In sea turtles, CYP2-like protein expression correlated with the rate of hydroxylation of testosterone at all three positions, with the strongest correlation with the 16α metabolite, while CYP3-like protein expression

correlated most highly with 6β-hydroxytestosterone production, but also with 16α-hydroxytestosterone formation. These results suggest that sea turtle CYPs, like those of other vertebrates, show overlapping catalytic activities. The hydroxylation rate of PCB 52 correlated with both CYP2-like and CYP3-like protein expression, but the correlation was stronger with CYP2-like protein expression. CYP2-like proteins may thus be responsible for hydroxylation of PCBs with vicinal *meta-para* protons in sea turtles, as is the case in mammals (Shimada et al. 1981; Mills et al. 1985; Ishida et al. 1991; Koga et al. 1996). However, biotransformation of PCB 54, a *meta-para* unsubstituted tetrachlorinated biphenyl, has been shown to undergo hydroxylation catalyzed by rat CYP3A1, albeit at a much lower rate than CYP2B1 (Edwards 2006). CYP3A4 is thought to contribute to the biotransformation of *meta-para* unsubstituted congeners in humans (Letcher et al. 2000a). Thus, CYP3-like proteins, in addition to CYP2-like proteins, may also contribute to the metabolism of *meta-para* unsubstituted PCBs in sea turtles.

Variation between the rates of hydroxylation in sea turtle species and individuals could be caused by biological or environmental factors. Sea turtles have adapted distinct dietary patterns that could influence not only induction of biotransformation and bioaccumulation of PCBs and other contaminants, but also evolutionary defense mechanisms against naturally occurring dietary compounds. Loggerhead and olive ridley sea turtles feed on fish, salps, crustaceans, and molluscs; green sea turtles feed on sea grass and algae; and hawksbill sea turtles feed on sea sponges (Bjorndal 1997). From a trophic standpoint, loggerhead and olive ridleys would be expected to show higher accumulation. However, significant differences in PCB bioaccumulation were not found

between loggerhead, olive ridley, and green sea turtles (Chapter 2). In the present study, some green and all hawksbill sea turtles had higher rates of PCB 52 metabolism than the other species. Algae, sea grass, and sponges have been shown to produce a wide variety of secondary metabolites as chemical defense against consumers (Hay 1996). Dietary exposure to natural compounds can influence the expression and capacity of biotransformation enzymes through evolutionary adaption of higher basal levels, or through xenobiotic responsive induction mechanisms. For example, a species of butterflyfish (*Chaetodon capistratus*) that preferentially feeds on chemically-rich gorgorian corals had higher total CYP content and CYP2B-like and CYP3A-like enzymes than a cogeneric species that avoids eating gorgonians (Vrolijk et al. 1994). Thus, natural compounds may influence the biotransformation capacity of green and hawksbill sea turtles, leading to higher rates of PCB 52 hydroxylation.

Hydroxylation of PCB 77 was not detected in hepatic microsomes of any of the four species of sea turtles. However, hydroxylation rates of BNF-treated rat microsomes were within the literature reported values of 18-50 pmol/min/mg for treated rats (Mills et al. 1985; Ishida et al. 1991; Borlakoglu and Wilkins 1993). The lack of PCB 77 hydroxylation, a reaction catalyzed by CYP1A in rodents, is consistent with the lack of cross-reactivity between sea turtle hepatic proteins and a CYP1A antibody that recognizes a conserved peptide sequence of vertebrate CYP1A (Chapter 2). This antibody recognizes CYP1A from mammals, birds, and fish, and therefore should allow detection in reptiles as well. The lack of CYP1A cross-reactivity was found despite the presence of dioxin-like PCBs in loggerhead, green, and olive ridley turtles at TEQ

(toxicity equivalency quotient) levels near the EC_{50} for 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) induced EROD activity in snakes (Hecker et al. 2006). In Kemp's ridley sea turtles, low EROD activity was observed in four partially purified CYP enzymes from the liver of wild Kemp's ridley turtles (Goldman and McClellan-Green 2001). Taken together, these results suggest recalcitrance of CYP1A induction in sea turtles or differences between mammal and sea turtle CYP1A catalytic activities.

Liver microsomes from painted turtle treated three times with 25 mg/kg, then once with 50 mg/kg BNF, hydroxylated PCB 77 at a rate 9-15 fold lower than that of rat treated with Aroclor 1254 (Schlezinger et al. 2000) or BNF (present study), and was comparable to the lower rates detected in sea turtles in the present study. Painted turtle liver microsomes had the highest CYP1A protein expression (scup-equivalents) as compared to all other induced species, including rat induced with Aroclor 1254, but showed very low EROD activity, which was comparable to levels in non-induced chicken (*Gallus domesticus*), gull (*Larus argentatus*), and eel (*Anguilla rostrata*) (Schlezinger et al. 2000). In this same species of turtle, a single treatment with 5 mg/kg PCB 77 failed to induce CYP1A expression, while repeated dosing with much higher concentrations of this and other CYP1A inducers caused up to a 20 fold increase in CYP1A induction (Yawetz et al. 1998). Further evidence of recalcitrance in CYP1A expression and catalytic activity has been found in other species of reptiles. Liver microsomes from freshwater turtles (*Mauremys caspica rivulata*) collected from sewage oxidation ponds failed to show CYP1A induction, although induction was observed in other species collected from the ponds (Yawetz et al. 1997). In alligators treated 3 times with 35

mg/kg 3MC, benzo-a-pyrene hydroxylase (BPOH), and EROD (and other O-dealkylase) activities were induced compared to control alligators, but were comparable with uninduced rat (Jewell et al. 1989). Additionally, based on O-dealkylase activities characteristic of various CYP isoforms, the authors suggested that 3MC induced unique CYP isoform expression patterns or equivalent isoforms with distinct catalytic activities in reptiles as compared to mammals and fish. BPOH activity in alligator was inhibited by alpha-naphthoflavone (ANF), whereas snake BHOH showed profound insensitivity to this classic CYP1A inhibitor (Jewell et al. 1989), suggesting variation of CYP expression patterns or activity between reptilian species as well. In African clawed frogs (*Xenopus laevis*), as well as some adapted fish populations, recalcitrance of CYP1A induction by TCDD and other classic inducers has been linked to low binding affinity for TCDD to the aryl hydrocarbon receptor (AhR), the transcription factor responsible for CYP1A induction (Bello et al. 2001; Lavine et al. 2005). A photoaffinity labeling study of radiolabeled TCDD binding to AhR demonstrated the presence of AhR in two out of three painted turtles, but not in the one alligator examined in the study (Hahn et al. 1994).

In contrast to PCB 77, some sea turtles hydroxylated PCB 52, a substrate for rat CYP2B with *meta-para* vicinal protons. Preferential metabolism of *meta-para* unsubstituted PCBs was supported by congener-specific PCB accumulation patterns in green, loggerhead, olive ridley, and Kemp's ridley turtles which showed lower concentrations of CYP2B substrate PCBs relative to CYP1A substrate PCBs (Chapter 2; Keller et al. 2004). Metabolism of PCBs with *meta-para* vicinal protons has been suggested in a variety of animals, including worms, fish, birds, amphibians, and

mammals, based on accumulation patterns and/or elimination (e.g., Boon et al. 1989). The predominant metabolite of PCB biotransformation was a hydroxylated compound. OH-PCBs have been detected in a variety of organisms ranging from fish to humans (Letcher et al. 2000b). Differences in individual OH-PCB concentrations, total OH-PCB levels, and ratios of OH-PCB to total PCBs vary between and within species, highlighting variability of biotransformation and elimination in wild organisms. For example, despite the fact that fish have been shown to be slow metabolizers of PCBs, levels of OH-PCBs in the plasma of benthic and pelagic fish from the highly contaminated Detroit River were in the ppm range, and in some fish OH-PCB levels were higher than PCB concentrations (Li et al. 2003; Valters et al. 2005). Additionally, although it is generally believed that most organisms, especially mammals, preferentially metabolize *meta-para* unsubstituted PCBs, hepatic microsomes from beluga whales preferentially metabolize *ortho-meta* unsubstituted PCBs (White et al. 2000; McKinney et al. 2006). Concentrations of OH-PCBs may represent biotransformation capacity, but also may be influenced by maternal transfer via placenta (Sinjari and Darnerud 1998; Meerts et al. 2002) or yolk (e.g., Jorundsdottir et al. 2010), as well as by accumulation from abiotic or biotic sources (Ueno et al. 2007; Letcher et al. 2009). Nevertheless, formation of OH-PCBs seems to be a biotransformation capacity possessed by vertebrates, including reptilian species such as sea turtles.

The metabolism of PCBs, primarily *in vivo*, has been demonstrated in a variety of organisms and generally shows the following pattern of biotransformation capacity: mammal > bird > fish (Matthews and Dedrick 1984). Studies of PCB biotransformation

in leopard (*Rana pipiens)* and green (*Rana clamitans*) frogs suggest that amphibians have a higher PCB metabolic capacity than fish but less than mammals (Safe et al. 1976; Leney et al. 2006). Direct comparison between other vertebrates and reptiles is confounded by variations in methodologies used to determine PCB biotransformation (i.e., *in vivo* versus *in vitro* systems, single PCB congener versus mixture exposures, and loss of parent compound versus metabolite formation determination). In the present study, sea turtles had rates of PCB 52 hydroxylation up to half of that in rats, and Schlezinger et al. (2000) showed that painted turtle PCB hydroxylation rates were higher than fish and similar to birds. Thus, rates of amphibian and reptilian CYP-catalyzed biotransformation, like birds, fall in between the rates of fish and mammals.

Measurements of hepatic *in vitro* PCB 52 and PCB 77 oxidation rates by sea turtles support inferences of CYP2-like metabolism as a factor of elimination of PCBs with *meta-para* vicinal protons. Species-specific oxidation rates and PCB accumulation profiles in sea turtles (Chapter 2; Keller et al. 2004) support a model in which rates of hepatic biotransformation may determine elimination and relative concentrations of some PCBs in reptilian tissues.

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Chapter 5: Interaction of 4,5-Dibromopyrrole-2-Carboxylic Acid with Hawksbill Sea Turtle (*Eretmochelys imbricata*) Glutathione *S*-Transferase

Abstract

While anthropogenic halogenated organic compounds (HOCs) are notorious for a multitude of toxic effects, exposure to naturally produced HOCs, thousands of which are also known to exist and have recently been recognized as persistent and bioaccumulative, may also cause adverse effects. As is the case for anthropogenic compounds, the toxicity of natural HOCs is not an inherent trait of the compound, but rather depends on the biochemical interactions, such as biotransformation, between the compound and the consumer. The endangered hawksbill sea turtle (*Eretmochelys imbricata*) feeds primarily on marine demosponges, including *Agelas* species, which produce natural HOCs, such as 4,5-dibromopyrrole-2-carboxylic acid (DBPC), as deterrents against predation. In the present study, we investigated the potential mechanisms of tolerance to DBPC in hawksbill sea turtle liver subcellular fractions, with particular focus on glutathione *S*-transferase enzymes (GST) because this family of biotransformation enzymes has been implicated as a major factor in adaptive processes that prevent toxicity to consumers by chemically-defended prey. Hepatic cytosol from green (*Chelonia mydas*), loggerhead (*Caretta caretta*), and olive ridley (*Lepidochelys olivacea*) sea turtles, as well as phenobarbital (PB)-treated rats were included for comparative studies. To investigate the possibility of GST-catalyzed GSH conjugation, inhibition of GST catalytic activity was examined in subcellular fractions of the liver of hawksbill sea turtles. The potential biotransformation of DBPC was evaluated by loss of parent compound from *in vitro*

incubations of DBPC with hawksbill (and PB-treated rat) liver subcellular fractions using HPLC/UV, as well as by formation of ^{35}S-GSH metabolites from similar *in vitro* incubations using HPLC/RD. The lack of detectable metabolism of DBPC by hawksbill sea turtle subcellular fractions indicates that biotransformation may not be the primary mechanism of tolerance. However, non-competitive binding of DBPC to hawksbill GST, in a similar manner to bilirubin, indicated ligandin-like function of hawksbill GST. These results indicate that the non-substrate binding and sequestration of DBPC by GST may provide protection from sponge HOCs found in the diet of hawksbill sea turtles.

Introduction

Halogenated organic compounds (HOCs), such as dichlorodiphenyl-trichloroethane, chlordane (DDT), pentachlorophenol, toxaphene, lindane, chlordane, polychlorinated dibenzo-p-dioxins, dibenzo-p-dioxin-furans, polybrominated diphenyl ethers (PBDEs), and polychlorinated biphenyls (PCBs), are some of the most hazardous environmental contaminants documented by environmental toxicologists. Because of the wide range of toxic effects in many different organisms, as well as their bioaccumulative and persistent properties, the manufacture and use of these anthropogenic HOCs has been banned in many countries.

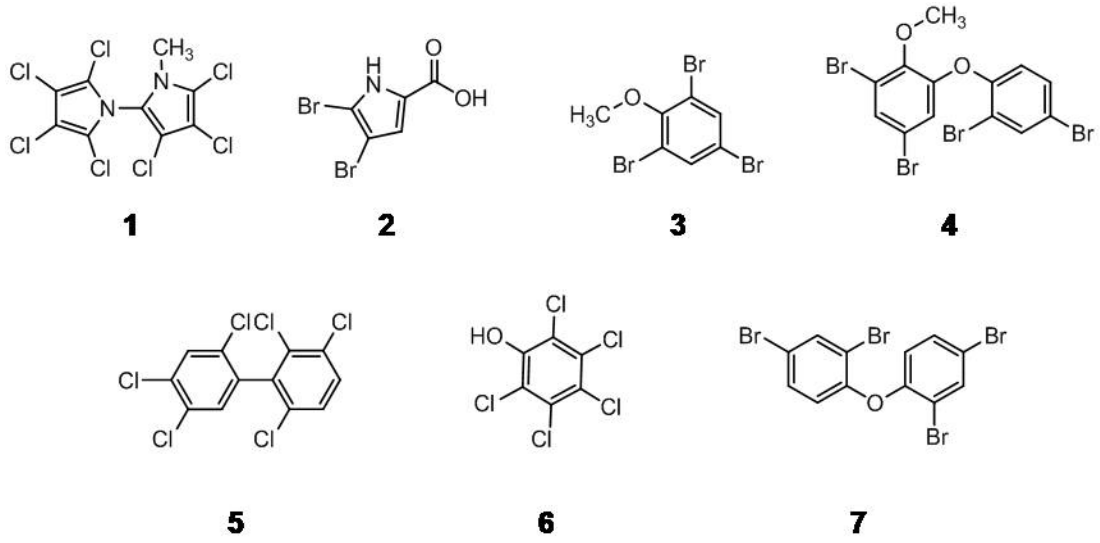

Figure 5.1. Representative structures of natural (upper panel) and anthropogenic (lower panel) halogenated organic compounds (adapted from Vetter et al. 2004). **1:** 2,3,3',4,4',5,5'- heptachloro-1'-methyl-1'-1,2'-bipyrrole; **2:** 4,5-dibromopyrrole-2-carboxylic acid; **3:** 2,4,6-tribromoanisole; **4: :** 4,6-dibromo-2-(2',4'-dibromo) phenoxyanisole (a MeO-PBDE) **5:** 2,2',3,4',5',6-hexachlorobiphenyl; **6:** pentachlorophenol; **7:** 2,2',4,4'-tetrabromodiphenyl ether

Thousands of naturally produced HOCs are also known to exist in the environment (Gribble 2003), which show structural similarities to anthropogenic HOCs (Figure 5.1). The detection of hydroxy- (OH)- and methoxy (MeO)- PBDEs in wildlife was thought to result from biotransformation of anthropogenic PBDE (see Figure 5.1, structure **4** for an example of MeO-PBDE). However, about 25 different OH- and MeO-PBDEs have been isolated from marine demosponges, primarily of the *Dysidea* order (Gribble 2000). Radiocarbon dating of MeO-PBDEs found in a cetacean indicated the MeO-PBDEs accumulated from natural sources (Teuten et al. 2005). Furthermore, PBDE metabolites have been reported at concentrations higher than PBDEs in marine animals (Vetter et al., 2002). Various modes of toxicity, including neurologic and endocrine effects, have been

associated with both OH- and MeO-PBDEs, often with greater potency than analogous PBDEs (e.g., Schultz 2002; Dingemans et al. 2008). Other natural HOCs which have garnered recent attention are the halogenated dimethyl-2,2'-bipyrroles (HDBPs), an example of which is shown in Figure 5.1. structure **1**. The halogens of HDBPs can include bromine atoms, chlorine atoms, or a combination of the two (Vetter 2006). Several lines of evidence, including radiocarbon dating (Reddy et al. 2004), indicate that the source of HDBPs is natural (Vetter 2006). The physiochemical properties of HDBPs are within the range of those for PCBs (Tittlemier et al. 2004), and stable nitrogen isotope analysis was used to show that these compounds, in fact, biomagnify with trophic level in marine ecosystems (Tittlemier et al. 2002). In chicken embryo hepatocytes, HDBPs bind to the aryl hydrocarbon receptor (AhR), and induce CYP1A1 activity and porphyrin accumulation (classic indicators of dioxin-like effects), illustrating that (some) natural HOCs act in a similar manner as anthropogenic HOCs(Tittlemier et al. 2003). In sessile benthic creatures, including algae, corals, and sponges, HOCs are considered secondary metabolites (SM) which provide a defense mechanism against predation (Gribble 2003). For example, 2,3,4-tribromopyrrole, isolated from a marine hemichordate worm, *Saccoglossus kowalevskii*, deters fish predation (John et al. 2004; Kicklighter et al. 2004).

