Intestinal Bioavailability and Biotransformation of 3,4,3',4'-Tetrachlorobiphenyl in the Channel Catfish, Ictalurus Punctatus.

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INTESTINAL BIOAVAILABILITY AND BIOTRANSFORMATION
OF 3,4,3',4'-TETRACHLOROBIPHENYL
IN THE CHANNEL CATFISH, ICTALURUS PUNCTATUS

A Dissertation

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Doctor of Philosophy

in

The Interdepartmental Program
in Veterinary Medical Sciences through
the Department of Veterinary Physiology,
Pharmacology and Toxicology

by
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August 2000
This dissertation is
dedicated to
my parents
Noriko and Hiroto Doi,
for all the support and
unconditional love.
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ABSTRACT

These studies examined the role of micelle fatty acid composition, contaminant body burden, biotransformation, and non-specific toxicity on the systemic bioavailability of 3,4,3',4'-tetrachlorobiphenyl (TCB) from the intestine of catfish, as well as the effect of TCB upon the vectorial transporter p-glycoprotein (pgp). Initial in vitro experiments demonstrated that the solubility of [14C]-TCB in micelles was favored by longer chain fatty acids, with higher [14C]-TCB solubility correlating to greater systemic bioavailability of [14C]-TCB in an in situ preparation. The systemic bioavailability of [14C]-TCB was demonstrated to be affected by in vivo exposures to TCB, in a dose-independent manner, and apparently unrelated to P4501A-related induction or [14C]-TCB metabolism. The latter was detectable, but quantitatively a minor event. Bioavailability decreases in TCB-treated animals resulted from a combination of lowered [14C]-TCB transport to the blood, and retention in the lumen, which was consistent with permeability changes resulting from toxicity, or most likely, alterations in [14C]-TCB gradients resulting from unlabeled TCB accumulation in the tissues. Dietary pretreatment with the prototypic CYP1A inducer β-naphthoflavone (BNF) confirmed the lack of effect of intestinal AHH activity induction on the systemic bioavailability of [14C]-TCB. Histological alterations seen with BNF exposures, indistinguishable from those seen with TCB, did not appear to influence [14C]-TCB bioavailability. These latter findings reinforced the assumptions that the effect of TCB preexposure on the subsequent bioavailability of [14C]-TCB is diffusion-related. The pgp-related transporter identified in the catfish intestine was not
inducible by dietary exposures to the P4501A inducers TCB, BNF, benzo(a)pyrene, or the pgp inducer vincristine, under the conditions examined. In addition, functional analysis demonstrated that catfish pgp may exhibit some classic MDR transport abilities. These studies suggest that TCB intestinal bioavailability can be modulated by micelle fatty acid composition, as well as TCB exposure history. First-pass intestinal metabolism appears to be an evident, but minor event in the biotransformation of dietary TCB. Furthermore, studies with catfish pgp suggested that induction of CYP1A and pgp appear to be unrelated events in the catfish intestine.
INTRODUCTION

Anthropogenic contaminants are found in many of the world ecosystems. Since seventy percent of the globe’s surface is covered with water, and land areas are washed by water, a great portion of the environmental load of contaminants is distributed to aquatic environments. Organisms living in those environments are exposed to contaminants, and are in turn consumed by humans. Aquatic species may accumulate xenobiotics by various routes. The relative importance of each route of exposure is mostly associated with the nature of the chemical. The dietary route of exposure is often most important for relatively nonpolar compounds, and the toxicity and residue implications of these compounds are tied to compound bioavailability and processing by the intestine. Polychlorinated biphenyls (PCBs) are lipophilic pollutants widely distributed in aquatic ecosystems (Kalmaz and Kalmaz, 1979; Tanabe, 1988; Fowler, 1990) which accumulate in aquatic species and undergo food chain biomagnification (Thomann and Connolly, 1984; Connolly, 1991). 3,4,3',4'-Tetrachlorobiphenyl (TCB) is an important component of PCB commercial mixtures with respect to toxicity and environmental distribution. The abundance of TCB in tissues of higher organisms suggests that this compound may be subjected to dietary transfer.

The exact mechanism of lipophilic xenobiotic uptake in the gastrointestinal tract is not fully understood, but evidence indicates that it may be associated with movement of dietary lipids (Vetter et al., 1985; Van Veld, 1990). Lipid soluble compounds released from the diet are believed to associate with micelles formed by bile salt solubilization of dietary lipids, in order to move through the aqueous luminal
environment, and reach the absorptive epithelium. Studies have suggested that the incorporation of commercial PBC mixtures into lipid micelles is dependent on their fatty acid composition (Laher and Barrowman, 1983). It remains to be determined whether constituent fatty acids would similarly affect the micellar solubilization of individual PCB congeners, such as TCB, or whether or not the solubilization in micelles would influence chemical delivery to, and through, the intestinal wall.

Once in the intestinal mucosa, PCBs may be metabolized before being transferred to the systemic circulation. The initial oxidative metabolism of PCBs is mediated by the cytochrome P450 enzyme system. Recent studies with TCB have provided evidence that this compound may be a substrate for P4501A (CYP1A) hepatic metabolism in fish species (White et al., 1997), in addition to being an Ah (aryl hydrocarbon) agonist, and effecting CYP1A induction in a number of fish species (Monosson and Stegeman, 1991; Wirgin et al., 1992; Lindstrom-Seppa et al., 1994; Sleiderink and Boon, 1996; Otto et al., 1997). Most research on the reciprocal interaction of TCB and CYP1A, however, have focused on the liver, and much is to be learned regarding the interactions of PCBs with monooxygenases of other organs, including the gastrointestinal tract.

Intestinal biotransformation often, but not always, results in the formation of more polar products, which may differ from the parent compound on its rate of transfer to the blood. The modulatory effect of intestinal biotransformation on systemic bioavailability of xenobiotics has been recently examined in fish, using the polycyclic aromatic hydrocarbon benzo(a)pyrene (BaP) (Kleinow et al., 1998). These
earlier studies indicated that CYP1A-dependent intestinal metabolism, inducible by Ah receptor agonists, substantially affected both the extent and the composition of absorbed BaP. The impact of induction of intestinal biotransformation on the systemic uptake of chemicals such as PCBs has not been investigated in fish species to date.

Another feature recently recognized as a major modulator of intestinal bioavailability of orally administered compounds is transport by the membrane-bound p-glycoprotein (pgp) (Lown et al., 1997; Sparreboom et al., 1997; van Asperen et al., 1997). Active secretion by pgp prevents systemic absorption of drugs through active efflux back to the intestinal lumen (Hsing et al., 1992; Saitoh and Aungst, 1995). Studies in our laboratory (Kleinow et al., 2000), as well as others (Hemmer et al., 1995), indicate that pgp is a prominent feature in the fish intestine. Broad overlap in substrate and inhibitor specificities indicate that pgp may act in coordination with, or complimentary to cytochrome P450 enzymes, in a protective mechanism against toxicity (Burt and Thorgeirsson, 1988; Salphati and Benet, 1998; Tateishi et al., 1999). Environmental pollutants containing CYP1A inducers (e.g. Diesel-2 oil) have been shown to modulate pgp-related transport in aquatic species. Likewise, evidence has been provided that lipophilic environmental contaminants, such as BaP, may be substrates for pgp transport (Yeh et al., 1992; Penny and Campbell, 1994). Regional disposition of BaP, demonstrated in the catfish intestine (Kleinow et al., 1996), suggests that pgp may play a modulatory role in the intestinal uptake of contaminants that are CYP1A inducers (e.g. TCB, BaP) in this fish species.
The overall objective of these studies was to investigate factors which would, alone or in combination, modulate the systemic bioavailability of the lipophilic contaminant TCB from the intestine of the channel catfish. Included among the factors examined herein were luminal lipid micelle composition, contaminant body burdens, intestinal biotransformation, non-specific toxicity, and the vectorial action of the transporter p-glycoprotein in the intestine.

The first hypothesis examined was that fatty acid composition determines the solubility of TCB in lipid micelles, and modulates the extent of TCB systemic bioavailability from the catfish intestine. The second hypothesis was that exposures to the Ah receptor agonists TCB and β-naphthoflavone (BNF) decrease the systemic bioavailability of TCB from the catfish intestine, in a dose-dependent manner, through increases in intestinal cytochrome P450-dependent biotransformation, contaminant body burden, and non-specific toxicity. The third hypothesis was that expression and activity of intestinal p-glycoprotein are inducible by the Ah agonists TCB, BNF and BaP, with a potential role in lipophilic xenobiotic bioavailability.

In order to address these questions, specific objectives of these studies were:

1. To examine the effects of alterations in fatty acid composition on (1) the in vitro solubility of [14C]-TCB in lipid micelles, and (2) the intestinal bioavailability of [14C]-TCB in an in situ preparation.
2. To investigate the effect of TCB body burden, resulting from 10-day in vivo dietary exposures, on subsequent intestinal bioavailability and biotransformation of [14C]-TCB.
3. To investigate the effects of *in vivo* dietary exposures to TCB and BNF on intestinal integrity, using histological analysis.

4. To examine the effects of alterations of intestinal metabolic status, resulting from *in vivo* dietary exposure to TCB and BNF on subsequent intestinal $[^{14}C]$-TCB bioavailability and biotransformation in an *in situ* preparation.

5. To assess the effect of *in vivo* dietary exposures to the CYP1A inducers TCB, BaP and BNF, and the pgp inducer vincristine (VIN), on pgp-related immunoreactivity in the catfish intestine using immunoblot analysis.

6. To elucidate the pgp-related transport capabilities of intestinal membrane vesicles collected from the intestine of controls, and BNF- and VIN-treated catfish, using the prototypic pgp substrate vinblastine, and the inhibitor verapamil.

The first chapter of this dissertation provides a literature overview of the distribution, food chain transfer, and biotransformation of polychlorinated biphenyls in aquatic systems, as well as a discussion of factors that would modulate their intestinal bioavailability. The second chapter investigates both issues associated with micelle lipid composition, as well as TCB *in vivo* exposure, in relation to the *in situ* intestinal biotransformation and systemic bioavailability of $[^{14}C]$-TCB. The third chapter provides an account of the effects of BNF *in vivo* exposure on the systemic bioavailability of $[^{14}C]$-TCB using an *in situ* intestinal preparation. The fourth chapter examines the inducibility of pgp by Ah agonists, as well as the functional abilities of this protein, in context with their role in intestinal bioavailability. The fifth chapter provides an overall summary and general conclusions.
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of cytochrome P4501A in adult and larval fathead minnow *Pimephales promelas*. *Aquatic Toxicology*. 28: 147-167.


CHAPTER 1: LITERATURE REVIEW

POLYCHLORINATED BIPHENYLS

PROPERTIES, USAGE, AND ENVIRONMENTAL DISTRIBUTION

Polychlorinated biphenyls (PCBs) are a collection of 209 synthetic congeners and isomers produced by the chlorination of biphenyl, using iron filings or ferric chloride as catalysts (Hutzinger et al., 1974; Brinkman and de Kok, 1980). The empirical formula of PCBs is $C_{12}H_{10-n}Cl_n$ ($n = 1-10$), and the conventional numbering and positional nomenclature of the central structure are depicted in Figure 1.1.

The industrial production of PCB mixtures began in the late 1920s in the U.S.A., and spread to industrial countries worldwide. Commercial PCBs were marketed under various trade names, including Aroclor (Monsanto, U.S.A. and Great Britain), Clophen (Bayer, Germany), Kanechlor (Kanegafuchi, Japan), Phenochlor or Pyralene (Prodelec, France), and Fenclor (Caffaro, Italy). Although 209 congeners exist, approximately 100 individual isomers can be found in all commercial mixtures, each containing from 40 to 70 different compounds (Pomerantz et al., 1978; Brinkman and de Kok, 1980; Rappe and Buser, 1980). The commercial PCB mixtures are identified according to their degree of chlorination. For example, the commercial Aroclor is identified by a four digit number, where the first two numbers designate the biphenyl structure, and the last two digits denote the percentage of chlorine content by weight.