As is the case for anthropogenic compounds, the toxicity (or feeding deterrence) of natural HOCs and other SMs is not an inherent trait of the compound, but rather depends on the biochemical interactions between the compound and the consumer (Sotka et al. 2009). Pharamacological mechanisms responsible for xenobiotic processing within an organism include absorption, distribution, metabolism, and elimination (ADME), and

metabolism (or biotransformation) is particularly important in the process as it influences all of the other mechanisms (Klassen and Watkins 2003). Understanding the ADME of SMs in terrestrial systems has provided great insight into consumer-prey interactions, including adaptive processes that prevent toxicity in consumers in terrestrial systems (Li et al. 2007). Biotransformation enzymes responsible for tolerance of SMs in consumers include cytochrome P450 monooxygenases (CYPs) and glutathione *S*-transferases (GSTs), as well as ATP binding cassette (ABC) transporters, which do not catalyze chemical transformations, but are responsible for the export of the metabolites from the cell (Sotka and Whalen 2008).

GSTs are a superfamily of catalytically versatile and diverse enzymes which play a critical role in the detoxification of both exogenous and endogenous electrophiles (Hayes and Pulford 1995). GST enzymes are highly conserved and have been identified in all examined taxa, including bacteria, plants, insects, invertebrates, and vertebrates (Zimniak and Singh 2007). GSTs are constitutively expressed, but are also subject to regulation in response to cellular stress, and xenobiotic exposure (Chapter 1). The basic reaction catalyzed by GST is the nucleophilic attack by GSH on a diverse group of hydrophobic compounds that contain an electrophilic carbon, nitrogen, or sulfur atom. GST conjugation of HOCs can result in reductive dehalogenation or halogen displacement as is the case for GST catalyzed biotransformation of dichloromethane to formaldehyde, as well as GSH conjugation to 1,2-dichloro-4-nitrobenzene (DCNB), 1-chloro-2,4-dinitrobenzene (CDNB) , and *para*-nitrobenzyl chloride (NBC), all of which are classic indicators of GST catalytic activity (Chapter 1; Zimniak 2007). The ability of

terrestrial insects to tolerate SMs in host plants is attributed, at least in part, to high GST catalytic activity (Li et al. 2007), and in marine organisms which prey on chemically-defended organisms, GST activity has been postulated to provide similar tolerance (DeBusk et al. 2000; Whalen et al. 2008).

The endangered hawksbill sea turtle (*Eretmochelys imbricata*) feeds primarily on marine demosponges (Meylan 1988; Leon and Bjorndal 2002), including *Agelas* species (M.T. Hamann, personal communication). Previous research has shown that the biotransformation capacity of hawksbill sea turtle livers varies from other species of sea turtles (which do not feed on sponges). The rate of 2,2',5,5'-tetrachlorobiphenyl (PCB 52) hydroxylation by hawksbill liver microsomes was approximately 10-fold higher than that of carnivorous olive ridley (*Lepidochelys olivacea*) and loggerhead (*Caretta caretta*) sea turtles (Chapter 4). The rate of GST catalyzed NBC conjugation was 3-4 fold higher in hepatic cytosol from hawksbills than in that from green (*Chelonia mydas*), loggerhead, or olive ridley sea turtles (Chapter 3). These species-specific differences in biotransformation capacity may be indicative of adaptive tolerance of hawksbill sea turtles to natural HOCs found in their diet. The natural HOC, 4,5-dibromopyrrole-2-carboxylic acid (DBPC), has been identified in several sponges of the *Agelas* and *Axinella* genera (Forenza et al. 1971; Barrow and Capon 1993; Assmann et al. 2000). Fractionation studies of sponge tissue, as well as use of synthetic compound, identified DBPC as the primary deterrent against fish predation (Chanas et al. 1996). DBPC and other brominated pyrrole alkaloids affect calcium influx and levels in neurons, which may reflect chemoreception and/or potential toxicity (Bickmeyer et al. 2004; Bickmeyer

et al. 2005). However, the mechanism(s) of tolerance in hawksbill sea turtles to DBPC and other natural HOC found in their sponge-rich diet has not been investigated.

In the present study, we investigated the biotransformation of DBPC by sea turtle liver subcellular fractions. Green, loggerhead, and olive ridley sea turtle samples, as well as phenobarbital (PB)-treated rat samples were included for comparative studies. The biotransformation of DBPC was evaluated by loss of parent compound from *in vitro* incubations of DBPC with hawksbill (and PB-treated rat) liver subcellular fractions using HPLC/UV, as well as by putative formation of radiolabeled metabolites from *in vitro* incubations with ^{35}S-GSH using HPLC/RD. Incubations failed to catalyze metabolite formation, but kinetic studies indicated species specific ligandin activity which may provide hawksbill sea turtles a mechanism of tolerance towards DBPC toxicity.

Methods

Reagents

DBPC was synthesized following the method of Ponasik et al. (1998). Nicotinamide adenine dinucloetide phosphate (NADPH), reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), and 4-nitrobenzyl chloride (NBC) were purchased from Sigma Chemical (St. Louis, Mo, USA). Bilirubin was purchased from Frontier Scientific Inc. (Logan, Utah, USA). ^{35}S-glutathioine (^{35}S-GSH, 894 Ci/mmol) was obtained from Perkin-Elmer (Waltham, MA, USA). The ^{35}S-GSH was aliquoted to single-use amounts to avoid repeated freeze-thaw cycles associated with volatilization of

^{35}S and stored, after evacuation of oxygen with nitrogen gas, at -80°C to avoid degradation of the compound. Various buffers, salts, and cofactors used were purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma Chemical.Chromatography grade formic acid and methanol were obtained from Fisher. All solvents used for HPLC, including E-PURE (Barnstead, Thermo Scientific, Waltham, MA, USA) water, were further purified by filtration through 0.2 μm nylon membranes (Millipore, Billerica, MA, USA) prior to use.

Sea turtle and rat samples

Methods for the collection and preparation of samples of liver tissue from wild loggerhead, green, olive ridley, and hawksbill sea turtles were described elsewhere (Chapters 2 and 3). Additional samples of liver tissue from wild hatchling hawksbill sea turtles were collected in the summer of 2007 from nesting beaches near El Cuyo, Yucatan, Mexico from animals for which time of death could be approximated within 6 hours, and sub-cellular fractions (microsomes and cytosol) were prepared as previously described (Chapters 2 and 3). Microsomes and cytosol from rats treated with phenobarbital (PB) were obtained from Xenotech (Lenexa, KS, USA).

GST catalytic activity inhibition studies with DBPC and bilirubin

Cytosolic GST catalytic activity was assessed spectrophotometrically using CDNB, and NBC as substrates as described previously (Chapter 3). DBPC inhibition was evaluated by pre-incubation of cytosolic protein with DBPC (in ethanol, final concentration of ethanol in reaction was 0.1%, and negative controls received an equal volume of ethanol) before assaying CDNB or NBC catalytic activity. Initially, equimolar

concentrations of DBPC and CDNB (500 μM) or DBPC and NBC (250 μM) were employed. The kinetics of GST CDNB activity inhibition by DBPC and bilirubin were also evaluated. GST CDNB concentrations were varied as described in Chapter 3 over a range of DBPC concentrations (50 to 2500 μM), and bilirubin concentrations (50 to 200 μM). Prism 5 for Windows software (Graphpad Software Inc., La Jolla, CA, USA) was used for kinetic analyses.

In vitro incubations for biotransformation studies

DBPC (100 μM) was incubated with rat or hawksbill cytosol, or both microsomes and cytosol and various cofactors in 200 μL reaction volumes as follows: cytosol reactions contained 0.3 mg/mL cytosolic protein and 10 mM GSH; and microsome plus cytosol reactions contained 0.3 mg/mL microsomal protein, 0.3 mg/mL cytosolic protein, 10 mM NADPH, and 10 mM GSH. Negative controls excluded GSH for cytosolic incubations or NADPH for microsome plus cytosol incubations, or contained boiled protein. The incubations were initiated by the addition of NADPH, except cytosolic incubations which were initiated with GSH, and incubated for up to 2 hours at 25°C or 37°C, for turtle and rat, respectively. The addition of methanol (4°C, 50 μL) terminated the reactions. The incubations were then centrifuged (15 min at 13,000 *g*) and the supernatant was subjected to high pressure liquid chromatography/UV detection (HPLC/UV) analysis. *In vitro* incubations with ^{35}S-GSH contained either radiolabeled GSH as100 nCi with 0.56 nM total GSH in each reaction or 10 μCi/μmol with 200 μM total GSH in each reaction. Extracts were analyzed by HPLC/radioactivity detection (RD).

HPLC/UV and HPLC//RD analysis

The loss of parent compound from *in vitro* biotransformation incubations was assessed by HPLC/UV. The HPLC/UV consisted of an Agilent1100 Series HPLC system (Wilmington, DE, USA) equipped with a Hypersil ODS C18 column (5 um, 250 x 4.6 mm), protected by a Phenomenex Security Guard cartridge system equipped with a C18 4x3 mm cartridge. The mobile phase consisted of A, 0.1% formic acid and 5% methanol in water, and B, 100% methanol. The HPLC system was operated at a flow rate of 1.0 mL/min with a linear gradient of 0% B to 80% B over 30 minutes, then held at 80% B for 5 minutes before returning to 100% A over 5 minutes, and then held at 100% A to stabilize the column prior to subsequent injection. UV/Vis absorbance was monitored at 254 nm.

The appearance of ^{35}S-DBPC metabolite(s) from *in vitro* biotransformation incubations was evaluated by HPLC/RD. The HPLC/RD Shimadzu SCL-10AVP HPLC system (Columbia, MD, USA) connected to an on-line radioactivity detector (β-ram Model 3, IN/US Systems Inc., Tampa, FL, USA) using In-Flow 2:1 (IN/US Systems Inc.) as scintillation cocktail. The column/guard column, mobile phase, and gradient program were identical to those used for HPLC/UV analysis. Radioactivity was monitored from the start of the run until 30 minutes, which allowed the monitoring of all peaks between reduced glutathione and the DBPC.

Data analysis

Sample sizes ranged from n=3-6 for each species (replicates of 2-3) in each experiment, except rat, which represents 50 pooled individuals (3-5 replicates).

Statistical analyses were performed with Prism 5. A p value of less than 0.05 was considered significant. ANOVA and Tukey's tests were performed in order to determine differences among species. Two-tailed T tests were performed in order to determine inhibition and loss of parent compound within a species. Results are presented as mean ± standard error.

Results

As shown in Table 5.1, DBPC caused significant inhibition of hawksbill GST CDNB activity, but did not significantly inhibit GST CDNB activity in any of the three other species of sea turtles. While hawksbill sea turtles demonstrated higher (3-4 fold) GST NBC activity than the other 3 species of sea turtles, DBPC did not significantly inhibit GST NBC activity in any species of sea turtle (Table 5.1). Inhibition of CDNB activity in hepatic cytosol from hawksbill sea turtles and PB-treated rat by bilirubin, a non-competitive inhibitor of mammalian GST, was also carried out. Incubations containing 500 μM CDNB and 1 mM GSH, 500 μM DBPC significantly inhibited 45% of hawksbill and 51% of rat GST activities, while 50 μM bilirubin significantly inhibited 22% of hawksbill and 45% of rat activities (Figure 5.2). Kinetic analysis revealed that DBPC showed non-competitive, concentration-dependent inhibition of hepatic cytosolic GST CDNB activity from hawksbills with a calculated K_i of 560 μM (Figure 5.3). Kinetic analysis also demonstrated that bilirubin showed non-competitive, concentration-dependent inhibition of hawksbill cytosolic GST CDNB activity with a calculated K_i of 90 μM (Figure 5.4).

Table 5.1. Sea turtle cytosolic GST catalytic activity (nmol/min/mg protein) towards CDNB and NBC with and without DBPC.

	CDNB		NBC	
Species	Control	+DBPC	Control	+DBPC
Hawksbill	247 ± 15.9	139 ± 11.2*	110 ± 6.82‡	99.9 ± 8.62‡
Loggerhead	398 ± 86.5	283 ± 76.3	26.6 ± 9.76	24.7 ± 10.9
Olive ridley	391 ± 87.8	239 ± 34.9	29.6 ± 9.01	27.5 ± 11.1
Green	510 ± 74.2	426 ± 66.4	34.0 ± 4.13	31.6 ± 6.26

* indicates significant inhibition by DBPC as compared to control (two-tailed T test, $p=0.002$)

‡ indicates significantly higher activity than the three other species within treatment group (ANOVA,Tukey's test, $p<0.001$)

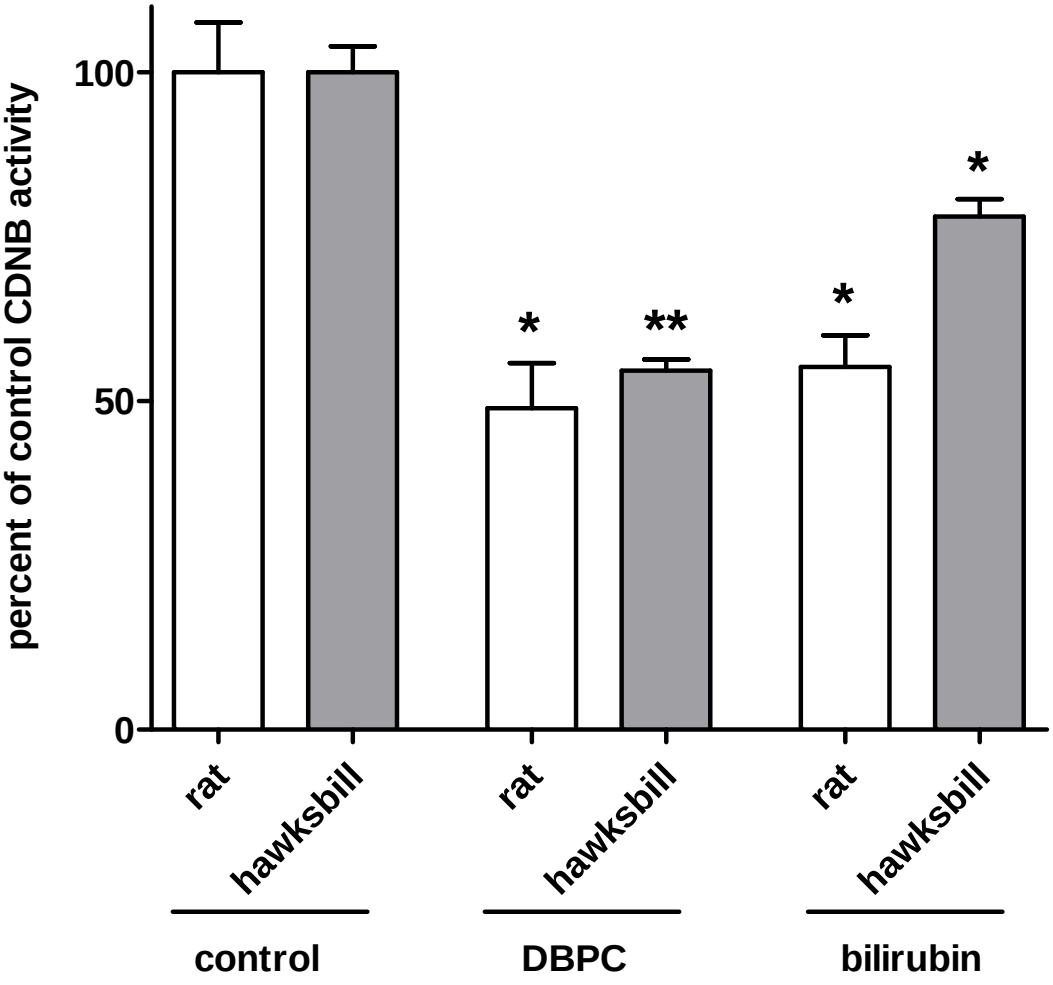

Figure 5.2. Inhibition of hawksbill and rat GST CDNB activity by DBPC and bilirubin. * indicates significant inhibition, $p \leq 0.01$, ** indicates significant inhibition, $p \leq 0.001$, as compared to control (two-tailed T tests)