The physicochemical properties of PCBs are associated with their degree and position of chlorination (Hutzinger et al., 1974; Pomerantz et al., 1978; Safe, 1994).
Figure 1.1 - (A) Numbering and (B) positional nomenclature of biphenyl.
Lower chlorinated mixtures (e.g. Aroclor 1221, 1232, 1242, and 1248) are crystalline, mobile oils, while higher chlorinated ones (e.g. Aroclor 1254, 1260, 1262) are viscous or sticky resins (Hutzinger et al., 1974; Brinkman and de Kok, 1980). Such differences in physical properties illustrate the diversity of PCBs, which lead to the numerous industrial applications (Brinkman and de Kok, 1980). The high chemical stability made PCBs ideal as plasticizers in rubbers and resins, one of their main open-ended uses. Dielectric features suited PCBs for electric insulators in transformers and capacitors, the latter representing the single largest PCB application. Chemical stability along with low vapor pressure qualified PCBs as perfect lubricants, heat-transfer fluids, hydraulic fluids, cutting oils and vacuum pump oils. Additionally, PCBs were used as adhesives, flame retardants (in polyethylene, polystyrene, and polyurethanes), employed as protective coating for swimming pools and maritime equipment, and incorporated into pesticides, dust inhibitors, immersion oils for microscopes, carbonless copy paper, ink, and packaging materials.

Some of the commercial applications of PCBs resulted in the direct contamination of the environment (for example, by agricultural run-off), and industrial careless disposal practices or unintentional leakage further increased their environmental burden (Kalmaz and Kalmaz, 1979; Kimbrough, 1980; Safe et al., 1987). The first report of PCBs detection in environmental samples was by Jensen (1966), a Swedish scientist, who discovered several unknown peaks while quantitating DDT using gas chromatography. A few years later, the health effects of PCBs went public with the Yusho incident, a mass food poisoning caused by ingestion of PCB contaminated rice
that occurred in Japan (Kuratsune, 1980). Since then, numerous studies have shown that PCBs can be found in most compartments of the environment (water, air, soil, sediments and biota) in various regions of the world (Kalmaz and Kalmaz, 1979; Kimbrough, 1980; Tanabe, 1988; Fowler, 1990), and a lot of attention has been paid regarding human and wildlife toxicity by PCBs (Mayer et al., 1977; Sleight, 1983; Safe, 1984; Parkinson and Safe, 1987).

It has been estimated that approximately 1.2 million metric tons of PCBs have been produced globally from the late 1920s until their production was terminated in the 1970s (Tanabe, 1985; Hansen, 1987). Of the total PCB production, nearly one third appears to be distributed in the environment, where the same chemical properties (high chemical stability, resistance to degradation, etc.) that favored their wide industrial use result in the extended persistence of PCBs. Approximately 97% of the total environmental load are distributed in aquatic ecosystems (Tanabe, 1985; Hansen, 1987), in association with the large area encompassed by world waters. For example, the Great Lakes account for 95% of the total surface freshwater of the U.S.A, and it is responsible for over 97% of the country's aquatic PCB (Hansen, 1987). A compilation of global PCB concentrations has shown that PCBs are mostly distributed in the Northern hemisphere, with high concentrations in coastal areas, probably a reflection of industrial development (Tanabe and Tatsukawa, 1986; Fowler, 1990).

**AQUATIC FOOD CHAIN TRANSFER**

In both open ocean and coastal areas, PCBs in water tend to accumulate in the surface microlayer, because of its higher concentration of lipids. Limited information
is available regarding the concentration of PCBs in open ocean sediments, but data from coastal areas indicate that PCBs accumulation in sediments are dependent on location and sediment physiochemical characteristics (Segar and Davis, 1984; Fowler, 1990). PCBs in surface waters and sediments may be taken up by a number of aquatic species, including phytoplankton and benthic species. Phytoplankton play an important role in pelagic food chains, and bioconcentration (direct uptake from the water) of PCBs have been shown to occur in these species (Harding Jr and Phillips Jr, 1978; Brown et al., 1982). Benthic organisms in contact with contaminated sediments have been shown to bioaccumulate PCBs (through direct contact or ingestion of sediments) and have been included in a number of models examining their food chain transfer (Oliver and Niimi, 1988; Connolly, 1991; Iannuzzi et al., 1996). Independent of the pathway by which PCBs enter the food web, they may progress along the various trophic levels, ultimately reaching humans.

Higher levels of PCBs have been found in top predators, suggesting that these compounds undergo biomagnification along the food chain (Mayer et al., 1977; Rasmussen et al., 1990). Biomagnification refers to the increases in tissue residues of those chemicals taken up by the animals, with progression through one or more trophic levels of the food chain. The relative contribution of dietary routes of xenobiotic exposure over direct uptake from the water to the overall biomagnification of PCBs has been a matter of debate over the years. Studies with a number of organochlorines have implied that tissue accumulation can be explained exclusively by direct partition between water and tissues (Scura and Theilacker, 1977; Macek et al., 1979;
Increased xenobiotic accumulation in top predators was attributed to the relative lipid content of these organisms. For that reason, the correlation of the bioconcentration factor (BCF) (constant of proportionality between the concentration of chemical in the fish and in the water) and the octanol-water partition coefficient ($K_{ow}$) (a measure of the lipophilic properties of a chemical), was examined by many authors. It was suggested that the $K_{ow}$ values could be used to predict the bioconcentration potential of those chemicals with log $K_{ow}$ up to 6, but not for higher $K_{ow}$ values (Hamelink et al., 1971; Neely, 1979; Veith et al., 1979).

Contrasting studies in Lake Ontario ecosystems have shown that increases in total PCB tissue concentrations with increases in trophic levels greatly surpassed those expected by bioconcentration alone, indicating that diet is a major contaminant source (Mackay, 1982; Oliver and Niimi, 1988). In these studies, a greater divergence from the BCF-$K_{ow}$ line was seen with each trophic level, independent from tissue lipid content. Additional evidence supporting the role of dietary exposure in the overall biomagnification of PCBs in aquatic food chains has been provided by several authors (Bruggeman et al., 1984; Thomann and Connolly, 1984; Thomann, 1989). Mechanistic studies utilizing variables such as the uptake efficiency from the water, assimilation efficiency from the food, and excretion rates have suggested that food chain exposure can account for 90 to 99% of contaminant accumulation in Lake Michigan lake trout (Thomann and Connolly, 1984; Thomann, 1989). Likewise, studies by Bruggeman et
al. (1984) comparing different routes of uptake in guppies have shown that dietary uptake of PCBs was much more efficient than from water.

A few models have examined the extent of PCB dietary transfer in relation to the food chain structure. A model by Connolly (1991), examining PCB concentrations in predators of different feeding habits (flounder and lobster), have shown that even though dietary uptake of PCBs by both species exceeded gill uptake, greater accumulation occurred for those predators more closely associated with the benthic elements of the food chain. The length of the food chain, as much as its structure, may influence the extent of PCBs bioaccumulation (Rasmussen et al., 1990; Evans et al., 1991; Bentzen et al., 1996). Concentrations in tissues of trout in the Great Lakes were shown to increase with the length of the food chain (Rasmussen et al., 1990; Bentzen et al., 1996), with a contribution of 3.5-fold biomagnification factor for each trophic component (Rasmussen et al., 1990). Likewise, Evans et al. (1991) have showed an increase from 1.3 to 12.9-fold for PCBs in a Lake Michigan food web, for successive trophic levels.

The controversy involving the relative importance of the various routes of exposures appears to be based on methodological differences. Comparisons of lipophilic chemical bioaccumulation in different systems should take into account as many variables as possible, including chemical properties, conditions of exposure, food chain structure, metabolism, excretion and the refinement of modeling. Utilization of solvent carriers, as well as concentrations beyond solubility limits have been cited as factors that could result in overestimation of chemical uptake from the water (Harding

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et al., 1981; Lederman and Rhee, 1982). Likewise, it has been stated that omission of parameters such as gill area and fish feeding rate when modeling bioaccumulation could otherwise result in the overestimation of dietary intake (Barber et al., 1991).

**BIOCHEMICAL EFFECTS**

Studies in mammals have shown that the biochemical, as well as the toxic effects of polychlorinated biphenyls (PCBs), are determined by the position of their halogen substitutions (Safe et al., 1985; McFarland and Clarke, 1989). Those PCBs containing either non- or mono-ortho chlorines can assume a coplanar configuration, and bind to the cytosolic aryl hydrocarbon (Ah) receptor. Upon ligand binding, the Ah receptor dissociates from a heat shock protein (hsp90), and associates with the aryl hydrocarbon receptor nuclear translocator (ARNT) (Swanson and Bradfield, 1993; Schmidt and Bradfield, 1996). The Ah receptor-ARNT complex, in turn, binds to specific sequences in promoter regions of the target genes, effecting CYP1A induction and other toxic effects. The molecular mechanism of CYP1A induction in fish has not been fully clarified, but the Ah receptor has been identified in several fish species (Hahn et al., 1992; Hahn et al., 1997; Roy and Wirgin, 1997), and recent studies have also identified a functional ARNT protein in killifish (Powell et al., 1999).

Induction of hepatic CYP1A parameters by controlled laboratory exposures to PCB mixtures has been demonstrated in several fish species, including rainbow trout (Elcombe and Lech, 1979; Melancon and Lech, 1983), sheepshead (James and Little, 1981), and carp (Melancon et al., 1981; Melancon and Lech, 1983). These studies have suggested that individual coplanar PCB congeners present in the PCB mixtures
account for the CYP1A-related induction effects of the commercial products. In sheepshead, induction of metabolizing enzymes by 3,3',4,4',5,5'-hexachlorobiphenyl was similar to observed with Aroclor 1254 (James and Little, 1981). Similar results were seen in rainbow trout and carp, where 3,4,3',4'-tetrachlorobiphenyl induced monooxygenase activities comparable to Aroclor 1254 exposures (Melancon and Lech, 1983). Studies with individual isomers have additionally demonstrated a lack of effect of non-coplanar PCBs on CYP1A-related parameters (Melancon et al., 1981; Gooch et al., 1989). Gooch et al. (1989) showed that parenteral CYP1A-related parameters were induced in scup liver following administration of a coplanar PCB, while three other ortho-chlorinated PCBs failed to do so. Likewise, no CYP1A-related effects were seen in carp treated with the non-coplanar PCB 2,2',4,4'-TCB (Melancon et al., 1981).

The coplanar 3,4,3',4'-tetrachlorobiphenyl (TCB) has been widely used as a model Ah receptor ligand in fish, and has been shown to induce hepatic CYP1A in rainbow trout (Melancon and Lech, 1983; Otto et al., 1997), dab (Sleiderink and Boon, 1996), winter flounder (Monosson and Stegeman, 1991), scup (Gooch et al., 1989; White et al., 1997a), fathead minnow (Lindstrom-Seppa et al., 1994), carp (Melancon and Lech, 1983), and Atlantic tomcod (Wirgin et al., 1992). The hepatic induction of CYP1A by TCB in fish appears to be dose-dependent, with increases in CYP1A catalytic activity, as well as CYP1A transcription and protein expression both in vitro and in vivo (Gooch et al., 1989; Hahn et al., 1993; Lindstrom-Seppa et al., 1994). Above a maximum induction level, a decline in CYP1A catalytic activities is
observed, apparently caused by increased TCB tissue levels that result in inhibition of CYP1A catalytic activity (Monosson and Stegeman, 1991; Hahn et al., 1993; White et al., 1997a).

**METABOLISM**

Early studies have suggested that fish were limited in their ability to metabolize polychlorinated biphenyls (PCBs) (Hutzinger et al., 1972). In these studies, brook trout orally dosed with three PCB congeners (4-chlorobiphenyl, 4,4'-dichlorobiphenyl, 2,2',5,5'-tetrachlorobiphenyl, and 2,2',4,4',5,5'-hexachlorobiphenyl) failed to demonstrate hydroxylated metabolites in the water, within four days of exposure. Subsequent investigations have demonstrated formation of polar metabolites of 2,2',5-trichlorobiphenyl, 2,2',5,5'-tetrachlorobiphenyl, and 2,2',4,5,5'-pentachlorobiphenyl in whole body preparations of the green sunfish (Sanborn et al., 1975), and conjugated metabolites of 2,2',5,5'-tetrachlorobiphenyl in bile from rainbow trout (Melancon and Lech, 1976).

Depletion of PCB congeners from tissues, and the relative abundance of PCB congeners in different trophic levels of the aquatic food chain have also been used to investigate the metabolism of PCBs in aquatic species. Niimi and Oliver (1983) examined the temporal elimination of 31 different PCB congeners from muscle and whole body of rainbow trout, following a single oral dose. It was noted that elimination was enhanced for those PCB congeners of lower chlorine content, no ortho-chlorine substitutions, and vicinal unsubstituted carbons. Similar selectivity was observed in studies with winter flounder from New Bedford Harbor for those PCB congeners.
containing adjacent unsubstituted meta-para carbons (Elskus et al., 1994). Comparisons of relative abundance in the food chain have shown that in general, unsubstituted vicinal ortho-meta positions with no more than one ortho substitution, and unsubstituted vicinal meta-para positions appear to favor metabolism in lower aquatic organisms (Boon et al., 1992; Walker, 1992).