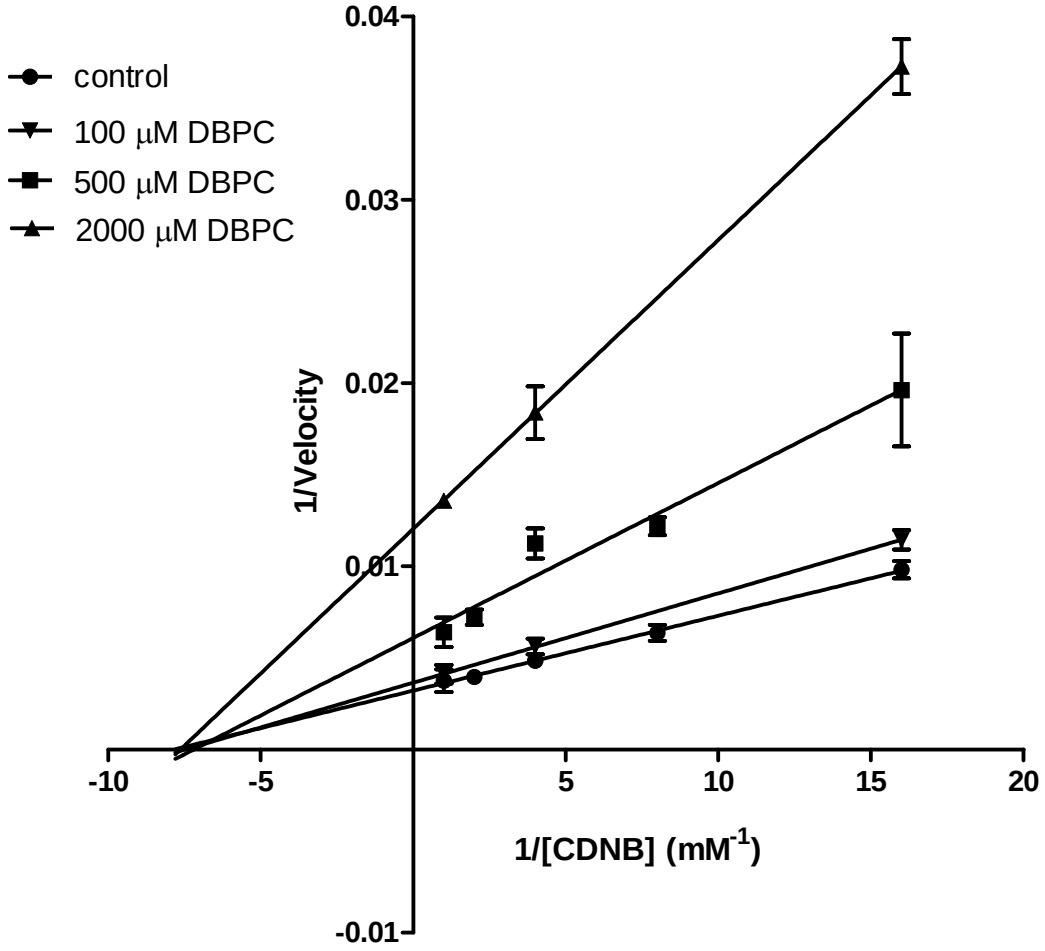

Figure 5.3. Double reciprocal plots of the inhibition of hawksbill GST by DBPC in the presence of varying concentrations of CDNB. The graph demonstrates that DBPC is a non-competitive inhibitor, showing a decrease in Vmax (1/y-intercept) and little or no change in Km (-1/x-intercept).

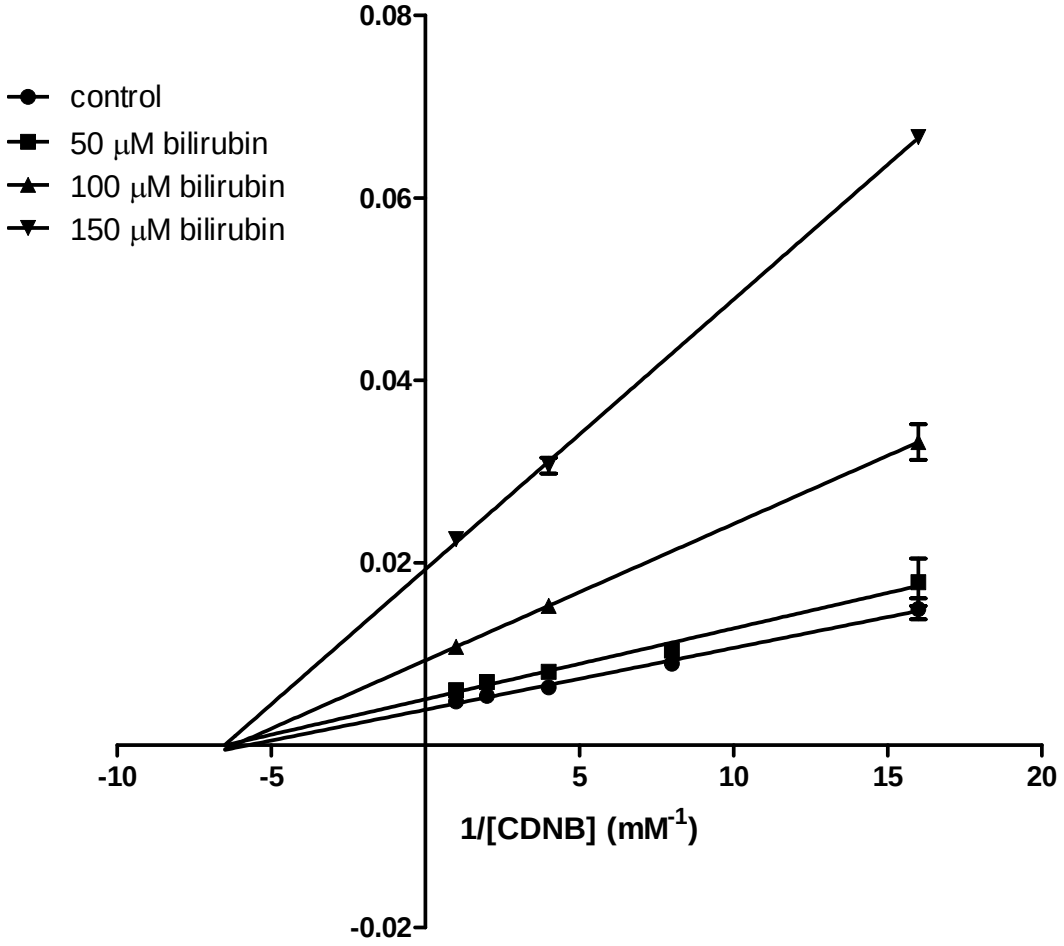

Figure 5.4. Double reciprocal plots of the inhibition of hawksbill GST by bilirubin in the presence of varying concentrations of CDNB. The graph demonstrates that bilirubin is a non-competitive inhibitor, showing a decrease in Vmax (1/y-intercept) and little or no change in Km (-1/x-intercept).

For *in vitro* biotransformation studies, HPLC/UV analysis was employed to examine the loss of parent compound. Using this system, the detection limit for DPBC was 9 pmol/min/mg protein (14.5 pmol, or 0.6% of starting DBPC concentration). As shown in Figure 5.5, significant loss of DBPC was not detected in any incubation with hawksbill or rat subcellular fractions (T test, $p>0.05$). Further *in vitro* biotransformation studies were analyzed by HPLC/RD to evaluate the putative formation of radiolabeled metabolite(s). The HPLC/RD system provided much greater sensitivity because levels as low as 0.2 fmol of ^{35}S could be detected by the radioactivity detector (see Appendix B for detailed information regarding detection limits). Peaks indicative of ^{35}S metabolites were not detected in any incubation with hawksbill or rat subcellular fractions (see Figure 5.6 for example). However the system was able to detect ^{35}S labeled metabolites from incubations of PB-treated rat cytosol with styrene oxide and ^{35}S-GSH (see Figure B.4 for representative chromatogram of metabolites).

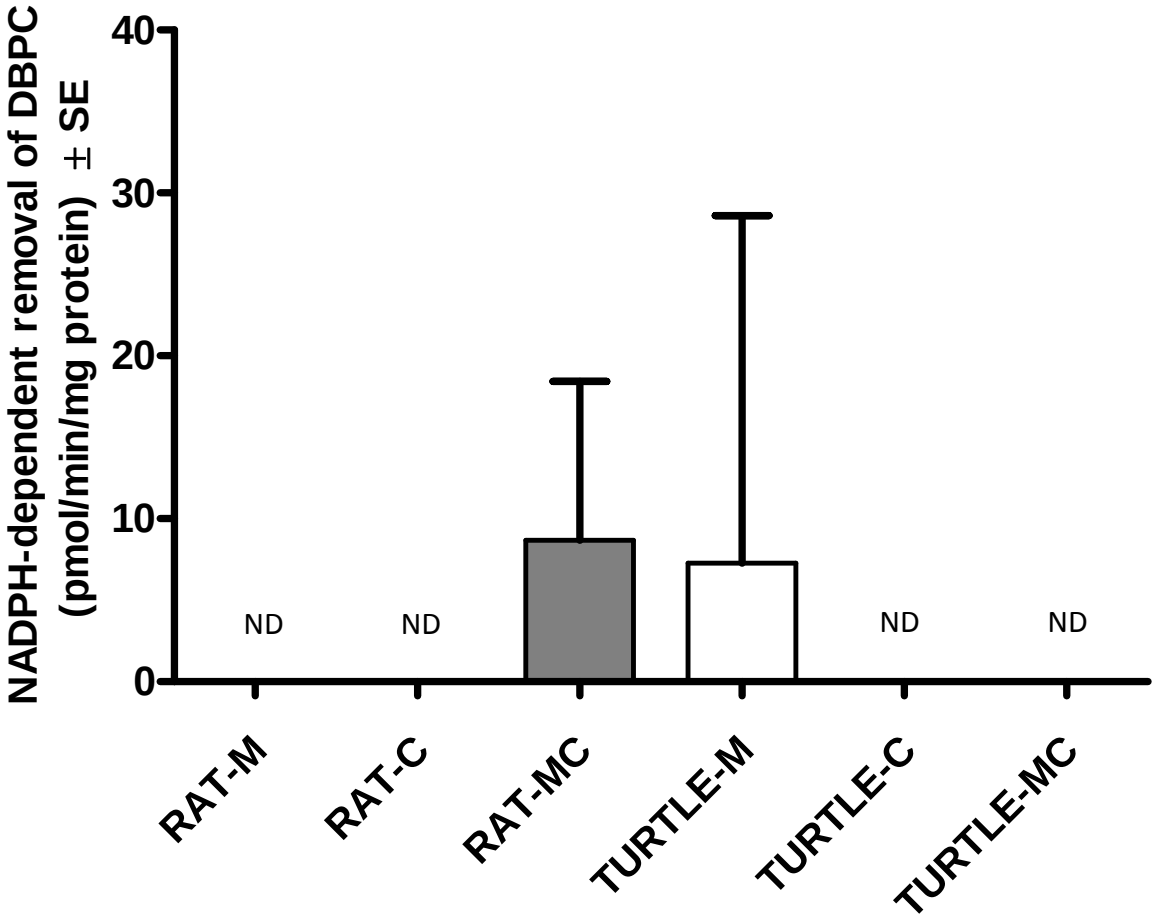

Figure 5.5. Change in DBPC concentration after 90 min incubation with various subcellular fractions from rat and turtle (M=microsomes, C=cytosol, MC=microsomes+cytosol, ND=not detected).

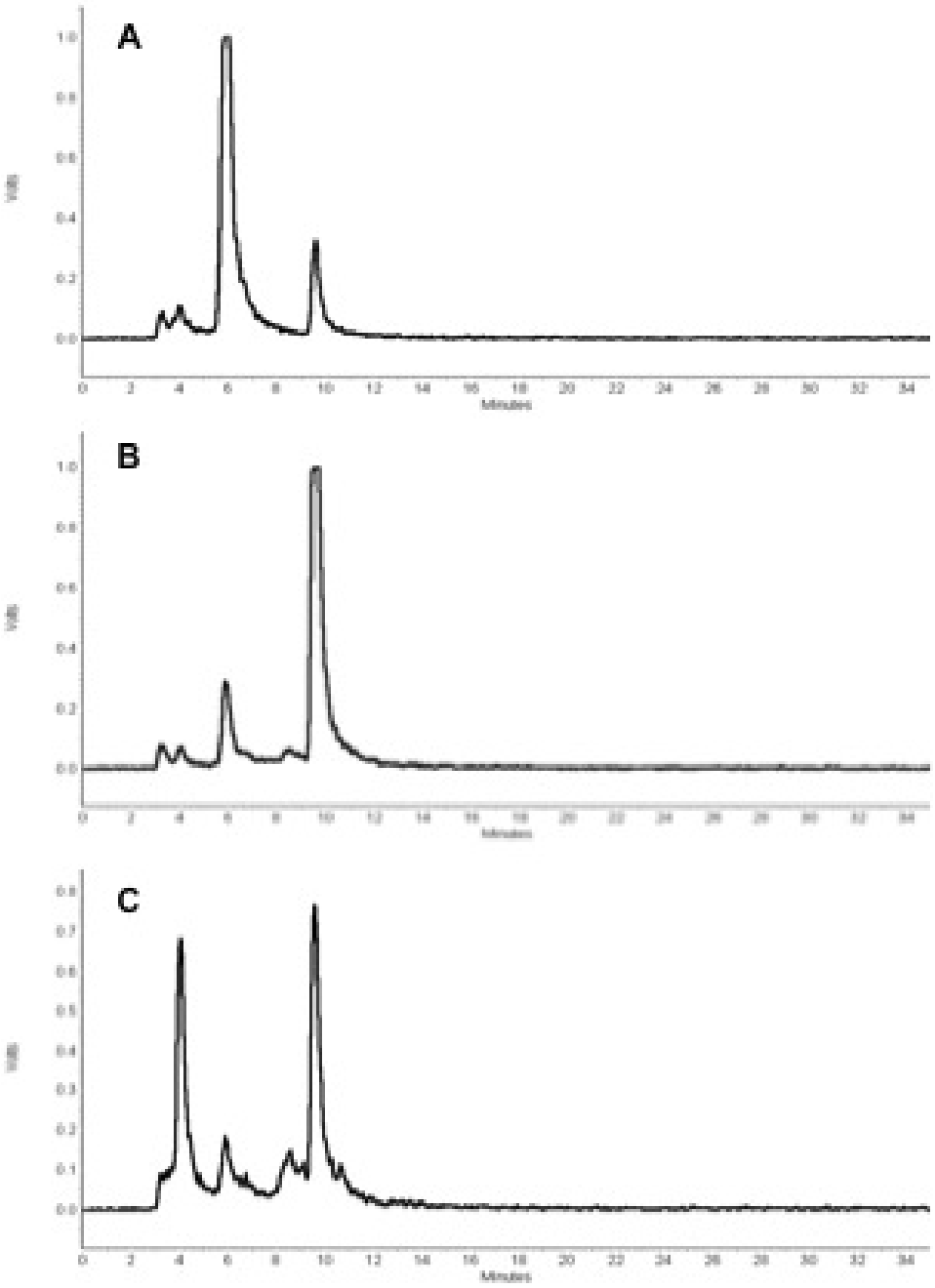

Figure 5.6. Representative radio-chromatograms of a)*in vitro* incubation of hawksbill cytosol with DBPC and ^{35}S-GSH, and b) similar incubation containing boiled proteins as a negative control, and c) similar incubation without DBPC added to the incubation as a negative control. The peaks at minute 3-11 represent reduced and oxidized glutathione.

Discussion

The present study represents the first investigation of the potential mechanism(s) of tolerance in hawksbill sea turtles to DBPC and other natural HOC found in their sponge-rich diet. The results of this study indicated that biotransformation by GST (with or without phase I biotransformation by CYP) may not contribute to tolerance. However, inhibition studies indicated that DBPC, like bilirubin, caused non-competitive inhibition of hawksbill GST, suggesting that GST enzymes may play a protective role through the sequestration of DBPC.

Ageleas sponges contain DBPC at levels ranging from 0.35 to 2.0 mg/mL (Chanas et al. 1996; Assmann et al. 2000), illustrating the high levels of natural HOCs present in the diet of hawksbills. Little is known about hawksbill sea turtle biochemistry, but CYP and GST catalytic activities have been demonstrated in hepatic tissues (present study, Chapters 3 and 4). Initial studies indicated that hawksbill GST (CDNB activity) was non-competitively inhibited when DBPC was added to the incubation. However, i*n vitro* incubations with hawksbill sub-cellular fractions that are responsible for NADPH- and GSH- catalyzed biotransformation failed to show detectable metabolism of DBPC. It is plausible that *in vivo*, hawksbills metabolize DBPC in extrahepatic tissues. Alternatively, distribution and bioavailability, rather than biotransformation, of DBPC may be more important for hawksbill sea turtle tolerance to DBPC.

Bilirubin also demonstrated non-competitive inhibition of hawksbill GST. DBPC caused similar inhibition of both rat and hawksbill GST (51% and 41% of control activity, respectively), suggesting that the GST isoform(s) inhibited by DBPC may be

present in both mammals and reptiles. Bilirubin caused more dramatic reduction of rat GST than of hawksbill GST (55% and 78% of control activity, respectively). The rate of bilirubin inhibition of rat GST in this study was similar to that reported previously (Fukai et al. 1989). Hawksbill GST inhibition by bilirubin was similar to that reported for oysters and quahogs (69-82% of control activity), two other marine organisms which may also need to deal with marine SM in their diet (Blanchette and Singh 1999).

In addition to GSH conjugation activity, several cytosolic GSTs, such as alpha-class GSTs, have shown ligandin function, or non-covalent binding of compounds without GSH conjugation (Habig et al. 1974; Ketley et al. 1975; Boyer et al. 1984). Compounds that display non-competitive inhibition of GST are considered "ligandin inhibitors" (Mahajan and Atkins 2005). The ligandin function of GST may serve to modulate cellular uptake and distribution of hydrophobic compounds, with anionic functional groups, such as bilirubin (Litwack et al. 1971). Bilirubin, a highly hydrophobic compound, is transported to the liver via albumin, and then transported to the ER of hepatocytes via human GSTA2 for conjugation with glucuronic acid by UGT enzymes (Akizawa et al. 2008). While the ligandin binding site of GST has not been uniquely identified, several lines of evidence suggest that it may be a large contiguous hydrophobic site that spans the intersubunit cleft and the H-site (electrophilic substrate binding site), which may include discrete subsites that preferentially accommodate various hydrophobic ligands in partially overlapping regions(Le Trong et al. 2002; Mahajan and Atkins 2005). Affinity labeling of rat GSTA1-1 indicated the presence of a non-substrate binding site within the cleft between the two subunits of the dimer (Vargo

and Colman 2001). High resolution crystal structures of human GSTA1-1 revealed a solvent "puddle" or network between the two subunits of dimeric GST and the authors postulated that this site could serve as the ligandin binding site (Le Trong et al. 2002). The binding of a buffer (HEPES) molecule in the crystal structure of human GSTP1-1 also indicated a possible ligandin binding site near the H-site (Ji et al. 1997). Additional structures of hGSTP1-1 in complex with non-substrate ligands indicated that the ligandin binding site overlapped with the H-site (Oakley et al. 1999). Squid sigma-class GST has a ligandin-like binding site within the intersubunit cleft as well (Ji et al. 1996). Thus, various GST isoforms exhibit non-substrate binding sites, which may vary depending on the specific isoform.

Hawksbill GST isoforms have not been well characterized, so it is difficult to speculate as to which isoform(s) possess ligandin function. Hawksbill, green, loggerhead, and olive ridley turtles possess hepatic cytosolic GST enzymes with similar kinetic parameters (Chapter 3). Hawksbill turtles had higher catalytic activity towards NBC (indicative of theta-class, and mu-class to a lesser degree, activity in mammals) than the other 3 species examined (present study and Chapter 3). In the present study, NBC activity was not significantly inhibited in hawksbill sea turtles (or other species) by DBPC, and ligandin function has yet to be demonstrated for theta-class GSTs in any species thus far. However, kinetic studies of NBC inhibition with varying concentrations of DBPC may reveal inhibition, but a lack of available biomass precluded further analysis of the effect of DBPC on NBC activity.

In hawksbill sea turtles, non-competitive binding of DBPC to GST may represent a mechanism of tolerance by sequestering or transporting DBPC, thereby preventing the compound from reaching the target of toxicity. Tolerance of some insects to pyrethroid insecticides has been suggested to result from non-catalytic binding of pyrethroid to GSTs (Kostaropoulos et al. 2001). In a screening of SMs from host plants of the fall armyworm (*Spodoptera frugiperda*), many of the compounds non-competitively inhibited GST activity (Yu and Abo-Elghar 2000). The natural HOC lanosol (1,2-dihydroxy-3,4-dibromo-5-(hydroxymethyl)-benzene) demonstrated non-competitive inhibition of gumboot chiton (*Cryptochiton stelleri*), a marine gastropod known to feed on algae which produce lanosol (DeBusk et al. 2000). Ligandin function of GST in the marine gastropod *Cyphoma gibbosum* has been suggested to offer protection against their chemically-defended gorgonian coral diet (Whalen et al. 2010). Taken together, these results suggest that the ligandin function of GST may provide tolerance to HOCs and other SMs in a variety of organisms that feed upon chemically defended prey.

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Chapter 6: Summary and General Conclusions

Compared to other animals, data on reptilian toxicology is particularly sparse. For reptilian species, such as sea turtles, which are threatened with extinction, information on the accumulation and biotransformation of ubiquitous halogenated organic compounds (HOCs) may provide information on the potential health hazards these compounds present. Thus, this study was undertaken to gain insight into the accumulation and biotransformation of HOCs in several species of sea turtles - loggerhead (*Caretta caretta*), green (*Chelonia myda*s), olive ridley (*Lepidochelys olivacea*), and hawksbill (*Eretmochelys imbricata*).

For this study, polychlorinated biphenyls (PCBs) were selected as a group of representative anthropogenic HOCs that are present in the marine ecosystems of sea turtles. These persistent, ubiquitous, bioaccumulative HOCs have been linked to a wide array of toxic effects in mammals, thereby providing a basis for comparison between the well understood mammalian models and the under-studied sea turtles. PCBs are notorious for dioxin-like toxic effects, which are induced via planar (or dioxin-like) PCBs binding to aryl hydrocarbon receptor (AhR). However, PCB metabolites, including hydroxylated and methylsulfone derivatives, are also persistent, bioaccumulative, and toxic.

Chapter 2 described PCB congener profiles in the livers of sea turtles, and the initial examination of the biotransformation enzymes, such as cytochrome P450 (CYP), involved in the formation of persistent PCB metabolites. The results showed similar levels of total PCBs, individual PCB congeners, and dioxin-like PCB congeners between three species (loggerhead, green, and olive ridley turtles), and

somewhat variable levels within each species. The turtles were shown to express CYP2- and CYP3-like proteins, but not CYP1A-like proteins. The presence of relatively high levels of dioxin-like congeners and the lack of expression of CYP1A, which is induced via the AhR and involved in the biotransformation of some PCBs, suggested that sea turtles may accumulate PCBs metabolized by CYP1A, including some dioxin-like PCBs. Furthermore, similarities in PCB levels between the 3 species, which demonstrate different feeding strategies, suggested that bioaccumulation may be affected by other toxicokinetic processes, such as biotransformation.

In Chapter 3, we examined glutathione *S*-transferases (GSTs), which are a family of enzymes involved not only the biotransformation of HOCs (e.g., the formation of methyl-sulfone PCB metabolites involves GST) and other xenobiotics, but also plays a role in general oxidative stress response. The results showed that hawksbill, loggerhead, olive ridley, and green sea turtles possess functional GST enzymes with similar kinetic parameters. However, GST activity towards various reference substrates elucidated differences between species, which may be reflective of sea turtle evolutionary adaptations to diverse feeding strategies. Furthermore, different GST catalytic activities in various sea turtles may alter susceptibility to the potential health impacts of environmental chemicals, such as HOCs, by various sea turtle species.

After establishing that sea turtles are exposed to PCBs, and that they possess biotransformation enzymes involved in the formation of persistent metabolites, we investigated the capacity of sea turtles to biotransform PCB *in vitro*. Initially, we aimed to examine metabolism by both CYP and GST enzymes, however the lack of

detectable GST-produced metabolites from rat tissues for use as controls precluded the investigation of PCB biotransformation by sea turtle CYP (Appendix B). Therefore, we focused on CYP-catalyzed oxidation of PCBs. In mammals and other animals, the number and position of chlorine atoms of a PCB congener determines the degree to which the congener can be biotransformed. As discussed in Chapter 4, the selection of 3,3',4,4'-tetrachlorinated biphenyl (PCB 77), an *ortho-meta* unsubstituted rodent CYP1A substrate PCB and 2,2',5,5'-tetrachlorinated biphenyl (PCB 52), a *meta-para* unsubstituted rodent CYP2 substrate PCB as representative congeners would provide insight into the overall PCB biotransformation capacity of sea turtles. Sea turtles showed no detectable hydroxylation of PCB 77, consistent with the lack of CYP1A-like proteins (Chapters 2 and 4). Yet, sea turtle livers showed levels of dioxin-like PCBs near the EC_{50} for dioxin induced EROD activity in other reptilian species (Chapter 2). These results suggested that sea turtle CYP1A, as suggested for other reptilian species, may be recalcitrant to induction, or that its function differs dramatically from that of mammals and other animals.

Sea turtles showed variable rates of PCB 52 metabolism, and the rates suggested that reptiles, like birds, possess of CYP-catalyzed biotransformation capacity that of fish and mammals. Correlation analyses between PCB 52 hydroxylation rates, testosterone hydroxylase activities (Chapter 4), and CYP2- and CYP3-like protein expression supported inferences of CYP2-like biotransformation as a factor of elimination of PCBs with *meta-para* vicinal protons, as is the case for mammals. Taken together, the results of Chapters 2 and 4 supported a model in which rates of hepatic biotransformation may determine elimination and relative concentrations of PCBs in reptilian tissues. Furthermore, the potential for PCB to

impact the health of sea turtles is two-fold: 1) the turtles accumulate CYP1A substrate PCBs, some of which are dioxin-like PCBs that are toxic in the parent form, presenting the possibility for toxicity mediated by the AhR; and 2) some PCB metabolites have been linked to endocrine disruption, so there is also the potential toxicity to the endocrine system of sea turtles with PCB biotransformation.

While anthropogenic HOCs are well-known and the toxicological properties of these compounds are relatively well understood, thousands of naturally produced HOCs are also found in marine ecosystems and present the potential for persistence and toxicity. In sessile benthic creatures, including algae, corals, and sponges, HOCs provide a defense mechanism against predation. As is the case for anthropogenic compounds, the toxicity (or feeding deterrence) of natural HOCs is determined by the biochemical interactions between the compound and the consumer. In Chapter 5 we investigated the mechanisms of tolerance to the representative sponge HOC, 4,5-dibromopyrrole-2-carboxylic acid (DBPC), in the hawksbill sea turtle. The results of this study indicated that biotransformation may not play an important role in tolerance to natural sponge HOCs. However, non-substrate binding of DBPC and bilirubin to hawksbill GST suggested the potential for tolerance to sponge and possibly other HOCs via GST sequestration or transport. It is plausible that hawksbill sea turtle biotransformation enzymes reflect adaptations to their diet of natural HOCs, and, therefore, their susceptibility to toxicity from HOCs may differ from that of other sea turtle species. Furthermore, the results of Chapter 5 suggests that future studies on GST non-substrate binding, especially in organisms that feed upon chemically defended prey, may provide information on xenobiotic tolerance provided by non-catalytic functions of GST.

To conclude, this research offers valuable insight into the role biotransformation enzymes play in sea turtle susceptibility to HOCs. The information presented here provides critical knowledge to connect toxic effects of HOCs to sea turtle population decline. Much research still needs to be done in order to mitigate the risk of pollution to sea turtle populations, but the results of these studies provide a basis for future studies on the health impacts of HOCs of both natural and anthropogenic origins in sea turtles.

Appendix A: PCB Congeners

Table A.1. Polychlorinated biphenyl congener numbers, nomenclature, and chemical formulas.

Congener Number[†]	IUPAC Name	Chemical Formula
1	2-Chlorobiphenyl	$C_{12}H_9Cl_1$
2	3-Chlorobiphenyl	$C_{12}H_9Cl_1$
3	4-Chlorobiphenyl	$C_{12}H_9Cl_1$
4	2,2'-Dichlorobiphenyl	$C_{12}H_8Cl_2$
5	2,3-Dichlorobiphenyl	$C_{12}H_8Cl_2$
6	2,3'-Dichlorobiphenyl	$C_{12}H_8Cl_2$
7	2,4-Dichlorobiphenyl	$C_{12}H_8Cl_2$
8	2,4'-Dichlorobiphenyl	$C_{12}H_8Cl_2$
9	2,5-Dichlorobiphenyl	$C_{12}H_8Cl_2$
10	2,6-Dichlorobiphenyl	$C_{12}H_8Cl_2$
11	3,3'-Dichlorobiphenyl	$C_{12}H_8Cl_2$
12	3,4-Dichlorobiphenyl	$C_{12}H_8Cl_2$
13	3,4'-Dichlorobiphenyl	$C_{12}H_8Cl_2$
14	3,5-Dichlorobiphenyl	$C_{12}H_8Cl_2$
15	4,4'-Dichlorobiphenyl	$C_{12}H_8Cl_2$
16	2,2',3-Trichlorobiphenyl	$C_{12}H_7Cl_3$
17	2,2',4-Trichlorobiphenyl	$C_{12}H_7Cl_3$
18	2,2',5-Trichlorobiphenyl	$C_{12}H_7Cl_3$
19	2,2',6-Trichlorobiphenyl	$C_{12}H_7Cl_3$
20	2,3,3'-Trichlorobiphenyl	$C_{12}H_7Cl_3$
21	2,3,4-Trichlorobiphenyl	$C_{12}H_7Cl_3$
22	2,3,4'-Trichlorobiphenyl	$C_{12}H_7Cl_3$

Congener Number[†]	IUPAC Name	Chemical Formula
23	2,3,5-Trichlorobiphenyl	$C_{12}H_7Cl_3$
24	2,3,6-Trichlorobiphenyl	$C_{12}H_7Cl_3$
25	2,3',4-Trichlorobiphenyl	$C_{12}H_7Cl_3$
26	2,3',5-Trichlorobiphenyl	$C_{12}H_7Cl_3$
27	2,3',6-Trichlorobiphenyl	$C_{12}H_7Cl_3$
28	2,4,4'-Trichlorobiphenyl	$C_{12}H_7Cl_3$
29	2,4,5-Trichlorobiphenyl	$C_{12}H_7Cl_3$
30	2,4,6-Trichlorobiphenyl	$C_{12}H_7Cl_3$
31	2,4',5-Trichlorobiphenyl	$C_{12}H_7Cl_3$
32	2,4',6-Trichlorobiphenyl	$C_{12}H_7Cl_3$
33	2,3',4'-Trichlorobiphenyl	$C_{12}H_7Cl_3$
34	2,3',5'-Trichlorobiphenyl	$C_{12}H_7Cl_3$
35	3,3',4-Trichlorobiphenyl	$C_{12}H_7Cl_3$
36	3,3',5-Trichlorobiphenyl	$C_{12}H_7Cl_3$
37	3,4,4'-Trichlorobiphenyl	$C_{12}H_7Cl_3$
38	3,4,5-Trichlorobiphenyl	$C_{12}H_7Cl_3$
39	3,4',5-Trichlorobiphenyl	$C_{12}H_7Cl_3$
40	2,2',3,3'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
41	2,2',3,4-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
42	2,2',3,4'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
43	2,2',3,5-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
44	2,2',3,5'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
45	2,2',3,6-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
46	2,2',3,6'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
47	2,2',4,4'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$