The involvement of the different P450 isoforms in the metabolism of PCBs has been deduced from comparison of PCB congener distributions in the aquatic fauna to those patterns seen in mammalian species (Brown, 1992). Patterns in aquatic animals were classified as CYP1A-like and CYP2B-like, according to resemblance to alterations effected by those P450 isozymes in mammals. Of the fish species examined, CYP1A-like alterations were frequently observed, while CYP2B-like alterations were seen only in a few species. Recent studies with scup, examining the hepatic metabolism of 3,4,3',4'-tetrachlorobiphenyl (TCB), provide the first measured rates of TCB metabolism in fish, in addition to evidence that CYP1A may be involved (White et al., 1997b). These authors demonstrated that the rates of metabolism of TCB in scup are very low in comparison to previous reports in mammalian species.

INTESTINAL XENOBIOTIC ABSORPTION

CHEMICAL UPTAKE OF LIPID-SOLUBLE COMPOUNDS

Transfer of lipophilic compounds across the intestinal membrane may occur through passive or carrier-mediated diffusion, active transport and pinocytosis. Of these transcellular processes, passive diffusion is the mechanism most likely to be involved in the absorption of lipophilic xenobiotics in the intestine (Thomson and
Dietschy, 1981; Kuksis, 1984). The mechanism by which lipophilic xenobiotics traverse the intestine in fish is not completely understood, but evidence indicates that it is closely associated with digestion and absorption of dietary fat. Facilitated absorption of lipophilic compounds in the presence of triglycerides have been demonstrated to occur in fish (Van Veld, 1990), an association previously described for lipid-soluble drugs and vitamins in mammals (Bloedow and Hayton, 1976; Kuksis, 1984; Yoshitomi et al., 1987). Triglycerides are hydrolyzed in the intestine by pancreatic lipases, and the released fatty acids are solubilized by bile salts forming micelles. Highly lipophilic compounds appear to be incorporated into lipid micelles in order to reach the absorptive epithelium (Vetter et al., 1985). In vitro studies by Laher and Barrowman (1983), utilizing the PCB mixture Aroclor 1242, have shown that the PCB carrying capacity of micelles is favored by long chain polyunsaturated fatty acids, compared to shorter chains or monounsaturated components. These latter studies suggest that stereochemical factors might be influential in the association of chemicals and micelles, so that micelle carrying capacity will be dependent both on the lipid composition of the micelle, and the compound to be solubilized.

The movement of lipophilic compounds across the intestinal membrane in fish species is thought to occur according to either one of two proposed models. The model proposed by Gobas (Gobas et al., 1993) suggests that lipophilic xenobiotics diffuse across the intestinal mucosa following a fugacity gradient. The leaving tendency (fugacity) of xenobiotics in the lumen is believed to increase following food volume reduction by digestive processes, and by the reduction of lipid content in the lumen. A
second model proposes that lipophilic xenobiotics and dietary lipids are codigested, codispersed, and coassimilated across the intestinal epithelium (Vetter et al., 1985). In the latter, following absorption, xenobiotics are incorporated into fat vacuoles which are formed by the products of lipid digestion that are resynthesized to triglycerides within the smooth endoplasmic reticulum.

**MODULATORS OF BIOAVAILABILITY**

**Intestinal Biotransformation**

Once in the enterocyte, xenobiotics may be excreted back to the intestinal lumen by counter-transport processes, may enter the systemic circulation as parent compounds, or undergo metabolism prior to absorption or elimination. Biotransformation usually, but not always, results in the formation of a more polar product, which often differs from parent compound in its ability to cross biological membranes. Studies have shown that the intestine of fish is capable of most phase I and phase II biotransformation reactions, including cytochrome P450-dependent monooxygenase reactions and phase II conjugation (Lindstrom-Seppa et al., 1981; Van Veld et al., 1988b; James et al., 1997). In comparison to liver, the major organ in xenobiotic biotransformation, the intestine expresses quantitatively lower levels of biotransformation enzymes, due to its relative lower mass. Intestinal metabolic activity in fish play a role in the transformation of those chemicals present in low levels in the environment, but may be overwhelmed by very high levels of exposure (James and Kleinow, 1994). Intestinal enzymes, however, may reach levels comparable to liver following exposure to environmental inducers, when significant first-pass
biotransformation of environmental contaminants may occur (Van Veld et al., 1987; Van Veld et al., 1988a; Van Veld et al., 1988b; Van Veld et al., 1991; James et al., 1997; Kleinow et al., 1998).

Cytochrome P4501A (CYP1A) has received the most attention in relation to the intestinal oxidative metabolism of xenobiotics in fish. CYP1A activity is commonly measured using assays such as ethoxyresorufin-O-deethylase (EROD), which represents primarily CYP1A activity (Stegeman and Hahn, 1994), and benzo(a)pyrene-3-hydroxylase (AHH), which is a measure of P450 isozymes that include CYP1A. Low CYP1A concentrations and related activities have been demonstrated in the intestine of a variety of uninduced fish species, including catfish, spot, toadfish, mummichog, and Atlantic tomcod (Van Veld et al., 1988a; Van Veld et al., 1988b; Van Veld et al., 1988b; Husoy et al., 1994; James et al., 1997; Van Veld et al., 1997; Kleinow et al., 1998). Induction of intestinal CYP1A-related parameters have been shown to occur in various fish species, following exposure to a variety of Ah agonists (Van Veld et al., 1987; Van Veld et al., 1988a; Van Veld et al., 1988b; Van Veld et al., 1991; James et al., 1997; Kleinow et al., 1998). Van Veld et al. (1987) demonstrated that oral administration of benzo(a)pyrene (BaP) (16 mg/kg diet) resulted in a 1.9-fold increase in total P450 content, as well a 8.5-fold increase in AHH activities in the intestine of spot. Similar results were seen with 10 mg/kg BaP or 3-methylcholanthrene (3-MC) exposures in spot, which effected respective 14- and 17-fold increases in intestinal AHH activities, 17- and 36-fold increases in EROD activities, and 2- and 1.9-fold increases in intestinal total P450 content (Van Veld et
Similarly, studies with catfish have demonstrated increases in both intestinal EROD and AHH activities following administration of β-naphthoflavone (BNF) (10 mg/kg diet) (James et al., 1997; Kleinow et al., 1998). EROD activities in control catfish ranged from 1.9 to 3.6 pmol/min/mg protein, and increased to ~12 pmol/min/mg protein following BNF exposure. AHH activities increased from ranges of 12.5 to 23.1 pmol/min/mg protein, in control animals, to ~70 pmol/min/mg protein in BNF-treated animals. These changes occurred without corresponding increases in total P450 content (James et al., 1997), which suggested that CYP1A possibly represents a small amount of total P450s in the catfish intestine. Overall, CYP1A-related activities appeared to be lower in catfish in comparison to other species examined. In addition, high doses of dietary BNF at 100 mg/kg diet resulted in inhibition of EROD and AHH activity in catfish (James et al., 1997), while induction of EROD activity could be seen with much higher doses (250 mg BNF/kg diet) in mummichog (Van Veld et al., 1991). The inhibitory effect seen in catfish was attributed to the presence of residual BNF in the catfish intestine, which could have inhibited both EROD and AHH activities. These studies demonstrate that although dietary Ah agonists markedly affect the status of biotransformation enzymes in fish, the degree of induction may vary with species, inducer, and experimental differences.

Induction of intestinal biotransformation activities has been shown to modulate the systemic bioavailability of xenobiotics. In catfish, administration of dietary BNF at 10 mg/kg diet for 15 days altered the degree of metabolism of a subsequent BaP dose, which affected the profile of metabolites transferred to the intestinal circulation.
(Kleinow et al., 1998). Treatment with BNF resulted in an increase in the proportion of the potentially hazardous products BaP-7,8 and 9,10 dihydrodiols, as well as in a higher percentage of unconjugated metabolites. Exposure to BNF, as well as higher BaP dose increased the systemic bioavailability of both parent BaP, as well as its metabolites. In studies with toadfish, exposure to dietary BaP at 10 mg/kg diet resulted in a higher percentage of BaP metabolites being transferred to the blood, in comparison to untreated animals (Van Veld et al., 1988a).

**P-glycoprotein**

P-glycoprotein (pgp) is a transmembrane energy-dependent efflux transporter initially discovered in tumor cells and associated with the multidrug resistance (MDR) phenotype (Juliano and Ling, 1976). Pgp is overexpressed following exposure to anticancer drugs, and renders MDR resistance by preventing intracellular accumulation not only of the initial drug it was exposed to, but also to other structurally unrelated compounds. MDR genes have been shown to be highly conserved across species, being described in a wide variety of organisms, including rodents (Ng et al., 1989), nematoid worms (Broeks et al., 1995), and fish (Chan et al., 1992). The discovery of pgp expression in non-transformed tissues with secretory, excretory or barrier function has lead to the belief that the physiological function of pgp is associated with protection from natural toxins (Gottesman and Pastan, 1988; Ford and Hait, 1990). Normal tissues shown to express pgp include the intestine, liver, adrenal gland, brain capillaries, and kidney (Fojo et al., 1987; Thiebaut et al., 1987). In aquatic animals, a
pgp-related multixenobiotic resistance (MXR) mechanism has been associated with defense against pollution (Kurelec, 1992).

Studies in mammals have suggested that intestinal pgp, in concert with first-pass biotransformation, may play a role in the variability seen in the systemic bioavailability of orally administered drugs. Studies with human colonic adenocarcinoma cell line (Caco-2) demonstrated the vectorial transport of the pgp substrate vinblastine, as well as other compounds (β-blockers and quinidine) (Terao et al., 1996). Yumoto et al. (1999), utilizing both in vitro and in vivo techniques, demonstrated that the intestinal transport of the pgp substrate Rhodamine 123 was inhibited by both CYP3A- and pgp-related compounds (e.g. midazolam, ketoconazole, verapamil, and quinidine). Corroboration of a pgp role in the intestinal bioavailability of drugs was provided by kinetic studies in mice that showed that previous treatment with the pgp blocker SDZ PSC 833, a non-immunosuppressive cyclosporine analogue, decreased the systemic bioavailability of paclitaxel (van Asperen et al., 1997). Further evidence was provided by studies with MDR knockout mice, where animals which did not express functional pgp maintained higher systemic concentrations of paclitaxel following oral administration (Sparreboom et al., 1997). Immunohistochemistry studies have shown that pgp is prominent in the intestine of various fish species (Hemmer et al., 1995; Hemmer et al., 1998; Kleinow et al., 2000), but a modulatory role of pgp in the intestinal bioavailability of chemicals in fish has not been established.

Mammalian MDR genes have been shown to be divided in two classes which code for different pgps, an inducible form involved in drug resistance, and a
constitutive apparently uninducible phosphatidylcholine translocator (Van Helvoort et al., 1996). Partial cloning of two fish pgp genes in winter flounder have shown that the 3' ATP binding site and 3' terminal exon are homologous to mammalian genes, but no specific relation to particular mammalian pgp isoforms could be established (Chan et al., 1992). Cursory studies in mammals have suggested that pgp and cytochrome P4501A (CYP1A) may work in coordination (Burt and Thorgerisson, 1988; Gant et al., 1991; Bain et al., 1997). Failure of model CYP1A inducers to elevate pgp expression in some experimental mammalian systems suggests that CYP1A and pgp are possibly coinduced, but are not coregulated. Studies in nonparenchymal rat liver epithelial cells have shown that exposure to 3-methylcholanthrene (3-MC) resulted in induction of MDR mRNA but not CYP1A1 or CYP1A2 expression (Fardel et al., 1996). Likewise, a lack of MDR induction was observed in primary hepatocyte cultures exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), with doses that induced maximum CYP1A induction (Gant et al., 1991). Induction of both MDR and CYP1A was observed in the latter system following exposure to 3-MC, in contrast to the nonparenchymal epithelial cell system previously mentioned. These results in mammalian systems have lead to investigations of the interaction of CYP1A and pgp in fish. Studies with blenny have shown that elevated expression of both hepatic pgp and CYP1A were detected in fish exposed to a pulp mill effluent, as well as to crude oil under laboratory conditions (Bard et al., 1998). These studies exhibited a significant correlation between pgp and CYP1A expression in the liver of fish exposed under laboratory conditions, indicating that both systems were responding against
contaminant exposure. Similar indications were observed with carp exposed to water contaminated with Diesel-2 oil (Kurelec et al., 1995). In this study, hepatic CYP1A-related activity was not altered by exposure to oil alone, but rapidly increased following addition of the pgp inhibitor verapamil. These results suggested that pgp inhibition resulted in increased uptake of Diesel-2 oil.