Congener Number[†]	IUPAC Name	Chemical Formula
48	2,2',4,5-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
49	2,2',4,5'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
50	2,2',4,6-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
51	2,2',4,6'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
52	2,2',5,5'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
53	2,2',5,6'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
54	2,2',6,6'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
55	2,3,3',4-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
56	2,3,3',4'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
57	2,3,3',5-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
58	2,3,3',5'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
59	2,3,3',6-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
60	2,3,4,4'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
61	2,3,4,5-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
62	2,3,4,6-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
63	2,3,4',5-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
64	2,3,4',6-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
65	2,3,5,6-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
66	2,3',4,4'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
67	2,3',4,5-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
68	2,3',4,5'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
69	2,3',4,6-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
70	2,3',4',5-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
71	2,3',4',6-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
72	2,3',5,5'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
73	2,3',5',6-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$

Congener Number[†]	IUPAC Name	Chemical Formula
74	2,4,4',5-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
75	2,4,4',6-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
76	2,3',4',5'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
77 *	3,3',4,4'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
78	3,3',4,5-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
79	3,3',4,5'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
80	3,3',5,5'-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
81 *	3,4,4',5-Tetrachlorobiphenyl	$C_{12}H_6Cl_4$
82	2,2',3,3',4-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
83	2,2',3,3',5-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
84	2,2',3,3',6-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
85	2,2',3,4,4'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
86	2,2',3,4,5-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
87	2,2',3,4,5'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
88	2,2',3,4,6-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
89	2,2',3,4,6'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
90	2,2',3,4',5-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
91	2,2',3,4',6-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
92	2,2',3,5,5'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
93	2,2',3,5,6-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
94	2,2',3,5,6'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
95	2,2',3,5',6-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
96	2,2',3,6,6'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
97	2,2',3,4',5'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
98	2,2',3,4',6'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
99	2,2',4,4',5-Pentachlorobiphenyl	$C_{12}H_5Cl_5$

Congener Number[†]	IUPAC Name	Chemical Formula
100	2,2',4,4',6-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
101	2,2',4,5,5'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
102	2,2',4,5,6'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
103	2,2',4,5',6-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
104	2,2',4,6,6'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
105 *	2,3,3',4,4'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
106	2,3,3',4,5-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
107	2,3,3',4',5-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
108	2,3,3',4,5'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
109	2,3,3',4,6-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
110	2,3,3',4',6-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
111	2,3,3',5,5'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
112	2,3,3',5,6-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
113	2,3,3',5',6-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
114 *	2,3,4,4',5-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
115	2,3,4,4',6-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
116	2,3,4,5,6-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
117	2,3,4',5,6-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
118 *	2,3',4,4',5-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
119	2,3',4,4',6-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
120	2,3',4,5,5'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
121	2,3',4,5',6-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
122	2,3,3',4',5'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
123 *	2,3',4,4',5'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
124	2,3',4',5,5'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
125	2,3',4',5',6-Pentachlorobiphenyl	$C_{12}H_5Cl_5$

Congener Number[†]	IUPAC Name	Chemical Formula
126 *	3,3',4,4',5-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
127	3,3',4,5,5'-Pentachlorobiphenyl	$C_{12}H_5Cl_5$
128	2,2',3,3',4,4'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
129	2,2',3,3',4,5-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
130	2,2',3,3',4,5'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
131	2,2',3,3',4,6-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
132	2,2',3,3',4,6'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
133	2,2',3,3',5,5'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
134	2,2',3,3',5,6-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
135	2,2',3,3',5,6'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
136	2,2',3,3',6,6'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
137	2,2',3,4,4',5-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
138	2,2',3,4,4',5'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
139	2,2',3,4,4',6-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
140	2,2',3,4,4',6'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
141	2,2',3,4,5,5'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
142	2,2',3,4,5,6-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
143	2,2',3,4,5,6'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
144	2,2',3,4,5',6-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
145	2,2',3,4,6,6'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
146	2,2',3,4',5,5'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
147	2,2',3,4',5,6-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
148	2,2',3,4',5,6'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
149	2,2',3,4',5',6-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
150	2,2',3,4',6,6'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
151	2,2',3,5,5',6-Hexachlorobiphenyl	$C_{12}H_4Cl_6$

Congener Number[†]	IUPAC Name	Chemical Formula
152	2,2',3,5,6,6'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
153	2,2',4,4',5,5'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
154	2,2',4,4',5,6'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
155	2,2',4,4',6,6'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
156 *	2,3,3',4,4',5-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
157 *	2,3,3',4,4',5'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
158	2,3,3',4,4',6-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
159	2,3,3',4,5,5'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
160	2,3,3',4,5,6-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
161	2,3,3',4,5',6-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
162	2,3,3',4',5,5'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
163	2,3,3',4',5,6-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
164	2,3,3',4',5',6-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
165	2,3,3',5,5',6-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
166	2,3,4,4',5,6-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
167 *	2,3',4,4',5,5'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
168	2,3',4,4',5',6-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
169 *	3,3',4,4',5,5'-Hexachlorobiphenyl	$C_{12}H_4Cl_6$
170	2,2',3,3',4,4',5-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
171	2,2',3,3',4,4',6-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
172	2,2',3,3',4,5,5'-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
173	2,2',3,3',4,5,6-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
174	2,2',3,3',4,5,6'-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
175	2,2',3,3',4,5',6-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
176	2,2',3,3',4,6,6'-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
177	2,2',3,3',4,5',6'-Heptachlorobiphenyl	$C_{12}H_3Cl_7$

Congener Number[†]	IUPAC Name	Chemical Formula
178	2,2',3,3',5,5',6-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
179	2,2',3,3',5,6,6'-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
180	2,2',3,4,4',5,5'-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
181	2,2',3,4,4',5,6-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
182	2,2',3,4,4',5,6'-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
183	2,2',3,4,4',5',6-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
184	2,2',3,4,4',6,6'-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
185	2,2',3,4,5,5',6-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
186	2,2',3,4,5,6,6'-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
187	2,2',3,4',5,5',6-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
188	2,2',3,4',5,6,6'-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
189 *	2,3,3',4,4',5,5'-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
190	2,3,3',4,4',5,6-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
191	2,3,3',4,4',5',6-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
192	2,3,3',4,5,5',6-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
193	2,3,3',4',5,5',6-Heptachlorobiphenyl	$C_{12}H_3Cl_7$
194	2,2',3,3',4,4',5,5'-Octachlorobiphenyl	$C_{12}H_2Cl_8$
195	2,2',3,3',4,4',5,6-Octachlorobiphenyl	$C_{12}H_2Cl_8$
196	2,2',3,3',4,4',5,6'-Octachlorobiphenyl	$C_{12}H_2Cl_8$
197	2,2',3,3',4,4',6,6'-Octachlorobiphenyl	$C_{12}H_2Cl_8$
198	2,2',3,3',4,5,5',6-Octachlorobiphenyl	$C_{12}H_2Cl_8$
199	2,2',3,3',4,5,5',6'-Octachlorobiphenyl	$C_{12}H_2Cl_8$
200	2,2',3,3',4,5,6,6'-Octachlorobiphenyl	$C_{12}H_2Cl_8$
201	2,2',3,3',4,5',6,6'-Octachlorobiphenyl	$C_{12}H_2Cl_8$
202	2,2',3,3',5,5',6,6'-Octachlorobiphenyl	$C_{12}H_2Cl_8$
203	2,2',3,4,4',5,5',6-Octachlorobiphenyl	$C_{12}H_2Cl_8$

Congener Number†	IUPAC Name	Chemical Formula
204	2,2',3,4,4',5,6,6'-Octachlorobiphenyl	$C_{12}H_2Cl_8$
205	2,3,3',4,4',5,5',6-Octachlorobiphenyl	$C_{12}H_2Cl_8$
206	2,2',3,3',4,4',5,5',6-Nonachlorobiphenyl	$C_{12}H_1Cl_9$
207	2,2',3,3',4,4',5,6,6'-Nonachlorobiphenyl	$C_{12}H_1Cl_9$
208	2,2',3,3',4,5,5',6,6'-Nonachlorobiphenyl	$C_{12}H_1Cl_9$
209	Decachlorobiphenyl	$C_{12}Cl_{10}$

† Congener numbers from Ballschmiter et al., 1992, and accepted by IUPAC

* Denotes dioxin-like congener

Appendix B: Experiments Investigating the Formation of Glutathione Conjugates of Polychlorinated Biphenyls

Abstract

Methyl sulfone metabolites of PCBs ($MeSO_2$-PCBs) are persistent, bioaccumulative, toxic compounds which have been detected in a variety of organisms. $MeSO_2$-PCBs are formed via a complex, multi-step biotransformation pathway from glutathione conjugates of PCBs (GS-PCBs). GS-PCB metabolites are hypothetically produced from PCB oxidation by microsomal cytochrome P450 to an arene-oxide intermediate followed by glutathione conjugation by cytosolic glutathione *S*-transferase. The aim of this study was to isolate and identify the rat produced GS-PCB metabolites, which could then be used to validate methods to examine the formation of GS-PCB in the livers of sea turtles. Therefore, we employed phenobarbital (PB)-treated rat liver microsomes, as well as CYP2B1 Supersomes and cytosol from rat in *in vitro* PCB incubations. A selection of chromatographic techniques was employed using the glutathione conjugate of styrene as a standard. However, GS-PCB metabolites were not detected in PCB incubations with rat enzymes or tissue extracts, precluding further experiments with sea turtle tissues. The results of this study suggest that PB-induced rat hepatic CYP isoforms may introduce oxygen to PCBs by direct hydroxylation, rather than an arene oxide intermediate (or the rate of arene oxide formation is less than detection limits). A discussion of the implications of the lack of a detectable glutathione metabolite from PCB is included for benefit of future research.

Introduction

Biotransformation of PCBs can result in the formation of two types of persistent metabolites of PCBs, hydroxylated (OH-) and methyl sulfone ($MeSO_2$-) PCBs. Phase I biotransformation of PCB involves the introduction of an oxygen to one of the aromatic rings by cytochrome P450 (CYP), which results in the formation of an arene oxide or OH-PCB (Letcher et al. 2000a). The arene oxide can be conjugated with glutathione (GSH) by glutathione *S*-transferase (GST) in a phase II reaction (Bakke 1989; Vermeulen 1996) and the glutathione conjugates may be further metabolized into mercapturic acids or $MeSO_2$-PCBs (Bakke et al. 1982; Bakke and Gustafsson 1986; Bakke 1989). CYP mediated biotransformation of PCBs can occur through 2 mechanisms: a hydroxyl group (OH) can be added directly or an arene oxide is formed, which is subject to rearrangement (see Figure 1.11, Preston and Allen 1980; Forgue and Allen 1982). The presence of vicinal protons on one aromatic ring is requisite for the formation of oxidative metabolites of PCBs, and the position of the vicinal pair allows specific CYP isoforms to catalyze the oxidation (Letcher et al. 2000a). It is generally accepted that phenobarbital (PB)-induced CYPs, e.g., CYP2B1, insert an oxygen molecule at the *meta-para* positions while 3-methylcholanthrene (3MC) -induced CYPs, e.g., CYP1A1, catalyze oxygenation at *ortho-meta* positions of PCBs (Parke 1985; Kannan et al. 1995). Metabolites from PB-induced enzymes go on to phase II biotransformation, and the formation of persistent $MeSO_2$-PCBs has been demonstrated for PCBs which possess *meta-para* vicinal protons (James 1987; (Letcher et al. 2000a). The exact GST isoform(s) responsible for GSH conjugation critical to the formation of $MeSO_2$-PCBs

have yet to be identified. As chlorination of a PCB increases, the ratio of OH to $MeSO_2$ metabolites increases but overall biotransformation decreases (Haraguchi et al. 1997).

Persistent *meta*- and *para*-$MeSO_2$-PCBs are preferentially retained in fatty tissues, especially the liver, and represent an abundant class of contaminants in wildlife (Letcher et al. 2000a). $MeSO_2$-PCB have been detected primarily in marine mammals (Jensen and Jansson 1976; Karlson et al. 2000; Letcher et al. 2000b; Gebbink et al. 2008) and humans (Guvenius et al. 2002), but also in the deepwater sculpin (*Myoxocephalus thompson*) (Stapleton et al. 2001) and guillemot (*Uria aalge*) (Jorundsdottir et al. 2006). In some marine mammals, levels of $MeSO_2$-PCBs can exceed that of PCBs, illustrating the potential impact of these toxicologically active metabolites (McKinney et al. 2006). Limited data exists on $MeSO_2$-PCBs in reptiles, but we have recently demonstrated that some sea turtles possess the biotransformation capacity to hydroxylate *meta-para* unsubstituted 2,2',5,5'-tetrachlorobiphenyl (PCB 52) (Chapter 4). Therefore we hypothesized that sea turtles may also produce $MeSO_2$-PCBs. However, the formation of $MeSO_2$-PCBs is a complex pathway involving entereohepatic circulation and biotransformation by gut microflora and thus, cannot be studied in *in vitro* systems. Instead, we focused on the two-step hepatic protein catalyzed formation of the glutathione conjugate of PCB (GS-PCB): 1) oxidation by microsomal CYP to an arene-oxide intermediate, followed by 2) GSH conjugation via arene oxide opening by cytosolic GST.

GS-PCB metabolites are not commercially available for use in method development or validation, so our initial aim was to isolate and identify the GS-PCB

metabolites in rat liver, which could then be used to validate methods to examine the formation of GS-PCB in the livers of sea turtles. Several lines of evidence support the formation of GS-PCB in PB-treated rat liver. The formation of the O-PCB 52 intermediate by PB-treated rat microsomes has been reported, as illustrated in Figure B.1 (Forgue et al. 1979; Forgue and Allen 1982). As shown in Figure B.2, cytosol from PB-treated rat has been shown to catalyze the formation of GS-PCB from the intermediate (Preston et al. 1984). In rodents exposed to PCB 52 and other *meta-para* unsubstituted PCB *in vivo,* methylsulfone metabolites were detected in tissues and feces (Mio et al. 1976; Haraguchi et al. 1997).

Therefore, we employed rat CYP enzymes to generate an appropriate concentration of O-PCB that could then undergo GSH conjugation following the subsequent addition of rat cytosolic GST. However, we were unable to identify a glutathione conjugate of PCB from rat tissues by a variety of chromatographic techniques, which were validated using the glutathione conjugate of styrene oxide. The lack of detectable metabolites from rat tissues precluded their use in method validation, and thus, further experiments with sea turtles were prohibited. A discussion of the implications of the lack of detectable glutathione metabolite from PCB is included for benefit of future research.

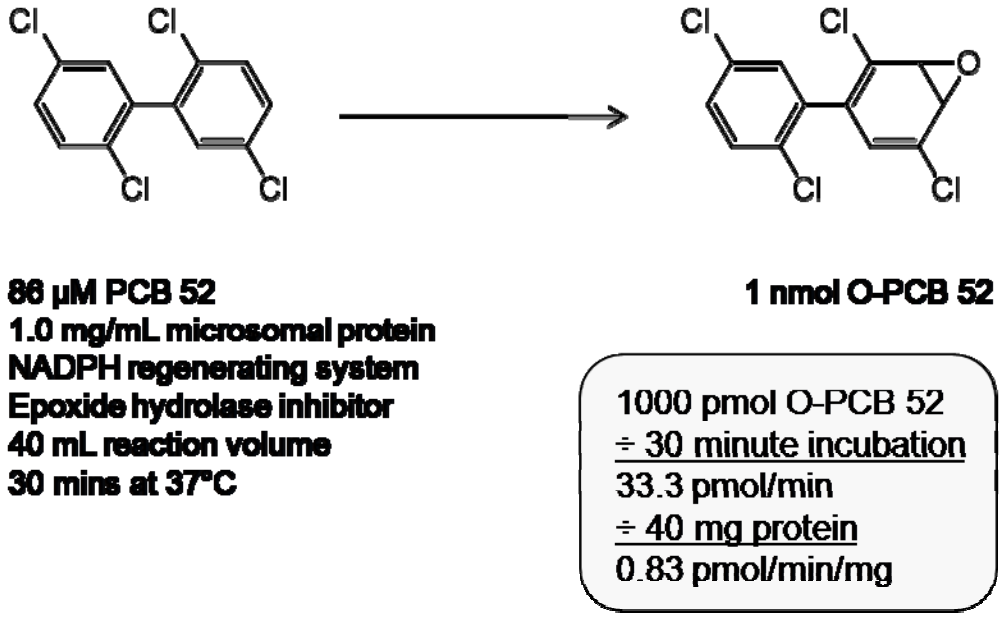

Figure B.1. Schematic summarizing the results of Forgue and Allen (1982). The shaded area includes calculations made with their data in order to obtain the rate of O-PCB 52 formation from PCB 52.

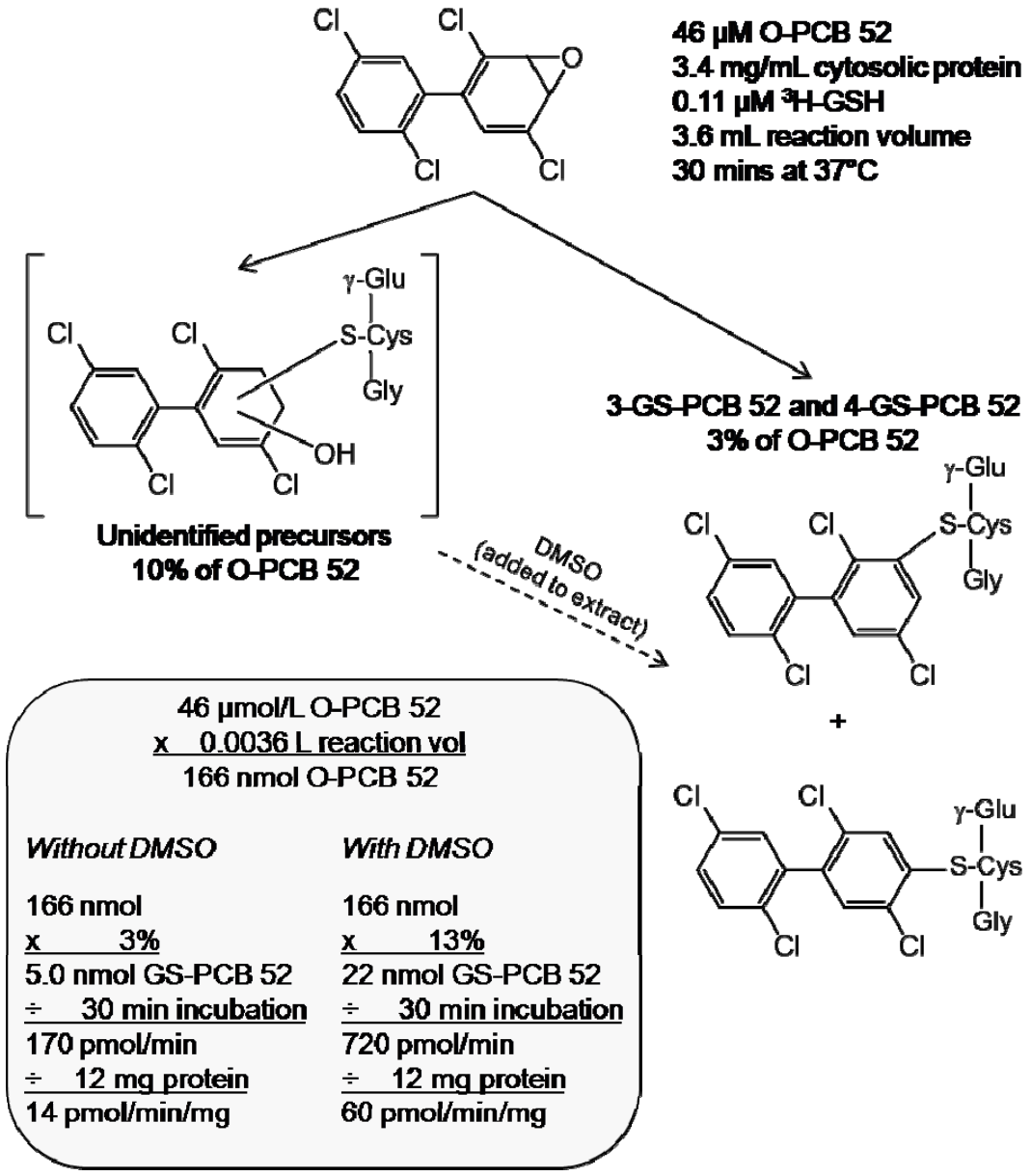

Figure B.2. Schematic summarizing the results of Preston et al. (1984). The shaded area includes calculations made with their data in order to obtain the rate of GS-PCB 52 formation from O-PCB 52.

Methods

Reagents

2,2',5,5'-tetrachlorinated biphenyl (PCB 52), 2,2',4,5'-tetrachlorobiphenyl (PCB 49), and 2,4',5-trichlorinated biphenyl (PCB 31) were purchased from Accustandard (New Haven, CT, USA). Styrene oxide (SO), nicotinamide adenine dinucloetide phosphate (NADPH), reduced glutathione (GSH), and ninhydrin were purchased from Sigma-Aldrich (St. Louis, MO, USA). ^{35}S-glutathioine (894 Ci/mmol) was obtained from Perkin-Elmer (Waltham, MA, USA). The SO was aliquoted to single-use amounts to avoid repeated freeze-thaw cycles associated with volatilization of ^{35}S and stored, after evacuation of oxygen with nitrogen gas, at -80°C to avoid degradation of the compound. OASIS-HLB solid-phase extraction cartridges (3 cc cartridges with 60 mg sorbent material of 30 μm particle size) were purchased from Waters Corporation (Milford, MA, USA). Chromatography grade formic acid and methanol were obtained from Fisher (Pittsburgh, PA, USA). All solvents used for HPLC or UPLC, including E-PURE (Barnstead, Thermo Scientific, Waltham, MA, USA) water, were further purified by filtration through 0.02 μm nylon membranes (Millipore, Billerica, MA, USA) prior to use. All other chemical were of high analytical grade and were purchased from Fisher or Sigma.

Subcellular fractions

Hepatic microsomes and cytosol from phenobarbital (PB)-treated rat, as well as hepatic cytosol from β-naphthoflavone (BNF)-treated rat were obtained from Xenotech (Lenexa, KS, USA). We also purchased rat CYP2B1 Supersomes (Gentest, Becton

Dickinson, Franklin Lakes, NJ, USA), which are microsomes derived from Baculovirus-insect cells expressing rat CYP2B1 with supplemental CYP-reductase and cytochrome b5. Cytosols from untreated rat liver and BNF-treated rainbow trout (50 mg/kg two times over 4 days) were prepared as described in Chapters 2 and 3.

Preparation of the glutathione conjugate of styrene oxide

The addition of GSH, a tri-peptide, to conjugation of GSH to a PCB arene oxide intermediate dramatically increases the aqueous solubility of the PCB (to predicted log p values less than zero), mandating specialized extraction procedures for preparation of samples for analysis. Furthermore, GS-PCB metabolites are not commercially available for use as a metabolite surrogate in extraction or analytical method development. Therefore, styrene oxide glutathione conjugate (GS-SO) was synthesized from commercially available styrene oxide and purified by preparative HPLC following the method of Luo and Guenthner (1994) and references within, with minor modification of the HPLC gradient to optimize separation of parent compound and metabolites. UPLC/TOF-MS (see below) was employed to confirm the purification of GS-SO in the collected fractions. The purified GS-SO was then evaporated to dryness, weighed to determine mass (0.5 ug reproducible balance was employed), and a standard solution was prepared in methanol.

Extraction procedures

OASIS-HLB solid-phase extraction cartridges (Waters Corporation, Milford, MA, USA) were selected for LC/MS sample clean-up because the HLB (Hydrophilic-Lipophilic Balance) sorbent allows for extraction of mixed polarity compounds, such as

GS-PCB. To develop an extraction procedure with OASIS HLB columns, various concentrations of GS-SO incubated in *in vitro* metabolism incubation mixtures containing 1.5 mg/mL total hepatic protein in buffer, were loaded onto OASIS HLB cartridges. A number of different aqueous and organic solvent combinations were employed for solid phase extraction. The extraction efficiencies were measured by HPLC-UV (see below). The procedure that yielded the highest recovery was as follows: after sample incubation, formic acid was added to adjust the pH of the reaction mixture to approximately 2.9 (and thus decrease ionization of the GS moiety), and then the samples were centrifuged to precipitate proteins (10 min at 13,000 *g*). The supernatant was applied to OASIS HLB cartridges pre-conditioned with 2 mL methanol and 2 mL water, adjusted to pH 3 with formic acid. The cartridges were then washed with 2 mL water (also at pH 3) and eluted with 2mL methanol. 50 uL of DMSO was added to the eluted sample [used to stabilize glutathione conjugate (Preston et al. 1984)], and then evaporated to a final volume of 50 µL under nitrogen.

In vitro biotransformation assays

Meta-para unsubstituted PCB 31 (2,4',5-trichlorinated biphenyl), PCB 49 (2,2',4,5'-tetrachlorobiphenyl), and PCB 52 were employed for *in vitro* biotransformatin assays. Mixed *in vitro* incubations contained 150 µM PCB with 0.5 to 2 mg/mL PB-induced rat liver microsomes or rat CYP2B1 Supersomes and 1 to 2 mg/mL cytosol from PB-induced rat liver, untreated rat liver, BNF-treated rat liver, or BNF-treated trout liver. The cofactors for CYP and GST enzymes, NADPH and GSH, respectively, were added at saturating conditions (1-2 mM) in 100 mM potassium phosphate buffer, pH 7.4, in a total

reaction volume of 200 µL. Negative controls included incubations with boiled proteins, as well as incubations lacking PCB or NADPH. Reactions were started by the addition of NADPH (or buffer in the case of minus NADPH reactions), and incubated at 37ºC for up to 2 hours. The reaction was stopped by the addition of ice cold DMSO or formic acid, and then subjected to centrifugation alone, or centrifugation followed by OASIS-HLB extraction, respectively, prior to analysis by TLC, HPLC/UV, HPLC/UV/RD or UPLC/MS. Mixed *in vitro* incubation experiments were also carried out, as described above, with ^{35}S-GSH. The ^{35}S-GSH experiments contained either all radiolabeled GSH (100 nCi, 0.56 nM in reaction) or 10 µCi/µmol GSH (200 µM in reaction), and were analyzed both with and without OASIS-HLB extraction by HPLC/UV/RD.

Further *in vitro* experiments were conducted in a two-step process in an attempt to build up higher concentrations of PCB oxide. Four sets of phase I *in vitro* incubations, adapted from Forgue and Allen (1982), were run: PB-treated rat microsomes with PCB 31, PB-treated rat microsomes PCB 52, CYP2B1 Supersomes with PCB 31, and CYP2B1 Supersomes with PCB 52. Each incubation tube contained 2 mg/mL microsomes or 0.6 mg/mL Supersomes and 86 µM PCB in 100 mM potassium phosphate buffer, pH 7.4, containing 1 mM $ZnSO_4$ to inhibit epoxide hydrolase activity (Draper and Hammock 1999). The incubations, held at 37°C, were initiated by the addition of NADPH (1 mM in reaction). NADPH was then added every hour to maintain saturating conditions of NADPH for the entire 24 hour incubations period, and then the reactions were stopped with 400 µL ethyl acetate. After vigorous vortexing, the samples were centrifuged (10 min at 13,000 *g*), and the organic layers from each tube in a set of incubations was

combined. The extraction process was repeated three more times. The phase I extract from each set was then evaporated to dryness and resuspended in a known volume of ethyl acetate. The organic extract from each phase I incibation type was then subjected to phase II incubations. Each extract was split into four tubes for each set – tubes 1 and 2: phase II incubations, tube 3 and 4: phase II incubation negative controls 1 contained boiled cytosolic protein, and 1 lacked protein) – and then the ethyl acetate was evaporated under nitrogen. Phase II incubations contained 2 mg/mL PB-treated rat microsomes and 10 μCi/μmol GSH (200 μM in reaction) in 200 μL 100 mM potassium phosphate buffer, pH 7.4, with 1 mM $ZnSO_4$. The phase II incubations were initiated by the addition of GSH and then held at 37°C for two hours. Reactions were stopped with the addition of 50 μL ice cold DMSO. After centrifugation (as above), the supernatants were analyzed by HPLC/UV/RD.

Additional biotransformation assays were performed to validate several methods. The catalytic activity of CYP2B1 Supersomes toward *meta-para* unsubstituted PCB 52 was confirmed by HPLC/MS, as described for PB-treated rat liver microsomes in Chapter 4. *In vitro* incubations with styrene oxide were conducted in order to validate HPLC/UV/RD methods. Various stock concentrations of styrene oxide were prepared in ethanol, which were diluted 100-fold in buffer before use so that the concentration of ethanol was less than 0.02% in the 200 μL final reaction volume. The SO incubations also contained 2 mg/mL PB-treated rat cytosol in 100 mM potassium phosphate buffer, pH 7.4, with 1 mM $ZnSO_4$, and were initiated by the addition of either all radiolabeled GSH (100 nCi, 0.56 nM in reaction) or 10 μCi/μmol GSH (200 μM in reaction).

Negative control incubations, as described above, were also included. The SO incubations were held at 37°C for two hours, and then stopped with the addition of 50 µL ice cold DMSO. After centrifugation, the supernatants were analyzed by HPLC/UV/RD.

Thin-layer chromatography (TLC)

Fluorescent silica TLC plates (Fisher) were spotted with various concentrations of *in vitro* incubation samples or GS-SO (20-40 uL total volume, added in 1 µL aliquots, which were allowed to dry before the next aliquot was spotted), and placed in a TLC chamber. The mobile phases, based on Luo and Guenthner (1994) and references within, were 12:3:5 butanol: acetic acid: water or 4:1:1:1 butanol: ethanol: ammonium hydroxide: water. The plates were visualized using UV light and ninhydrin (which allows colorimetric detection of amino groups).

HPLC/UV and HPLC/RD analysis

HPLC was conducted using a Shimadzu SCL-10AVP HPLC system (Columbia, MD, USA) equipped with a Hypersil ODS C18 column (5 um, 250 x 4.6 mm), protected by a Phenomenex (Torrance, CA USA) Security Guard cartridge system equipped with a C18 4x3 mm cartridge. The mobile phase consisted of A, 0.1% formic acid and 5% methanol in water, and B, 100% methanol. The HPLC system was operated at a flow rate of 1.0 mL/min with a linear gradient of 100% A to 100% B over 30 minutes, then held at 100% B for 5 minutes before returning to 100% A over 5 minutes, and then held at 100% A to stabilize the column prior to subsequent injection. The UV/Vis detector (UV) was set at 254 nm. For HPLC/RD, the HPLC system was connected to an on-line radioactivity detector (RD) (β-ram Model 3, IN/US Systems Inc., Tampa, FL, USA)

using In-Flow 2:1 (IN/US Systems Inc.) as the scintillation cocktail. Radioactivity was monitored for the first 35 minutes which allowed the monitoring of all eluent between GSH and the parent PCB (retention time of 30-35 minutes depending on PCB congener). When the ^{35}S-GSH conjugate of styrene oxide was analyzed, the identity of the metabolite was confirmed based on the retention time of purified GS-SO.

HPLC/MS analysis

The HPLC/MS system is described in Chapter 4. The HPLC/MS system was equipped with the same column and pre-column described for HPLC/UV and HPLC/UV/RD analysis. The mobile phases consisted of A, 0.1% formic acid and 5% methanol in water, and B, 100% methanol. The HPLC system was operated at a flow rate of 1.0 mL/min with a linear gradient of 10% A to 54% B over 15 minutes, then to 70% B over 3 minutes, held there for 1 minutes before returning to 10% A over 5 minutes, and then held at 100% A to stabilize the column prior to subsequent injection. The UV/Vis detector was set at 254 nm. The MS was equipped with an auxiliary isocratic pump, pumping 0.1% formic acid and 50% methanol in water at a flow rate of 0.2 mL/min. A switching valve between the UV and MS detectors allowed matrix diversion of LC flow to waste when peaks of interest were not eluting. The MS was operated in electrospray ionization (ESI), negative ionization mode. Selective ion monitoring of the molecular ion of GS-SO, *m*/*z* 426, was used for quantification. During the analysis, the ESI probe was held at 525ºC and ultra high purity nitrogen was used as the nebulizing gas (80 psi). The cone voltage was -110V, and full scan data for m/z 1-500 were also collected at this cone voltage.

UPLC/TOF-MS

UPLC/TOF-MS was conducted using an Waters Acquity UPLC system equipped with an Acquity BEH C18 column (1.7 μm, 2.1x150 mm), protected by VanGuard Acquity BEH C18 guard column (1.7 μm,2.1x5 mm), which was connected in-line to a quadrupole time of flight mass spectrometry instrument (TOF-MS) (Waters Corporation). The UPLC system was operated at a flow rate of 0.5 mL/min and the mobile phase consisted of A, 0.1% formic acid in water, and B, 0.1% formic acid, 5% water in acetonitrile. The separation was isocratic at 5% B for the first minute, followed by a linear gradient over 10 minutes to 95% B, then held at 95% B for 2 minutes, before returning to 5% B over 0.5 minutes and held there for 1.5 minutes before the injection of the next sample. Spectra were obtained by operating the ESI source in negative ion mode (although positive ion mode was also tested with parameters similar to these) using the following instrumental parameters: capillary voltage, 3 kV; sample cone voltage, 10 V; and a source temperature, 120 °C. Spectra were collected over a m/z range of 100−1000 using MassLynx 4.0 software (Waters Corporation).