REFERENCES


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CHAPTER 2: EFFECT OF MICELLE FATTY ACID COMPOSITION AND 3,4,3',4'-TETRACHLOROBIPHENYL (TCB) EXPOSURE ON INTESTINAL [14C]-TCB BIOAVAILABILITY AND BIOTRANSFORMATION IN CHANNEL CATFISH IN SITU PREPARATIONS*

INTRODUCTION

Polychlorinated biphenyls (PCBs), ubiquitous contaminants in the aquatic environment (Fowler, 1990; Kalmaz and Kalmaz, 1979), exhibit high chemical stability, resistance to degradation, and high lipophilicity (Safe, 1984). The high lipophilicity of PCBs results in the association of these chemicals with all components of the aquatic food chain (Connolly, 1991; James and Kleinow, 1994). PCBs undergo biomagnification through the food chain with higher trophic levels exhibiting greater accumulation than lower trophic levels (Biggs et al., 1980; Mayer et al., 1977; Oliver and Niimi, 1988; Rasmussen et al., 1990; Thomann and Connolly, 1984). The number of trophic steps in the food chain and composition of diet have been correlated to the magnitude of PCB transfer (Rasmussen et al., 1990). Mechanistic models suggest that 90 to 99% of lipophilic contaminants in higher trophic level fish may result from food chain exposure (Thomann, 1989; Thomann et al., 1986).

A variety of physiochemical factors modulate the dietary bioavailability of PCBs. For PCBs within the low to moderate $K_{ow}$ range an increasing partition coefficient has been shown to result in an increasing rate of uptake in fish (Chiou et al., 1977; Russell et al., 1995). In contrast, a compound $K_{ow}$ above 6 appears to limit bioavailability (Gobas et al., 1988; Opperhuizen and Sijm, 1990). Likewise,

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halogenation of PCBs has been shown to influence bioavailability with assimilation efficiencies of dietary PCBs inversely proportional to the chlorine content (Bruggeman et al., 1984; Tanabe et al., 1982). In general, compounds with lower molecular weights (Bruggeman et al., 1984; Niimi and Oliver, 1988) and small molecular volumes (Niimi and Oliver, 1988) tend to be more readily absorbed.

Fat in the diet as well as chemical solubility in triglycerides have been shown to facilitate absorption of lipophilic contaminants (Kuksis, 1984). Micelles, formed by bile salt solubilization of dietary fat, are involved in the transport of lipophilic xenobiotics to the intestinal wall (Gobas et al., 1993). Two models, both including lipid as an integral mechanistic component, have been proposed for the gastrointestinal uptake of lipophilic xenobiotics in fish. A fugacity (leaving tendency) based model proposed by Gobas et al. (1993) suggests that the driving force for chemical uptake is generated by digestive processes in the gastrointestinal lumen. Absorption of dietary fat in the form of fatty acids, through reduction of the volume (increasing the relative concentration) and the changing character of the luminal contents, increases the fugacity of lipophilic xenobiotics from the intestinal lumen. This action facilitates the movement of lipophilic contaminants to the intestinal mucosa and ultimately the systemic circulation. A second model proposes that lipophilic xenobiotics are co-assimilated from the gastrointestinal tract in direct association with dietary lipids (Vetter et al., 1985). These models suggest that dietary uptake of xenobiotics against an apparent concentration gradient, as with biomagnification, occurs in the consumer
at either the level of the intestine (model 1) or the tissues of deposition, with the latter occurring largely as a result of lipid incorporation or utilization (model 2).

PCB bioavailability along the food chain can be modulated by biotransformation. In general, higher trophic levels exhibit PCB patterns enriched in highly chlorinated congeners (Boon et al., 1989; Muir et al., 1988). PCBs with unsubstituted adjacent para and meta positions, and congeners with unsubstituted adjacent ortho and meta positions and less than one orthochlorine are more readily biotransformed by oxidative systems such as cytochrome P450 as compared to PCBs with other characteristics (Boon et al., 1992; Walker, 1992). Metabolic activity limits the availability of these lesser chlorinated compounds to higher trophic levels as a result of enhanced elimination by lower organisms. Recent \textit{in vitro} and \textit{in situ} studies also suggest that intestinal biotransformation of dietary xenobiotics by the consuming fish can appreciably alter chemical form and systemic bioavailability (Kleinow et al., 1998; Van Veld, 1990). Fish intestinal CYP1A activity, inducible by a variety of Ah agonists (James et al., 1997; Kleinow et al., 1998; Van Veld et al., 1987; 1988a,b; 1991), may approach or even exceed concurrent hepatic levels following dietary exposure, when examined on a per mg protein basis (James et al., 1997; Van Veld et al., 1988b; 1991). The relative activity of oxidative pathways in the intestine has been shown to influence the degree of metabolism and composition of absorbed xenobiotics. Similarly, phase II biotransformation pathways in the fish intestinal mucosa have been shown to be operative in the further modification of dietary xenobiotics, with dose and phase I activity as important determinants of phase II contributions (Kleinow et al., 1998).
3,4,3',4'-Tetrachlorobiphenyl (TCB), a coplanar PCB, is abundant in environmental samples and animal tissues (McFarland and Clarke, 1989). The presence of TCB in higher organisms suggests that the compound may be subject to the foregoing dietary processes. While not much is known regarding the fate of TCBs in the fish gastrointestinal tract, TCB has been shown to be an inducer of hepatic CYP1A in fish (Gooch et al., 1989; Lindstrom-Seppa et al., 1994; Monosson and Stegeman, 1991; Otto et al., 1997; Sleiderink and Boon, 1996; White et al., 1997a; Wirgin et al., 1992) and a substrate for fish hepatic CYP1A metabolism (White et al., 1997b). The induction of fish hepatic CYP1A by TCB has been shown to occur in a dose-dependent fashion in the low dosage range, with increases in CYP1A catalytic activity, as well as CYP1A transcription and protein expression both in vitro and in vivo (Gooch et al., 1989; Hahn et al., 1993; Lindstrom-Seppa et al., 1994). In contrast, high TCB concentrations have been reported to elicit an inhibitory effect upon hepatic CYP1A catalytic activity (Hahn et al., 1993; Monosson and Stegeman, 1991; White et al., 1997a).

The following studies examined [14C]-TCB bioavailability from the catfish intestine with alteration of 3 factors: luminal lipid composition, existing TCB body burdens and intestinal biotransformation. The specific objectives of this study were to examine the effect of micelle fatty acid composition upon the solubility of TCB in micelles, the effect of micelle composition on TCB intestinal bioavailability in an in situ preparation, the effect of a 10-day in vivo TCB exposure (0.5 or 5 mg TCB/kg diet) upon the bioavailability and biotransformation of subsequent [14C]-TCB
exposures using an *in situ* intestinal preparation, the effect of various TCB dosages upon CYP1A content and activity in the intestine, and the effect of TCB dietary exposure upon intestinal morphology and integrity.

**MATERIALS AND METHODS**

**CHEMICALS**

Radiolabeled (27.3 mCi/mM) $[^{14}\text{C}]-3,4,3',4'$-tetrachlorobiphenyl (TCB) (98% isomer specific) was graciously supplied by H.B. Matthews, NIEHS, Research Triangle Park, NC. Nonradiolabelled TCB was purchased from ChemService Inc., West Chester, PA. The standards for the known hydroxylated TCB metabolites were generously donated by Dr. L. W. Robertson, University of Kentucky, Lexington, KY. Tricane methane sulfonate (MS-222™) was obtained from Argent Chemical Company, Redmond, WA. TS-1™ tissue solubilizer was procured from Research Products International, Mount Prospect, IL. All other chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

**IN VITRO TCB SOLUBILITY IN MICELLES**

TCB solubility in micelles was examined *in vitro* in micelles of differing fatty acid composition using a modification of previously described methods (Hollander and Rim, 1978). The various fatty acids selected were forms commonly found in tissues of catfish held at 20°C as determined by previous assays (Toth et al., 1993). Micelle mixtures (1 ml) were formulated at 40°C, by gentle mixing of 0.9% saline, 10 mM sodium taurocholate (bile salt for fatty acid emulsification), plus 10 mM of either one or a combination of 4 fatty acids (each exhibiting different chain lengths and
degrees of saturation): (a) lauric acid (12:0); (b) myristic acid (14:0); (c) linoleic acid (18:2); and (d) a mixture of equal concentrations (2.5 mM) of myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), and linoleic acid (18:2). Micelle production using these methods was confirmed by optical clarity over time and filtration.

TCB and $[^{14}\text{C}]-\text{TCB}$ (0.05 μCi) (20 μM total) in a toluene carrier (50 μl) was added on top of 0.9% saline (1 ml) in a conical glass tube. The solvent carrier was slowly evaporated under nitrogen until only a thin surface layer of toluene with the TCB remained. This step removed excess toluene, which would otherwise precipitate the micelle mixture. Warm micelles (1 ml) as formulated above were slowly added below the surface of the toluene, allowing the micelle mixture contact with the underside of the toluene layer by temperature driven convection. TCB was allowed to partition into the micelles, as the remaining toluene was evaporated with nitrogen. Following complete evaporation of the toluene layer, the solution was rigorously gassed with nitrogen to remove any remaining toluene and then shaken. Due to the nature of the micelle mixture (micelles in aqueous saline) the hydrophobic TCB not associated with the micelles was partitioned by hydrophobic interactions to the edge of the aqueous phase onto the glass. Relative $[^{14}\text{C}]-\text{TCB}$ content in micelles of each treatment was measured by liquid scintillation counting of timed samples using 3 replicates (100 μl) from each micelle solution. Total TCB remaining in the micelle solution was expressed as a percentage of the total dose delivered.
IN SITU STUDIES

Animals

Channel catfish (*Ictalurus punctatus*) of either sex (1,379 ± 319 g) were obtained from the Louisiana State University Aquaculture Research Station at Baton Rouge, LA. Fish were kept on a 12-h light/dark photoperiod, in a flow through system supplied with dechlorinated tap water (pH 8.1 ± 0.2, temperature 19.4 ± 1.4°C, total hardness 27.1 ± 7.0 mg CaCO$_3$/l, alkalinity 114.3 ± 12.9 mg/l). All animals were acclimated to experimental conditions at least 2 weeks prior to use. Animals were fed a custom made, purified, semisynthetic catfish diet (Dyets Inc., Bethlehem, PA) composed of casein 32%, dextrin 29.8%, cellulose 19%, soybean oil 3%, Menhaden oil 3%, gelatin 8%, salt and vitamin mix 5%, and choline chloride 0.2%.

In Situ Intestinal Perusions

Catfish of both sexes were used for surgery and the *in situ* intestinal preparation. The first experiment examined the effect of micelle carrier composition on [14C]-TCB bioavailability in an *in situ* intestinal preparation of control animals. Following *in situ* intestinal preparation [14C]-TCB (60 μM) was delivered into the intestinal lumen in a micelle carrier composed of either (1) 2.5 mM monolauroyl-rac-glycerol (a common mono-acyl-glycerol which is an incomplete lipolysis product), 10 mM sodium taurocholate, and the mixture of 2.5 mM myristic acid, 2.5 mM palmitic acid, 2.5 mM stearic acid and 2.5 mM linoleic acid (n = 3), or (2) 2.5 mM monolauroyl-rac-glycerol, 10 mM sodium taurocholate, and 10 mM linoleic acid (n = 5). In a second experiment designed to examine the effect of TCB preexposure upon
subsequent $[^{14}\text{C}]-\text{TCB}$ intestinal bioavailability and metabolism, animals were exposed 10 days prior to the \textit{in situ} preparation to control diets ($n = 3$) or diets containing nonradiolabeled TCB at 0.5 mg ($n = 6$), and 5 mg TCB/kg diet ($n = 7$). For all these treatments, $[^{14}\text{C}]-\text{TCB}$ (60 $\mu$M) was delivered in the \textit{in situ} preparation, using the 10 mM linoleic acid micelle.

For the respective treatments of both experiments, control animals were maintained on the semisynthetic diet coated with corn oil (1 ml corn oil/100 g of diet) while for the second experiment TCB was delivered in corn oil applied as a coating on the semisynthetic diet (1 ml corn oil/100 g of diet). Both dietary groups (control and dietary TCB exposure) were maintained on designated experimental diets at 0.5% of fish body weight/day for 10 days prior to the \textit{in situ} intestinal preparation. Fish were fasted 24 hours prior to experimental manipulation.