Results

Mean recovery of GS-SO from the optimized OASIS-HLB extraction procedure was 88-90%. The recovery was calculated by HPLC/UV, which had a detection limit for GS-SO of 15 pmol/μL. TLC failed to detect glutathione conjugates. The minimal amount of detectable GS-SO using TLC was 416 pmol/spot. This limit of detection represents the lowest concentration of GS-SO which produced a ninhydrin-responsive

spot. Studies with combined and concentrated OASIS-HLB extracts of mixed *in vitro* PCB incubations yielded no ninhydrin responsive spot which was not also present in (negative) controls (see Figure B.3).

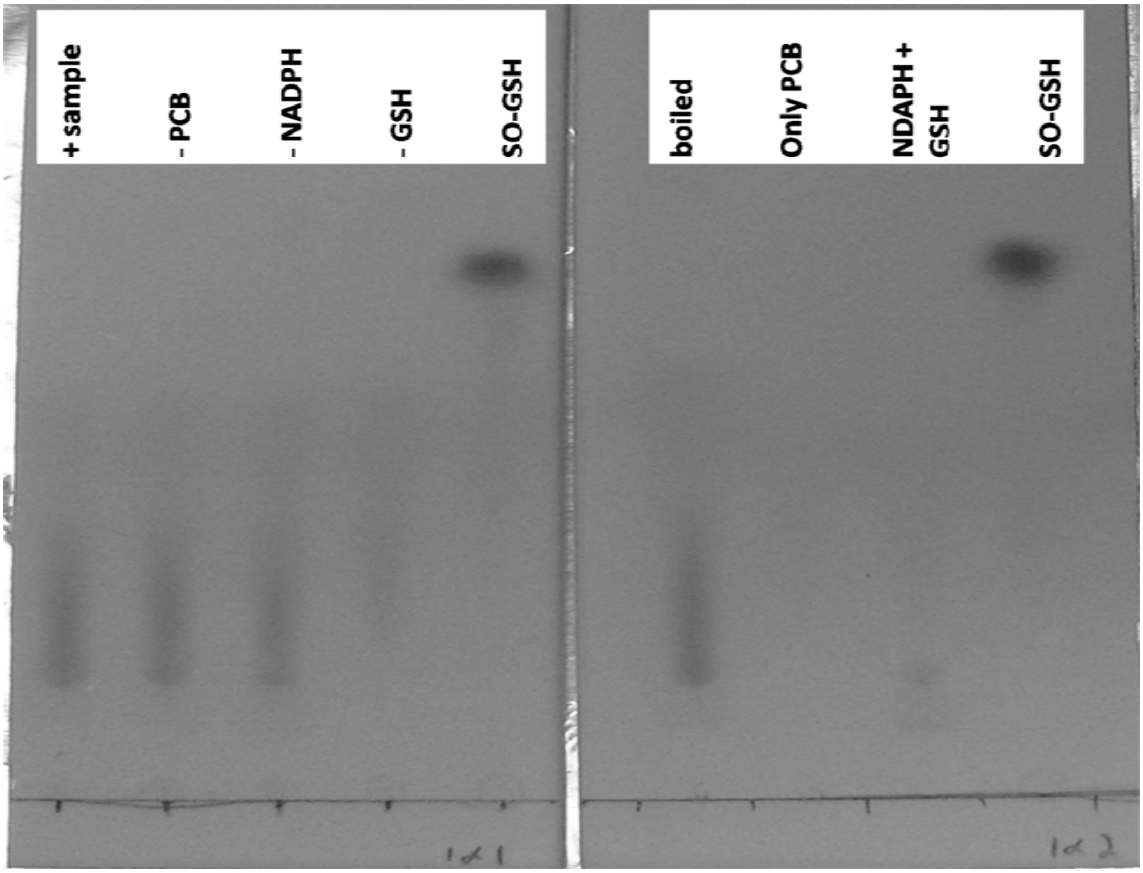

Figure B.3. Representative ninhydrin stained TLC plates from combined OASIS-HLB extracts from mixed PCB 52 *in vitro* incubation (+sample) and numerous controls (-PCB: no PCB 52 added to reaction mixture, -NADPH: no NADPH, -GSH: no GSH, only PCB: just PCB in buffer/DMSO, NADPH+GSH: just NADPH and GSH in buffer/DMSO, SO-GSH: positive control).

UPLC/TOF-MS analysis of the OASIS-HLB extracts from mixed *in vitro* incubations did not reveal GS-PCB metabolites. Spectra were examined for peaks with m/z corresponding to GS-PCB (and potential precursors) and phase I PCB metabolites. The presence of a chlorine isotopic cluster was considered indicative of a PCB metabolite. A hydroxylated PCB metabolite was the only PCB metabolite detected (spectra identical to those shown in Chapter 4). A detection limit on the UPLC/TOF-MS was not calculated, but the limit of detection for GS-SO on the HPLC/MS was 30 fmol/μL, and the UPLC/TOF-MS would be expected to have similar sensitivity.

GS-PCB metabolite(s) was not detected by HPLC/RD, however the system was able to detect both GS- (^{35}S)-SO metabolites, S-(1-phenyl-2-hydroxyethyl)-glutathione and S-(2-phenyl-2-hydroxyethyl)-glutathione (Watabe et al. 1981), formed from the *in vitro* cytosolic incubations (Figure B.4). The lowest concentration of styrene oxide that resulted in detectable GS-(^{35}S)-SO metabolites from 2 hour *in vitro* PB-treated rat cytosolic incubations was 75 μM (SO in the reaction). When the reaction contained all radiolabeled GSH (100 nCi, 0.56 nM in reaction), the metabolites represented 0.8% and 9% of the total radioactivity and GS-SO metabolites could be detected at concentrations as low as 4 amol/μL. When 10 μCi/μmol GSH (200 μM in reaction) was utilized, the metabolites represented 1% and 10% of the total radioactivity and GS-SO metabolites could be detected at concentrations as low as 1.6 pmol/μL. Mixed *in vitro* incubations analyzed by HPLC/RD yielded no radioactive metabolite peaks with (Figure B.5) or without OASIS-HLB extraction (Figure B.6). It is important to note that OASIS-HLB extracts showed greatly reduced levels of total radioactivity because most (greater than

80%) of GSH or GSSG passed directly through the cartridge during the load and wash steps, despite manipulations to the extraction procedure. HPLC/RD of two-step *in vitro* incubations also failed to yield radioactive metabolite peaks (Figure B.7). Finally, CYP2B1 Supersomes showed a mean PCB 52 hydroxylation rate of 42 pmol/min/mg, confirming that the recombinant system was catalytically active.

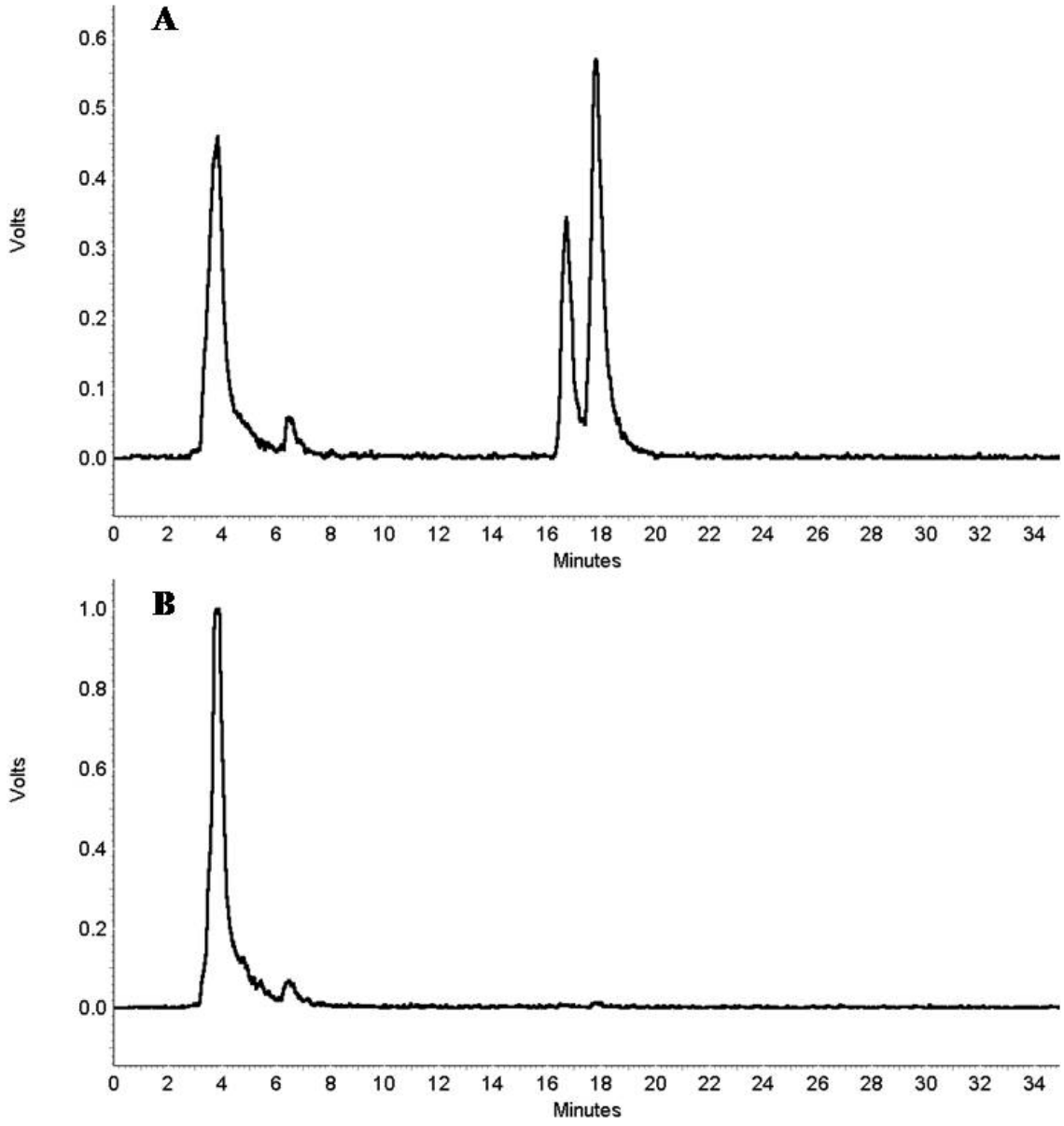

Figure B.4. Representative radio-chromatograms of A) *in vitro* incubation of PB-treated rat liver cytosol with styrene oxide and ^{35}S-GSH, and B) similar incubation containing boiled cytosolic proteins as the negative control. The peaks at minute 4-8 represent reduced and oxidized glutathione, while the peaks at minutes 17-19 represent GS-SO.

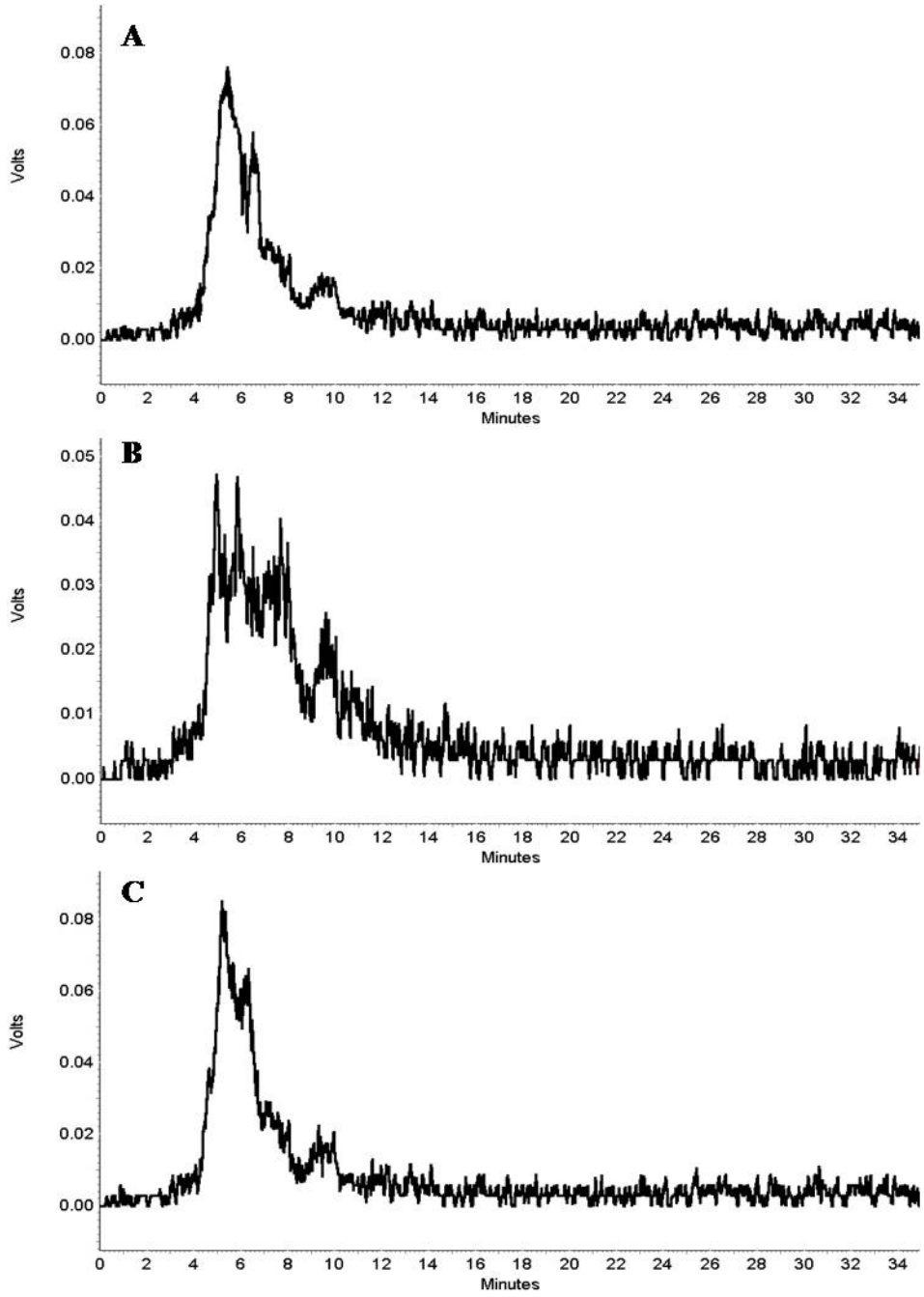

Figure B.5. Representative radio-chromatograms of OASIS-HLB extracted a) mixed *in vitro* incubation of CYP2B1 Supersomes and PB-treated rat liver cytosol with PCB, NADPH and ^{35}S-GSH, and b) similar incubation containing boiled proteins as a negative control, and c) similar incubation without NADPH added to the incubation as a negative control. The peaks at minute 4-8 represent reduced and oxidized glutathione.

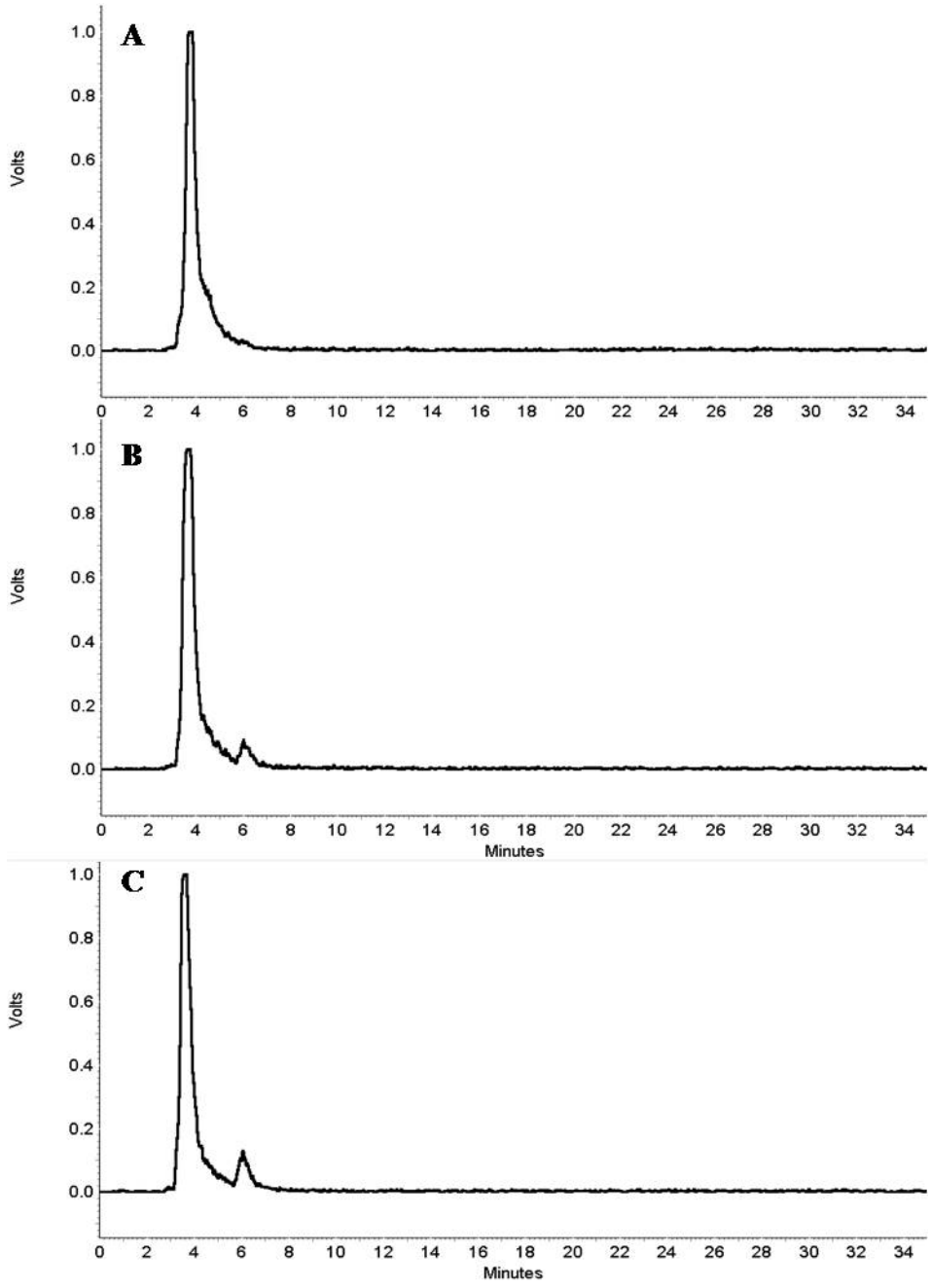

Figure B.6. Representative radio-chromatograms of non-extracted a) mixed *in vitro* incubation of PB-treated rat liver microsome and cytosol with PCB, NADPH and ^{35}S-GSH, and b) similar incubation containing boiled proteins as a negative control, and c) similar incubation without NADPH added to the incubation as a negative control. The peaks at minute 4-8 represent reduced and oxidized glutathione.

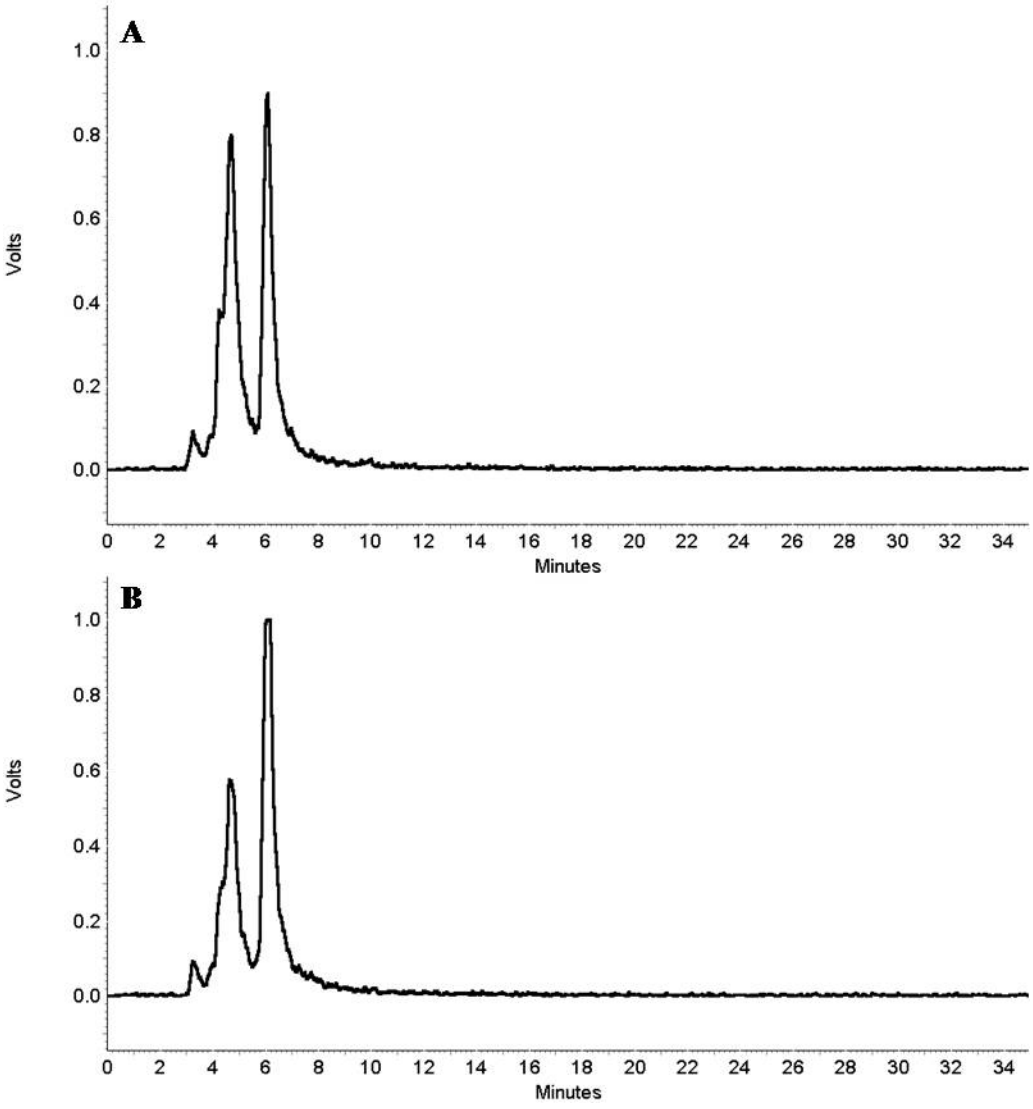

Figure B.7. Representative radio-chromatograms of samples from the two-step *in vitro* incubations. One-fourth of the extract from the phase I incubation of PB-treated rat liver microsomes with PCB and NADPH was a) incubated with PB-treated rat liver cytosol and ^{35}S-GSH for 2 hours, and b) similar incubation of extract containing boiled proteins as a negative control. The peaks at minute 4-8 represent reduced and oxidized glutathione.

Discussion

Methyl sulfone metabolites of PCBs are persistent, bioaccumulative, toxic compounds which have been detected in a variety of organisms (Letcher et al. 2000a). Several lines of evidence show that the $MeSO_2$-PCB metabolites result via a multi-step biotransformation pathway from GS-PCB metabolites (see Chapter 1, or Bakke 1989; Bakke 1990). Therefore, the focus of this research was to investigate the formation of GS-PCB in *in vitro* rat systems (with the hope that this would provide a methodological basis for the investigation of GS-PCB formation in sea turtles).

The formation of GS-PCB is generally thought to result from spontaneous or GST-catalyzed GSH addition to the 3,4-arene oxide PCB (O-PCB), which is produced by CYP. The detection of isomeric pairs (i.e., 3- and 4-substituted) of $MeSO_2$-PCB in humans, laboratory animals, and wildlife provide evidence for the arene oxide intermediate (Letcher et al. 2000a), while species and congener specific ratios of 3-$MeSO_2$-PCB to 4-$MeSO_2$-PCB support an enzyme-mediated (and not spontaneous) pathway. Furthermore, the concentration of glutathione derived metabolites in the feces of rats treated *in vivo* (*i.p.* injection) with O-PCB 52 were 10-fold greater than that of rats treated with an equimolar level of PCB 52 (Preston et al. 1984), supporting GSH conjugation to O-PCB 52. Additional *in vitro* experiments with PB-treated rat liver cytosol, along with non-enzymatic studies, confirmed the enzymatic formation of the GS-PCB 52 from O-PCB 52 and ^{3}H-GSH (Preston et al. 1984) - the calculated rate of formation of GS-PCB 52 from O-PCB 52 was 14 – 60 pmol/min/mg (see Figure B.2). The *in vitro* formation of O-PCB 52 by PB-treated rat liver microsomes has been

demonstrated by radiometric techniques, by gas chromatography-electron capture detection with co-chromatography of synthesized O-PCB 52, and by gas chromatography-mass spectrometry detection of rearranged and derivatized O-PCB 52 (i.e., derivatives of 3- and 4-hydroxy-PCB 52) (Forgue et al. 1979; Forgue and Allen 1982). The calculated rate of O-PCB 52 was 0.83 pmol/min/mg (see Figure B.1).

Table B.1 Predicted yields of O-PCB from 200µL mixed *in vitro* incubations and from the phase I portion of the two-step *in vitro* incubations.

In vitro incubation strategy	PB-treated microsomal protein concentration (mg/mL)	Incubation time (hour)	Predicted mass of O-PCB (pmol)[a]
Mixed incubation	0.5	2	10
	2	2	40
two-step incubation	2	24	1200[b]

a - based on calculated rate of O-PCB 52 formation from Forgue and Allen (1982), see Figure B.1

b – represents the theoretical amount of PCB-O delivered to each tube in the phase II portion of the two-step incubations

Table B.2 Predicted yields of GS- PCB from 200µL *in vitro* incubations.

Rate of GS-PCB formation (pmol/min/mg)[a]	PB-treated cytosolic protein concentration (mg/mL)	Incubation time (minutes)	Predicted mass of GS-PCB (pmol)[a]	Predicted concentration of GS-PCB (pmol/µL)[a]	
				Without OASIS extraction[b]	With OASIS extraction[c]
14	2	120	670	2.7	12
60	2	120	2900	12	51

a - based on calculated rate of GS-PCB 52 formation from Preston et al. (1984), see Figure B.2
b – 250 µL final volume
c – 50 µL final volume, corrected for 88% OASIS extraction efficiency

Yields from the *in vitro* incubations presented here were predicted based on the calculated rates of biotransformation (see above). The extract from the 24 hour phase I incubations should have delivered approximately 1.2 nmol of O-PCB 52 to each tube used in the phase II incubations (Table B.1). If O-PCB 52 was indeed delivered to the phase II incubation with PB-treated rat liver cytosol (2 mg/mL protein, 200 µL reaction volume, 2 hour incubation), the yield of GS-PCB should have been 670-2900 pmol (Table B.2). The predicted amount of GS-PCB would have resulted in concentrations (2.7 to 51 pmol/µL, see Table B.2) that were above the lowest level of detectable GS-SO (1.6 pmol/µL). However, the concentration of substrate affects the catalytic activity of enzymes – the lower the amount of substrate, the lower the rate of enzyme velocity. The phase I portion of the two-step incubations contained PCB 52 (or PCB 31) at the same

concentration (86 μM) used in Forgue and Allen (1982), so the rate of O-PCB 52 formation would be expected to be similar to the calculated rate. The theoretical concentration of O-PCB 52 added to each tube in the phase II step (1200 pmol/200 μL reaction = 6 μM) was about 8-fold lower than the 46 μM used in Preston and Allen (1984), so the rate of GS-PCB 52 formation in the present study may have been slower than the calculated rate of 14-60 pmol/min/mg. Sample size/cost precluded production of greater amounts of the arene oxide. The low level of predicted final O-PCB concentration in the mixed *in vitro* incubations may have cause GS-PCB formation by cytosolic GST to occur at a slower than predicted rate. To the author's knowledge, there is no kinetic data available on these PCB biotransformation rates, so the degree to which substrate concentration alters the velocity of the reaction cannot be predicted.

GS-SO was used as a surrogate for GS-PCB 52 during method development, and to establish detection limits. It is possible that the physiochemical differences between the conjugates of styrene oxide and the conjugates of PCB may have resulted in methodologies which were not optimized for GS-PCB, thereby precluding its detection. Styrene has one unsubstituted phenyl ring and GSH is conjugated to styrene at the aliphatic portion of the molecule whereas PCB has two chlorine substituted phenyl rings and GSH is conjugated directly to one of the aromatic rings of PCB. Yet, similar OASIS-HLB procedures have been successfully used for the extraction of diverse glutathione conjugates, e.g., conjugates of coumarin, diclofenac, and bile acids (Yu et al. 2005; Mitamura et al. 2009; Zhuo et al. 2009), suggesting that our methodology should have extracted GS-PCB as well. Additionally, even though the calculated rate of PCB 52

biotransformation to the arene oxide was above detection limits, we did not detect O-PCB 52 in incubations of PB-treated rat liver microsomes or CYP2B1 Supersomes by HPLC/MS or UPLC/MS (here and Chapter 4). However, it is possible that hydroxy-PCB 52 (OH-PCB 52) and O-PCB 52 were similarly retained on the C18 columns, and therefore the two metabolites, of equal nominal mass, could not be distinguished from one another. Also, despite the reported stability of O-PCB 52 (Reich et al. 1978), Preston et al. (1984) demonstrated that O-PCB 52 was reduced to PCB 52 during gas chromatography, indicating that similar degradation may have occurred, e.g., under the heat and pressure of the ESI source.

The results with CYP2B1 Supersomes were quite surprising. We selected CYP2B1 because PB-induced CYPs, insert an oxygen molecule at the *meta-para* positions of PCBs (Parke 1985; Kannan et al. 1995). In rats, the PB-induced CYP isoforms involved in PCB 52 biotransformation are CYP2B1 and CYP2B2, but the former shows much higher activity than the later (Ishida et al. 1991). Therefore, we predicted that CYP2B1 Supersomes, which, in essence, are concentrated microsomes with only one CYP present, would produce high concentrations of oxidized metabolites. However, in the present study, we saw no difference between PB-treated rat microsomes and CYP2B1 Supersomes in (the lack of) metabolite formation. When we attempted to ensure that the Supersomes were catalytically active by measuring their PCB 52 hydroxylation rate, we found that the mean rate of hydroxylation by Supersomes was less than half of that by PB-treated rat microsomes (42 pmol/min/mg versus 107 pmol/min/mg, respectively) (Chapter 4). A recent study of the *in vitro* hydroxylation of

another *meta-para* unsubstituted PCB, 2,2'6,6'-tetrachlorobiphenyl (PCB 54), PB-treated rat hepatic microsomes metabolized PCB 54 at a rate more than 100-fold greater than CYP2B1 Supersomes, and the inclusion of anti-CYP2B1 inhibited only 40% of PCB 54 hydroxylase activity in the PB-treated rat microsomes (Edwards 2006). Taken together, these results suggest that Supersomes do not possess the same catalytic activity as the native enzymes, and/or that other enzymes found in PB-treated rat liver microsomes, including CYP3A, contribute to the biotransformation of PCB 52. However, Edwards (2006) examined PCB 54 hydroxylase in several major PB-induced CYP isoforms, as well as several other CYPs (CYP1A2, CYP2A2, CYP2B1, CYP2C6, CYP2C11, CYP2C13, CYP3A1, CYP3A2, and CYP2D1 Supersomes), and the only two that exhibited detectable levels of PCB 54 biotransformation, CYP2B1 and CYP3A1, showed an additive rate of hydroxylation that was still more than 100-fold less than that of PB-treated rat liver microsomes. These results suggest that Supersomes do not possess the same catalytic activity as the native enzymes, and/or that other enzymes found in PB-treated rat liver microsomes contribute to the biotransformation of PCB.

In summary, we employed multiple chromatographic techniques to examine the formation of glutathione conjugates of PCBs, but were unable to detect the metabolite in a variety of *in vitro* incubation experiments. cand PCB congener metabolic groupings (e.g., *meta-para* unsubstituted PCBs are metabolized by PB-induced enzymes) may not represent the actual contribution of multiple CYPs in PCB biotransformation. PCBs are a

class of legacy contaminants, which have been the focus of toxicology research for almost 80 years (Safe 1994). While much is known about these compounds, the field of PCB biotransformation clearly requires more research.

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