\textit{In Situ} $[^{14}\text{C}]-\text{TCB}$ Dose Preparation

The micelle solution of defined composition used for intraluminal $[^{14}\text{C}]-\text{TCB}$ dosing of the intestinal preparation was formulated fresh daily as described in the \textit{in vitro} section. Micelle compositions containing 60 $\mu$M of $[^{14}\text{C}]-\text{TCB}$ (27.3 mCi/mmol) were formulated to a final volume of 3 ml.

Surgical Procedure and Dosing

Surgical preparations were performed as previously described (Kleinow et al., 1998). MS-222 buffered with NaHCO$_3^-$ was used for anesthesia at induction and maintenance dosages of 106 and 86 mg/l, respectively. The intestine was exteriorized and a ventral loop of proximal intestine of approximately 20 cm in length was selected.
for the preparation. Vessels perfusing the selected intestinal segment, including a branch of the coeliacomesenteric artery as the afferent (supply) vessel, the corresponding efferent (drainage) vessel and potential collateral vessels were identified and isolated. The afferent vessel was cannulated using a PE-50 tubing filled with saline treated with citrate anticoagulant. Once in place, oxygenated blood containing citrate anticoagulant was pumped into the afferent cannula at a flow rate of 0.1 ml/min. PE-60 tubing was used to cannulate the efferent vessel and collateral vessels were tied off. Integrity of the perfusion was verified through measurement of efferent blood volumes. Upon completion of the circuit the intestinal segment was transiently blanched with a small amount of saline containing citrate anticoagulant via the afferent cannula, in order to demarcate the length of intestine perfused by the preparation. Ligatures were placed on the intestine at the borders of the perfused region. Delivery of the micelle solution was performed by inserting the needle of the dosing syringe through the gut wall, securing the preplaced ligature over the needle and injecting the [14C]-TCB micellar dose into a closed intestinal segment. The injected dose was then massaged to allow distribution throughout the segment, and the time was then initiated.

**Sample Collection**

Throughout the 60-min perfusion, the intestine was moistened with saline, the segment periodically massaged, and a composite blood sample collected. After the 60-min perfusion, the isolated, ligated intestinal segment was drained and the postinfusate collected. Aliquots of the postinfusate were taken for liquid scintillation counting and
metabolite analysis. The intestinal segment was then cut open, washed copiously with ice cold saline and blotted dry. The mucosal layer was collected by scraping with a glass slide. Aliquots of weighed mucosal tissue were taken for liquid scintillation counting, metabolite analysis, and preparations for P450 content and activity. Liver, kidney, and anesthetic water samples were collected and counted for radioactivity, to verify, by the lack of counts, the integrity of the isolated preparation.

Blood, liver, kidney, mucosa and postinfusate samples (approximately 50 mg) were digested at 50°C in 0.5 ml of TS-1 Tissue Solubilizer for 24 h, neutralized with 18 µl of glacial acetic acid and counted in 4.5 ml of scintillant (Ultima Gold, Packard, Downers Grove, IL). Water and preinfusate samples were not digested prior to counting. Corrections for counting efficiency (~ 95%) and background were utilized for all samples. Preinfusate, postinfusate, intestinal mucosa, and blood sampled for metabolite analysis were snap frozen with liquid nitrogen, wrapped in foil, and kept at -80°C until analysis. Mucosal samples for P450 related analysis were suspended in buffer consisting of 0.25 M sucrose, 0.1 mM EDTA, 0.05 M Tris-HCl, pH 7.4, 0.1 mM phenylmethyl sulfonyl fluoride (PMSF), and similarly frozen.

**Enzyme Assays and P450 Content**

Mucosal samples were weighed and homogenized in 4 volumes of buffer (0.25 M sucrose, 5 mM EDTA, 0.05 M Tris-HCl, pH 7.4, 0.2 mM PMSF). Microsomal and cytosolic fractions were prepared using the procedure described by James and Little (1983). Total P450 content of microsomes was measured following the method of Estabrook et al. (1972), using a Shimadzu 265 spectrophotometer, as previously
described (James et al., 1988). Monooxygenase activity was measured by a fluorescence assay (AHH) of phenolic BaP metabolites (Nebert, 1978).

**Western Blot Analysis**

Microsomal protein fractions (40 µg for intestine, 20 µg for liver), incubated in sample buffer as recommended by BioRad, were resolved in a mini gel format (BioRad) on 4% stacking gel with 8.5% resolving gels, as described by Laemmli (1970). Unstained and prestained molecular weight standards in the range of 14,400 to 97,000 (BioRad low molecular weight range) were resolved at the same time as the SDS-treated microsomes. Gentest Supersomes™ expressing rat CYP1A were used to develop a standard curve for quantitation of the antibody response. Electrophoresis was carried out using a 25 mM Tris, 192 mM glycine, 0.10% SDS buffer at constant voltage of 200V. Western blot transfer of protein to nitrocellulose was performed as described by Towbin et al. (1979). The transfer was carried out at 100V in a mini Transblot system (BioRad) using a 25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3 transfer buffer. The remaining gel was stained with Coomassie blue as an indication of transfer effectiveness.

Immunodetection was carried out using monoclonal antibodies to scup CYP1A (courtesy of Dr. J. J. Stegeman). Transblotted nitrocellulose was rinsed in a 20 mM Tris, 500 mM NaCl, pH 7.5 buffer and nonspecific binding sites blocked with 5% (w/v) dried milk in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20 for 45 min. The membrane was washed 4 times with 20 mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.5 buffer. The primary antibody, diluted 1:10,000 in 5% (w/v) dried
milk in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20, was incubated
with the nitrocellulose for 2 h. The unbound antibodies were washed away in 20 mM
Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20 buffer and further incubated with a
1:1,000 dilution of secondary antibody in blocking agent (rabbit anti-rat antibody
conjugated to horseradish peroxidase) for 1 h. After washing with 20 mM Tris-HCl,
pH 7.5, 500 mM NaCl, 0.05% Tween 20, the immunoreactive proteins were detected
according to the Amersham Western Blotting kit for chemiluminescent detection and
the protein bands were visualized by fluorography on Kodak X-OMAT AR films.
Fluorograms were subsequently scanned and the protein bands were quantified by
scan-analysis densitometry.

**Metabolic Studies**

Samples of blood and postinfusate (1 ml) were extracted 3 times with (3 ml)
heptane:ethanol, 19:1, and the three extracts pooled. Intestinal mucosa (0.3 to 0.6 g)
was homogenized with 1 ml distilled water and extracted 3 times with (3 ml)
heptane:ethanol, 19:1. For each sample, the pooled heptane:ethanol extracts were dried
over anhydrous sodium sulfate, evaporated to dryness under nitrogen, and stored at -
80°C until HPLC analysis. Before analysis, the residue was dissolved in 0.1 ml 78%
methanol:water, the solution filtered (0.45 µm nylon filter) and 50 µl injected onto a
pre-equilibrated C18 reverse phase HPLC column. Initial HPLC conditions at a flow
rate of 1 ml/min were 78% methanol:22% water (held for 20 min) followed by a
gradient to 100% methanol over 5 min (held for 10 min), and then returned over 5 min
to 78% methanol. Analysis was accomplished using UV (262 nm) and radiochemical
detection (INUS detector). Under these conditions, baseline separation of the known TCB metabolites was achieved. 2-OH-3,4,3',4'-TCB eluted at 17.7 min, 4-OH-3,5,3',4'-TCB at 18.2 min, 5-OH-3,4,3',4'-TCB at 18.7 min, 6-OH-3,4,3',4'-TCB at 19.6 min, and 3,4,3',4'-TCB eluted at 22.6 min.

HISTOLOGICAL STUDIES

To assess if any morphological changes were induced in the intestine with TCB exposure, catfish (n = 18) were exposed to TCB following the same protocol as used for the in situ preparation. Animals were divided into 3 equal groups: control, 10-day dietary TCB exposure at 0.5 mg/kg diet, and 10-day dietary TCB exposure at 5 mg/kg diet. Following exposure, intestines were harvested, rinsed with ice cold 0.9% saline, and samples of proximal and distal sections collected for histological preparation. Samples were fixed in 10% formalin for at least 24 h, and 4 μm sections stained with hematoxylin-eosin using conventional techniques.

STATISTICAL ANALYSIS

Data analysis was completed using the statistical software SigmaStat (Windows, version 1.0, Jandal Corporation, San Rafael, CA). One way ANOVA, Student’s t-test, or corresponding nonparametric tests were used to examine for significant differences at \( p < 0.05 \). Multiple comparisons (Dunnet’s or Student-Newman-Keuls) were used when ANOVA demonstrated significant differences.

RESULTS

\(^{14}\text{C}\)-TCB carrying capacity of micelles varied, under in vitro conditions, with the fatty acid acyl side chain composition (Figure 2.1). As the fatty acid chain length
Figure 2.1 - Effect of micelle fatty acid composition on mean $[^{14}\text{C}]-3,4,3',4'-\text{tetra-chlorobiphenyl}$ (TCB) solubility. Results shown as a percentage of a 20 μM TCB dose incorporated into micelle carriers. Micelles were composed of 0.9% saline, 10 mM sodium taurocholate, plus 10 mM fatty acids: (a) lauric acid (12:0), (b) myristic acid (14:0), (c) linoleic acid (18:2), and (d) an equal mixture of myristic (14:0), palmitic (16:0), stearic (18:0), and linoleic (18:2) acids. Similar letters indicate significant differences ($p < 0.05$) (mean ± SD), ($n = 3$).
increased from C12 to C18, there was a trend towards increased $[^{14}\text{C}]-\text{TCB}$ solubility. $[^{14}\text{C}]-\text{TCB}$ mean solubilities were significantly higher (2.6-fold) in linoleic acid (18:2) compared to lauric acid (12:0) micelles (ANOVA, Student Newman-Keuls: $p < 0.05$), while myristic acid (14:0) micelles showed an intermediate solubility, not significantly different from the other 2 (ANOVA, Student Newman-Keuls: $p < 0.05$). Mixed micelles composed of equal amounts of myristic (14:0), palmitic (16:0), stearic (18:0), and linoleic (18:2) acids, exhibited a 3 to 7.6-fold lower $[^{14}\text{C}]-\text{TCB}$ solubility when compared to micelles composed of the individual fatty acids.

The influence of $[^{14}\text{C}]-\text{TCB}$ micelle solubilization upon systemic bioavailability was examined in the \textit{in situ} intestinal preparation using the micelle compositions exhibiting the greatest (linoleic acid) and least efficient $[^{14}\text{C}]-\text{TCB}$ solubilization (mixture). With equivalent $[^{14}\text{C}]-\text{TCB}$ dose and preparation techniques, linoleic acid micelles facilitated a significantly greater systemic $[^{14}\text{C}]-\text{TCB}$ bioavailability than the mixed micelle solution (Figure 2.2). The mean systemic bioavailability with linoleic acid micelles (895.9 ± 217.9 pmol $[^{14}\text{C}]-\text{TCB}/\text{ml blood}$) was approximately 2.2-fold higher than obtained with mixed micelle solution (401.9 ± 334.5 pmol $[^{14}\text{C}]-\text{TCB}/\text{ml blood}$) ($t$-test: $p < 0.05$).

TCB at both the 0.5 and 5mg/kg dosages when administered in the diet for 10 days significantly decreased subsequent $[^{14}\text{C}]-\text{TCB}$ systemic bioavailability to the blood of the \textit{in situ} preparation (Figure 2.3) (ANOVA, Student Newman-Keuls: $p < 0.05$). When compared to the control animals (895.9 ± 217.9 pmol $[^{14}\text{C}]-\text{TCB}/\text{ml of blood}$), TCB at 0.5 and 5 mg TCB/kg diets decreased blood concentrations of

53
Figure 2.2 - Scatter graph showing the concentration of $[^{14}\text{C}]-3,4,3',4'-\text{tetrachlorobiphenyl}$ (TCB) in the blood of in situ-isolated, perfused intestine of vehicle-treated (control) catfish with varying micelle carrier composition. Micelles were composed of 0.9% saline, 2.5 mM monooleoyl-rac-glycerol, 10 mM sodium taurocholate, and 10 mM of either an equal mixture of myristic, palmitic, stearic, and linoleic acids ($n = 3$), or linoleic acid alone ($n = 5$). $[^{14}\text{C}]-\text{TCB}$ systemic bioavailability was significantly greater with the linoleic acid micelles ($p < 0.05$).
Figure 2.3 - Scatter graph showing the concentration of [14C]-3,4,3',4'-tetrachlorobiphenyl (TCB) in the blood, mucosa and postinfusate of in situ-isolated, perfused intestines of individual vehicle-treated controls (n = 5) and 10-day TCB-preexposed catfish, dosed at either 0.5 mg TCB/kg diet (n = 6), or 5 mg TCB/kg diet (n = 7). Linoleic acid micelles were used for all treatments. Controls exhibited significantly greater [14C]-TCB concentrations in the blood compared to TCB pretreatments (p < 0.05).
[\(^{14}\text{C}\)]-TCB 47% (475.7 ± 134.4 pmol/ml of blood) and 45% (495.6 ± 160.2 pmol/ml of blood), respectively. There was no significant interdose effect of TCB dietary preexposure upon the intestinal systemic bioavailability of [\(^{14}\text{C}\)]-TCB.

[\(^{14}\text{C}\)]-TCB concentrations in intestinal mucosa, following the in situ perfusion, did not change with the increasing in vivo doses of the TCB preexposure (Figure 2.3). Mucosal [\(^{14}\text{C}\)]-TCB levels of 16.2 ± 5.6, 15.6 ± 7.9, and 13.9 ± 5.5 nmol/g mucosa were observed for control, 0.5 mg TCB and 5 mg TCB/kg-diet exposure groups, respectively. Mean postinfusate [\(^{14}\text{C}\)]-TCB concentrations were 19.6 ± 6.3, 28.4 ± 17.0, and 30.7 ± 9.5 nmol [\(^{14}\text{C}\)]-TCB/ml for the same treatments (Figure 2.3). The effects of TCB preexposure upon [\(^{14}\text{C}\)]-TCB concentrations were not significant for either the mucosa or postinfusate (ANOVA: \(p < 0.05\)).

For all treatments, the highest concentrations of [\(^{14}\text{C}\)]-TCB on a per-unit basis were found in the postinfusate, followed by the mucosa and then the blood. Postinfusate [\(^{14}\text{C}\)]-TCB concentrations were approximately 1.2, 1.8, and 2.2 times higher than mucosa concentrations in the controls, and animals preexposed to 0.5 and 5 mg TCB/kg diet, respectively. Mucosa concentrations as compared to blood were 18.1, 28.1, and 32.9 times greater for the same treatments. Consistent over all treatments, the largest contributor to the gradient difference from the intestinal lumen to the blood was that evident between the mucosa and the blood. The gradient differences observed between the lumen and blood were the lowest for controls (21.9×) rising nearly 3-fold for TCB-pretreated animals (59.7 to 61.9×). These results reflect the higher blood and
lower postinfusate $[^{14}\text{C}]$-TCB concentrations for the controls and the lower blood and higher postinfusate $[^{14}\text{C}]$-TCB concentrations for TCB-pretreated animals.

Microsomal and cytosolic fractions produced by differential centrifugation of mucosa were examined for $[^{14}\text{C}]$-TCB radioactivity. As compared to the microsomes, lower amounts of radioactivity were associated with the cytosol on a per mg protein basis. The microsomal fractions exhibited similar amounts of $[^{14}\text{C}]$-TCB-derived radioactivity for all treatment groups (0.196 ± 0.039, 0.201 ± 0.60 and 0.236 ± 0.049 nmol $[^{14}\text{C}]$-TCB/mg microsomal protein for control, 0.5 mg and 5 mg TCB/kg diet treatments, respectively). Control treatment cytosolic radioactivity values (65.6 ± 23.6 pmol $[^{14}\text{C}]$-TCB/mg cytosolic protein) were significantly higher than those for either the 0.5 mg TCB/kg diet (32.4 ± 6.0 pmol $[^{14}\text{C}]$-TCB/mg cytosolic protein) or 5 mg TCB/kg diet (41.5 ± 14.6 pmol $[^{14}\text{C}]$-TCB/mg cytosolic protein) treatments (ANOVA: $p < 0.05$).

Total P450 content, CYP1A content, and aryl hydrocarbon hydroxylase (AHH) activities were measured, to assess the effect of TCB preexposure upon the metabolic capabilities of the catfish intestine. Mean P450 concentrations (between 0.07 and 0.09 nmol/mg protein) were not significantly altered by TCB pretreatment (Figure 2.4) (ANOVA: $p < 0.05$). CYP1A cross-reactivity was not detected for either the controls or animals of the 0.5 mg TCB/kg diet treatments. CYP1A levels were variable for the 5 mg TCB/kg diet treatment, with values ranging from 0.14 to 24.11 pmol/mg protein (Figure 2.5). Composite AHH activities (Figure 2.6) were $2.46 ± 1.16$, $2.43 ± 1.58$ and $11.35 ± 10.25$ pmol/min/mg protein for the control, 0.5 and 5 mg TCB/kg diets,
Figure 2.4 - Scatter graph showing the P450 content in intestinal microsomes from control catfish (n = 4) or animals exposed for 10 days to either 0.5 (n = 6) or 5 mg TCB/kg diet (n = 6).
Figure 2.5 - Western blot showing cross-reactivity of microsomes from individual fish treated with a 5 mg TCB/kg diet for 10 days against a monoclonal antibody to scup CYP1A. Lanes 1-7 are intestinal microsomes, 40 μg per lane, except lane 3 (20 μg). Lane 8 shows hepatic microsomes (20 μg) from the fish whose intestinal microsomes are shown in lane 7.
Figure 2.6 - Scatter graph of intestinal aryl hydrocarbon hydroxylase (AHH) activity in control catfish and catfish exposed for 10 days to TCB on either 0.5 or 5 mg TCB/kg diets.
respectively. AHH activities of the 5 mg/kg treatment were not significantly greater than controls or the 0.5 mg/kg diet treatments due to the high standard deviation of the data (ANOVA: \( p < 0.05 \)). Four animals demonstrated large increases (~ 7-fold) in AHH activities, while 3 animals exhibited levels similar to the controls. AHH activity exhibited a strong correlation \( (r^2 = 0.96) \) with CYP1A cross-reactivity \( (y = 1.143x + 1.026) \) (Figure 2.7).

TCB was metabolized to a small degree (0.05 to 1.3\%) in the intestinal mucosa of individual catfish in \textit{in situ} preparations (Table 1). Analysis of tissue sample extracts by HPLC demonstrated 4 minor peaks which eluted before the parent. Three of these peaks were tentatively identified as 2-OH-3,4,3',4'-TCB, 4-OH-3,5,3',4'-TCB and 5-OH-3,4,3',4'-TCB by co-elution with standards. The fourth peak eluted much earlier (12.1 min) but remains unidentified.

TCB elicited few morphological effects in the intestinal tract of the 0.5 mg/kg diet dosage level whereas at 5 mg/kg, demonstrable changes were evident when compared to the controls. Among the changes consistently present were a narrowing or thinning of the submucosa with submucosal separation a common feature (Figure 2.8). Goblet cell numbers were also increased along the mucosal folds of TCB-treated catfish.

**DISCUSSION**

The solubilization of \([^{14}C]\)-TCB in micelles, when varying the chain length and degree of saturation of the component fatty acids showed a significant 156\% increase in \([^{14}C]\)-TCB solubility for micelles containing linoleic acid (18:2) as compared to
Figure 2.7 - Intestinal CYP1A content and AHH activity. This graph shows that CYP1A content, pmol/mg microsomal protein correlated in a linear fashion ($r^2 = 0.98$, $p < 0.001$) with AHH activity in intestinal microsomes of catfish exposed to a 5 mg TCB/kg diet and subsequently used for in situ studies of TCB bioavailability. The CYP1A content was determined by cross-reactivity with a scup CYP1A monoclonal antibody and quantitated relative to a rat CYP1A standard curve.
Table 2.1 - Metabolites of $[^{14}C]$-3,4,3',4'-tetrachlorobiphenyl (TCB) found in blood, mucosa and postinfusate after *in situ* perfusion, for control and catfish preexposed to 0.5 and 5 mg TCB/kg diet for 10 days.

<table>
<thead>
<tr>
<th></th>
<th>% of total metabolites&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% of 2-OH-3,4,3',4'-TCB&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% of 4-OH-3,5,3',4'-TCB&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.22 ± 0.61</td>
<td>N.D. to 0.15</td>
<td>N.D. to 0.53</td>
</tr>
<tr>
<td>0.5 mg TCB/kg diet</td>
<td>1.01 ± 0.62</td>
<td>N.D. to 0.43</td>
<td>N.D. to 0.52</td>
</tr>
<tr>
<td>5 mg TCB/kg diet</td>
<td>1.06 ± 0.74</td>
<td>N.D. to 0.59</td>
<td>N.D.</td>
</tr>
<tr>
<td><strong>Mucosa</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.55 ± 0.56</td>
<td>N.D. to 0.23</td>
<td>N.D. to 0.05</td>
</tr>
<tr>
<td>0.5 mg TCB/kg diet</td>
<td>0.57 ± 0.32</td>
<td>N.D. to 0.41</td>
<td>N.D. to 0.02</td>
</tr>
<tr>
<td>5 mg TCB/kg diet</td>
<td>0.35 ± 0.33</td>
<td>N.D. to 0.20</td>
<td>N.D. to 0.03</td>
</tr>
<tr>
<td><strong>Postinfusate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.24 ± 0.21</td>
<td>N.D. to 0.12</td>
<td>N.D.</td>
</tr>
<tr>
<td>0.5 mg TCB/kg diet</td>
<td>0.59 ± 0.15</td>
<td>N.D. to 0.11</td>
<td>N.D. to 0.04</td>
</tr>
<tr>
<td>5 mg TCB/kg diet</td>
<td>0.51 ± 0.08</td>
<td>N.D. to 0.24</td>
<td>N.D. to 0.11</td>
</tr>
</tbody>
</table>

Note: Peaks eluted before the parent compound were assumed to be metabolites. N.D., not detected, < 0.005% for mucosa and postinfusate, < 0.05% for blood.

<sup>a</sup> Total of all extracted radioactivity that eluted before the parent compound.

<sup>b</sup> Peaks were tentatively identified by co-elution with standards.
Figure 2.8 - The proximal intestine of the channel catfish following a 10-day exposure to (A) vehicle controls (63x) or (B) 5 mg TCB/kg diet (110x). Note thinning and separation of submucosa (SM) and increased numbers of goblet cells (G) in mucosal folds of TCB-treated catfish.
lauric acid (12:0). An increase in solubilization of TCB was noted with increasing fatty acid chain length (linoleic acid 18:2 > myristic acid 14:0 > lauric acid 12:0). Studies with other compounds including vitamins (Takahashi and Underwood, 1974) and the PCB Aroclor 1242 (Laher and Barrowman, 1983) have demonstrated increased solubilization in micelles composed of long acyl side chain fatty acids. Laher and Barrowman (1983) demonstrated that Aroclor 1242 solubility in linoleic (18:2) and oleic acid (18:1) micelles was 146 and 85% higher, respectively, compared to octanoic acid (8:0). In this regard, TCB behaves in a manner similar to Aroclor 1242. The current studies suggest that TCB delivery to the intestinal wall and even, perhaps, retention in foodstuff lipid may be dependent upon the fatty acid composition. Stearic factors between the size of the micelle and the compound's molecular volume have been forwarded as a possible explanation for similar findings (Takahashi and Underwood, 1974). Mixed micelles in the current study appear to support a lower TCB carrying capacity than micelles composed of a single fatty acid type. The reasons for these findings are unknown; however, it appears likely that such a mixed composition influences the determinant stearic factors. Fatty acid composition may be an important consideration for fishes, as chain length and even saturation status may change seasonally with temperature and with diet.

In the proximal intestinal segment, as used in our in situ study, the systemic bioavailability of [14C]-TCB molar equivalents (Meq) (composite of [14C]-TCB parent compound and metabolites) was significantly higher (~ 123%) when delivery was accomplished using linoleic acid micelles as when compared to micelles composed of
a fatty acid mixture (myristic, palmitic, stearic, and linoleic acids). These results indicate that the differences in bioavailability of [\(^{14}\)C]-TCB Meq in the catfish intestine may be related to the differences in micelle fatty acid composition and the TCB solubility in those micelles. Studies in the killifish indicate that the presence of fat in the diet facilitates absorption of lipophilic contaminants (Van Veld, 1990). Likewise, the solubility of PCB, DDT and BaP in triglyceride was directly correlated to intestinal absorption efficiency (Van Veld, 1990). The current studies take this concept one step further by suggesting that micelle composition may play a role in the modulation of lipophilic xenobiotic delivery to or through the intestinal wall. One mechanism which may contribute to the apparent differences in [\(^{14}\)C]-TCB Meq absorption with differences in micelle composition is the rate- and site-dependent intestinal absorption of fatty acids. Ockner et al. (1972), comparing absorption of linoleic acid micelles with palmitic acid micelles (19.2 mM fatty acid, 9.6 mM glycercyl-1-monooleate, 20 mM sodium taurocholate) in rats, demonstrated a greater uptake of linoleic acid compared to palmitic acid (~37%) in the proximal intestine. In addition, these studies showed that linoleic acid was absorbed primarily by proximal intestine, and palmitic acid by distal intestine. Absorption of triolein in killifish has been shown to occur primarily in the proximal intestine (Honkanen et al., 1985).

Blood [\(^{14}\)C]-TCB Meq concentrations were diminished, postinfusate levels were elevated and mucosal concentrations remained about the same for TCB-pre-treated animals as compared to the controls in the in situ preparations. These trends in tracer distribution suggest that after TCB preexposure at either dose, the subsequent
[14C]-TCB Meq dosage was not as effectively absorbed. The systemic bioavailability of [14C]-TCB Meq in the 60-min in situ preparation of control catfish was 2.9% of the total dose delivered in the linoleic acid micelles. Catfish preexposed to 0.5 and 5 mg TCB/kg diet exhibited, under identical in situ exposure conditions, [14C]-TCB Meq bioavailabilities of 1.41 and 1.51%, respectively. In vivo PCB accumulation studies with catfish have demonstrated diminished uptake and rate of uptake following PCB preexposure in the diet (Hansen et al., 1976) or by the sediments (Dabrowska et al., 1996). Similarly, models by Barber et al. (1991) and Clark et al. (1990) have described decreases in PCB uptake with increases in body burden. The in situ character of the current studies indicate that the decreases in [14C]-TCB uptake with TCB preexposure occur on a direct and temporally acute basis at the level of the intestine. Dietary pretreatment with unlabeled TCB effectively altered the [14C]-TCB gradient established by the end of the in situ preparation. The composite postinfusate to blood [14C]-TCB gradients were approximately 21:1 for controls and 59.5 to 61.5:1 for TCB-pretreated animals. These changes with TCB pretreatment appeared to occur by a combined effect of lowering transport to the blood and retention of [14C]-TCB in the postinfusate. These changes in systemic [14C]-TCB uptake were evident in this isolated preparation, although mucosa [14C]-TCB levels were not significantly different. For all treatments, the greatest [14C]-TCB Meq gradients at the end of the perfusion were between the mucosa and the blood (18- to 33-fold) rather than between the postinfusate and the mucosa (1.2- to 2.2-fold). This observation probably reflects the relative lipid content of these compartments.
A variety of mechanisms could decrease the systemic bioavailability of $[^{14}\text{C}]$-TCB Meq with TCB preexposure. Diffusion, one of those mechanisms, is often cited as a major means for the intestinal transfer of uncharged lipophilic xenobiotics in fish (McKim and Nichols, 1994). TCB preexposure may increase TCB tissue loads, which would decrease the diffusion gradient available to $[^{14}\text{C}]$-TCB from the intestinal lumen to the systemic circulation. Unfortunately, unlabeled TCB residues were not measured to directly confirm this hypothesis. While lower blood $[^{14}\text{C}]$-TCB concentrations with TCB pretreatment are consistent with this hypothesis, a couple of pieces of information do not wholly fit. The first is the lack of a clear dose-response relationship for $[^{14}\text{C}]$-TCB at the 0.5 and 5 mg TCB/kg dosages. Another confounding feature is the observation that mean mucosa $[^{14}\text{C}]$-TCB concentrations were very similar for all treatments. It would appear logical that the gradient effects would be clearly seen with $[^{14}\text{C}]$-TCB in this intermediate compartment. An interesting observation, which may relate to these discrepancies, is the significantly lower intestinal mucosa cytosolic $[^{14}\text{C}]$-TCB Meq values with TCB pretreatment. This effect may be due to unlabeled TCB occupying or modifying sites on proteins or lipids necessary for transport of $[^{14}\text{C}]$-TCB through the cytosol. In concert with the diffusional process, such a mechanism could impair $[^{14}\text{C}]$-TCB Meq transport to the systemic circulation. A number of cytosolic moieties have been described as transporters for xenobiotics including glutathione S-transferase (Dixit et al., 1982), fatty acid binding proteins (Larsen et al., 1991), nuclear transfer proteins (Tierney et al., 1980) and lipoproteins (Vetter et al., 1985). The lack of a clear relationship of the total mucosal $[^{14}\text{C}]$-TCB
counts to the gradient effects seen in other compartments is puzzling. Perhaps it is a reflection of the phenomenon observed with the microsomal membranes whereby $[^{14}\text{C}]-\text{TCB Meq}$ levels were held constant across treatments. Plasma membranes and other structures removed in the first differential centrifugation accounted for a large part of the total composite mucosal $[^{14}\text{C}]-\text{TCB Meq}$ and may have demonstrated a similar effect hiding any response to TCB exposure. The mechanism and the significance of such a response will require more investigation.

A number of studies have demonstrated that exposure of animals, including fish, to certain organochlorines alters the distribution of a subsequent tracer dose of the same $[^{14}\text{C}]$ organochlorine (Carpenter and Curtis, 1989; 1991; Gilroy et al., 1993; compounds dieldrin and chlordecone). A suggestion has been made that these effects, independent of changes in xenobiotic metabolism or total body lipid content, may be related to lipid composition. Qualitative changes in lipids have been correlated with organochlorine exposure, including changes in fatty acid profiles, alterations in triglycerides and phospholipids (Hansell and Ecobichon, 1974; Ishikawa et al., 1978), and modifications of plasma lipoproteins (Ishikawa et al., 1978). Plasma lipoprotein binding has been shown to facilitate organochlorine transport (including PCBs) in a variety of animal species (including rat, trout, human) (Maliwal and Guthrie, 1982; Plack et al., 1979; Skalsky et al., 1979; Soine et al., 1982; Spindler-Vomachka and Vodicnik, 1984). Given the changes in disposition induced by other organochlorines, the possibility exists that TCB may elicit similar effects altering either the temporary storage or transport by the intestine itself or by the blood.
Other processes that may be influential in xenobiotic bioavailability across the intestine may be inducible by TCB pretreatment. P-glycoprotein, an efflux transporter, has been identified in the catfish intestine (Kleinow et al., 1996). This transporter would reduce bioavailability by the transport of absorbed compound from the intestinal mucosa back into the intestinal lumen. Such an action could account for the observed findings. There currently exist conflicting results in the literature regarding the suitability of PCBs for transport by P-glycoprotein in marine organisms (Galgani et al., 1996; Cornwall et al., 1995). Likewise, there are even contrasting responses relative to the inducibility of P-glycoprotein in the catfish intestine with TCB, dependent upon the assay methodology (Kleinow, unpublished data). Induction of binding proteins by Ah receptor agonists have also been shown to alter the disposition of organochlorines, such as TCDD, in the liver of mice (Poland et al., 1989). The significance of such mechanisms relative to these results can be only speculative at this time.

Since TCB has been shown to be an inducer of CYP1A and its associated activity in the fish liver, pretreatment with TCB may also alter intestinal [\(^{14}\text{C}\)-TCB Meq bioavailability by enhancing metabolism and the production of polar metabolites. Metabolism data included herein indicates, however, that [\(^{14}\text{C}\)-TCB was very recalcitrant to intestinal metabolism, with only small changes in the metabolite profile from TCB pretreatment. Lack of a correlation between changes in bioavailability and the metabolites formed further suggest that metabolism does not, or at best can only minimally, account for the observed differences.
It is plausible that the decrease in the systemic bioavailability of [\(^{14}\text{C}\)]-TCB may be related in part to a non-specific effect, caused by a toxic action of TCB pretreatment on the intestine. The 10-day dietary preexposure in the current study, which provided a TCB dose of approximately 25 \(\mu\)g TCB/kg body weight/day (5 mg TCB/kg diet at 0.5% body weight), resulted in a thinning of the submucosa and an increase in mucus secreting Goblet cells. An increase in mucus production could contribute to a thickening of the unstirred layer along the intestinal wall, thus increasing the diffusion distance and decreasing the rate of uptake. Similar effects are known to modulate nutritive and xenobiotic absorption in mammals (Kellaway and Marriot, 1975; Smithson et al., 1981). Considerable variation in the gastrointestinal toxicity of PCBs have been reported for fishes. A PCB mixture containing 2-chlorobiphenyl, 2,2'-dichlorobiphenyl, 2,5,2'-trichlorobiphenyl and 2,5,2',5'-tetrachlorobiphenyl (125 \(\mu\)g PCB/kg body wt/day for 28 days) elicited an increase in mucosal exfoliation, cytoplasmic inclusions in the columnar cells and a reduction or absence of the mucosa brush border in some areas of the chinook salmon intestine (Hawkes et al., 1980). In contrast, channel catfish fed 600 \(\mu\)g PCB (Aroclor 1242)/kg body weight/day for more than a 100-day period exhibited no histopathological changes in the gastrointestinal tract (Hansen et al., 1976). It is unclear whether differences between studies are related to the PCBs used, the dosage, or other factors.

Intestinal AHH activity and CYP1A content were not affected by the 0.5 mg TCB/kg-diet pretreatment and showed induction, but great variability, with the 5 mg TCB/kg diet. The consistency of AHH activities at near-control values for the 0.5 mg
TCB/kg-diet dose, and the lack of detectable CYP1A, suggests that this dosage may be insufficient to induce intestinal AHH activity when fed at approximately 0.5% of body weight. Conversely, detectable CYP1A content and AHH activities ranges from control levels to more than 12-fold higher with the 5 mg TCB/kg diet dosage indicate that TCB is capable of intestinal induction. Previous studies have demonstrated dose-dependent induction and inhibition of fish hepatic CYP1A catalytic activities by TCB with induction at low and high doses while inhibition occurs only at high dosages (Gooch et al., 1989; Lindstrom-Seppa et al., 1994; White et al., 1997a). The differential response seen in the catfish intestine is most likely differential induction resulting from factors associated with compound ingestion and bioavailability. Direct correlation of AHH activity with CYP1A content for individual animals suggest that the variability was not related to TCB inhibitory effects. Studies determining TCB bioavailability and local TCB concentrations in the intestine following dietary exposure will be required to elucidate the dosimetry and extent of induction and in turn the inhibitory effects of TCB in the catfish intestine.

$[^{14}C]$-TCB, as administered in the in situ intestinal preparation, was poorly metabolized during the 60-min perfusion. In controls, approximately 0.55% of $[^{14}C]$-TCB Meq in the mucosa were metabolites while 0.57 and 0.35% of the $[^{14}C]$-TCB Meq in the 0.5 and 5 mg TCB/kg diet treatments were metabolites. Intestinal $[^{14}C]$-TCB metabolism was highly variable between animals, but in all cases was quantitatively a minor event. Of the small amount of metabolites formed in the mucosa, the predominate from tentatively identified by co-elution with standards for both the
controls and TCB-pretreated animals was 2-OH-3,4,3',4'-TCB. 4-OH-3,5,3',4'-TCB was found as a metabolite of [14C]-TCB in only 3 of 10 TCB-pretreated catfish. The catfish intestine exhibited no changes in metabolite profile with dose. In contrast, TCB metabolites in scup (Stenotomus chrysops) bile demonstrated a differing profile with the magnitude of the TCB dosage (White et al., 1997b). At a dosage of 0.1 mg TCB/kg, 5-OH-3,4,3',4'-TCB was the major metabolite (85%) followed by 4-OH-3,5,3',4'-TCB (13%) and 2-OH-3,4,3',4'-TCB and 6-OH-3,4,3',4'-TCB (1% each). Bile of scup treated with 5 mg TCB/kg contained higher amounts of 4-OH-3,5,3',4'-TCB, comparable to the amounts of 5-OH-3,4,3',4'-TCB. White et al. (1997b), in their study with scup, hypothesized that high doses of TCB inhibited CYP1A allowing other CYP to produce greater amounts of 4-OH-3,5,3',4'-TCB through a rearrangement of a 4,5-epoxide. Results of the current study suggest that CYP1A is a minor constituent of the total CYP in the catfish intestine. The foregoing line of reasoning would suggest that the intestine should proportionally produce more 4-OH-3,5,3',4'-TCB rather than less. Further studies are needed to determine if the differences with the current study are organ, species or experimentally related.

These studies have demonstrated (1) that fatty acid composition has an appreciable effect on the micelle-carrying capacity and subsequent intestinal bioavailability of [14C]-TCB, (2) that a previous TCB exposure may reduce the subsequent bioavailability of [14C]-TCB to the systemic circulation (this effect is not related to first pass intestinal metabolism), and (3) that first pass intestinal metabolism of TCB is
a minor event with the production of small amounts of metabolites including 2-OH-3,4,3',4'-TCB, 4-OH-3,5,3',4'-TCB, and 5-OH-3,4,3',4'-TCB.

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CHAPTER 3: INTESTINAL BIOAVAILABILITY AND BiotRANSFORMATION OF 3,4,3',4'-TETRACHLOROBIPHENYL IN IN SITU PREPARATIONS OF CHANNEL CATFISH FOLLOWING DIETARY TREATMENT WITH β-NAPHTHOFLAVONE

INTRODUCTION

Polychlorinated biphenyls (PCBs) are anthropogenic contaminants commonly found in aquatic ecosystems (Kalmaz and Kalmaz, 1979; Fowler, 1990). Aquatic animals can accumulate PCBs through exposure to contaminated diet, water or sediments. Lesser chlorinated PCB congeners frequently undergo oxidative metabolism, and are eliminated by lower trophic level organisms. Highly chlorinated PCB congeners have been shown to accumulate, and biomagnify, in the various trophic levels of the aquatic food chain (Thomann and Connolly, 1984; Muir et al., 1988; Oliver and Niimi, 1988; Boon et al., 1989). It appears that the position of the halogen substitution in the different PCB congeners, as much as the degree of chlorination, determines the susceptibility of PCBs to biotransformation. Structure-activity relationships for the oxidative cytochrome P450 (CYP) metabolism of PCBs in aquatic species have been deduced from relative comparisons of congener contents in the various trophic levels (Boon et al., 1992; Walker, 1992). Studies in mammals have further related the chemical structure to the pathways of PCB metabolism. The presence of unsubstituted vicinal ortho-meta positions, with at least one meta or para substitution, have been shown to favor CYP1A metabolism in rat liver microsomes (Mills et al., 1985; Klasson Wehler et al., 1989). Ortho substitutions (non-coplanar) have been shown to promote CYP2B metabolism in similar tissues, especially in
combination with unsubstituted vicinal meta-para positions (Kaminsky et al., 1981; Mills et al., 1985; Ishida et al., 1991).

Information regarding the metabolism of individual PCB congeners in fish is sparse, and little is known regarding the contribution of the various tissues to the overall biotransformation of PCBs. *In vivo* studies examining PCB metabolites in bile and tissues, or monitoring of tissue depletion of parent compound (Sanborn et al., 1975; Melancon and Lech, 1976; Hinz and Matsumura, 1977; Niimi and Oliver, 1983; Boon et al., 1989; Brown, 1992; Elskus et al., 1994) provide little information regarding the organs involved, as well as rates and pathways of PCB metabolism. Recent studies with the coplanar PCB, 3,4,3',4'-tetrachlorobiphenyl (TCB), an abundant contaminant in the environment (McFarland and Clarke, 1989), suggest that TCB is metabolized to a substantial extent in the liver of scup (White et al., 1997). Hepatic biotransformation in this fish species was shown to vary with the TCB dose administered, and the TCB metabolite profile appears to be influenced by CYP1A content and activity, indicating that TCB may be a substrate for CYP1A (White et al., 1997).

Intestinal CYP1A biotransformation has been demonstrated in a number of fish species following exposure to dietary Ah receptor agonists (Van Veld et al., 1988b; James et al., 1997). Studies with [3H]-benzo(a)pyrene (BaP) suggest that induction of intestinal biotransformation by such agonists may influence the profile of BaP derived compounds absorbed into the systemic circulation by the fish (Van Veld et al., 1988a; Kleinow et al., 1998). In contrast, recent *in situ* studies examining [14C]-TCB

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metabolism in the intestine of catfish have demonstrated little correlation between the
extent of metabolism or the profile of metabolites produced with induction of CYP1A
related activity (Doi et al., 2000). Likewise, there was little correlation of these factors
with apparent changes in $[^{14}\text{C}]$-TCB bioavailability following TCB in vivo
preexposure and induction with TCB (Doi et al., 2000). Non-specific toxicity and
changes in diffusion gradients (as a result of increased TCB burden) were suggested as
possible operative features in altered bioavailability (Kleinow et al., 1998; Doi et al.,
2000).

The objectives of the present study were to examine the effects of in vivo
dietary BNF exposure, on the intestinal bioavailability and biotransformation of $[^{14}\text{C}]$-
TCB, to further examine these issues. BNF was chosen for its known dose-dependent
CYP1A inducing abilities in a number of fish species (Melancon et al., 1981;
Andersson et al., 1985; Tate, 1988; Kloepper-Sams and Stegeman, 1989; James et al.,
1997), as well as its independence relative to TCB diffusion gradients. Histological
studies were performed to examine the correlation between BNF related structural
changes and modulation of $[^{14}\text{C}]$-TCB bioavailability. For this purpose, dietary BNF
was administered to catfish, and its effects on intestinal oxidative metabolism and
organ morphology were examined in context with changes in $[^{14}\text{C}]$-TCB systemic
bioavailability and metabolite profile. Control catfish and animals exposed in vivo for
10 days to different doses of dietary BNF (10 and 50 mg BNF/kg diet) were used in an
in situ perfused intestinal preparation.
MATERIALS AND METHODS

CHEMICALS

Radiolabelled (27.3 mCi/mM) $[^{14}C]$-3,4,3',4'-tetrachlorobiphenyl (TCB) was generously provided by H. B. Matthews, NIEHS, Research Triangle Park, NC. Standards for TCB hydroxylated metabolites (2-OH-3,4,3',4'-TCB, 4-OH-3,5,3',4'-TCB, 5-OH-3,4,3',4'-TCB, and 6-OH-3,4,3',4'-TCB) were a courtesy of Dr. L. W. Robertson, University of Kentucky, Lexington, KY. Tricaine methane sulfonate (MS-222™) was purchased from Argent Chemical Company, Redmond, WA. TS-2™ tissue solubilizer was supplied by Research Products International, Mount Prospect, IL. All other chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

ANIMALS

Male and female channel catfish (*Ictalurus punctatus*) were obtained from the Louisiana State University Aquaculture Research Station, Baton Rouge, LA. Fish (1,308 ± 345 g) were acclimatized to experimental conditions for at least 2 weeks prior to dosing. All animals were maintained on a 12 h light/dark photoperiod, in a dechlorinated tap water flow through system (pH 8.1 ± 0.2, temperature 19.7 ± 1.3°C, total hardness 24.4 ± 4.3 mg CaCO$_3$/l, alkalinity 111.7 ± 10.9 mg/l). A custom made semi-synthetic diet (Dyets Inc., Bethlehem, PA), composed of casein 32%, dextrin 29.8%, cellulose 19%, soybean oil 3%, Menhaden oil 3%, gelatin 8%, salt and vitamin mix 5%, and choline chloride 0.2%, was fed to animals throughout the acclimation period, and was used as the base diet in experimental treatments.
IN SITU PROTOCOL

Experimental Diets

Three dietary treatments were administered to separate groups of catfish, in order to examine the effects of BNF pretreatment upon the intestinal bioavailability and biotransformation of subsequent [\(^{14}\text{C}\)]-TCB exposures. Experimental diets were prepared by coating semi-synthetic diet with pure corn oil (1 ml corn oil/100 g diet) or corn oil containing BNF (1 or 5 mg BNF/1 ml corn oil/100 g diet). Control animals received semi-synthetic diet with corn oil vehicle (n = 5), while BNF-treated groups received either the 10 mg BNF/kg diet (n = 3), or 50 mg BNF/kg diet (n = 4). Diets were fed ad libitum, at 0.5% body weight, for 10 days prior to in situ intestinal preparations. All animals were fasted 24 h prior to surgery.

\[^{14}\text{C}\]-TCB Dose Preparation

A micelle solution (final volume of 3 ml) containing radiolabeled TCB was prepared according to previously described methods (Doi et al., 2000), and used for dose delivery into the in situ isolated perfused intestine. The constituents of the micelles were selected to allow for TCB solubility, as determined in previous studies (Doi et al., 2000). Micelles were formulated with 2.5 mM of monolauroyl-rac-glycerol, a product of incomplete lipolysis, 10 mM of the bile salt sodium taurocholate (used for lipid emulsification), and 10 mM of linoleic acid, a common constituent of catfish tissues (Toth et al., 1993). All constituents were added to 1.5 ml of 0.9% saline and gently stirred at 40°C. 60 mM of \[^{14}\text{C}\]-TCB (27.3 mCi/mM) in toluene carrier was loaded on top of 1.5 ml of 0.9% saline, and slowly evaporated under nitrogen until a
surface film of TCB in toluene remained. A fresh micelle mixture (1.5 ml) was slowly added below the toluene surface film, and the remaining toluene evaporated with nitrogen, facilitating the transfer of $[^{14}\text{C}]-\text{TCB}$ to the micelle solution. The micelles were then carefully gassed with nitrogen to ensure that no toluene remained, and gently vortexed. Liquid scintillation counting (LSC) of $[^{14}\text{C}]$ content (2 μl) was utilized to ensure proper dose formulation.

**Surgical Procedure and Dose Administration**

The surgical preparation of the intestinal segment and compound dosing were performed as described in detail in Kleinow et al. (1998). Animals were anesthetized (buffered MS-222, 106 and 86 mg/l for induction and maintenance, respectively), and a ventral loop of proximal intestine (~ 20 cm) isolated for the *in situ* preparation. After identification and isolation of the appropriate vessels, the afferent vessel (a branch of the coeliacomesenteric artery) was cannulated (PE-50 tubing) and connected to a syringe filled with blood containing citrate anticoagulant. Syringe was then connected to a pump and blood perfused at a flow rate of 0.1 ml/min. The corresponding efferent vessel was then cannulated using a PE-60 tubing, and all collateral vessels ligated. The final demarcation of the intestinal segment perfused by the selected vasculature was performed by blanching the segment through the afferent vessel with saline with citrate anticoagulant, followed by placement of ligatures at the proximal and distal borders of the demarcated segment. The distal ligature was securely tied and the radiolabelled $[^{14}\text{C}]-\text{TCB}$ dose (60 μM) in the 3 ml micelle solution delivered, by inserting the needle of the dosing syringe through the intestinal wall, into the isolated
intestinal segment, under the preplaced proximal ligature. The ligature was then tied over the needle and the micellar dose injected into the isolated segment. The dose was massaged throughout the segment to ensure complete distribution. The vascular integrity of the preparation was verified by monitoring the blood outflow, to ensure that all blood perfused was recovered.

Sample Collection

The isolated intestinal segment was perfused with blood for 60 min, following radiolabelled dose administration. A composite blood sample (one aliquot every 10 min) was collected during the 1-h perfusion for LSC, and the remaining perfused blood used for metabolite analysis. At the end of the experimental period, the perfused segment was harvested, the intestinal contents (postinfusate) were drained and collected. The intestine was then cut open, profusely washed with cold saline, blotted dry, and the mucosa harvested by scraping with a glass slide. Postinfusate and mucosa samples were aliquotted for metabolite analysis and LSC. Samples for metabolite analysis (blood, postinfusate and mucosa) were snap frozen in liquid nitrogen and stored at -80°C until analysis. Mucosa samples aliquotted for P450 analysis were similarly frozen and stored following suspension in storage buffer (0.25 M sucrose, 0.1 mM EDTA, 0.05 M Tris-HCl, pH 7.4, 0.1 mM phenylmethylsulfonyl fluoride). Samples of liver, kidney and anesthetic water were collected for LSC to verify the integrity of the intestinal preparation (in properly isolated preparations, these samples display no radioactivity). Samples for LSC were digested at 50°C in 0.5 ml of TS-2 tissue solubilizer for 24 h (with the exception of water), neutralized with 18 μl of
acetic acid and counted in 4.5 ml of scintillant (Optima Gold, Packard, Downers Grove, IL). Corrections for counting efficiency (~ 95%) and background were performed for all samples.

**Enzyme Assays and P450 Content**

Mucosa samples were weighed and homogenized in 4 volumes of buffer (0.25 M sucrose, 5 mM EDTA, 0.05 M Tris-HCl, pH 7.4, 0.2 mM PMSF). Microsomes and cytosol were obtained by differential centrifugation as described in James and Little (1983). Total cytochrome P450 content was determined by spectrophotometry following the method of Estabrook et al. (1972), using a Shimadzu 265 spectrophotometer (James et al., 1988). Aryl hydrocarbon hydroxylase (AHH) activity was measured by the fluorescence assay of phenolic BaP metabolites (Nebert, 1978).

**TCB Metabolite Analysis**

Samples of blood, mucosa and postinfusate were extracted 3 times with heptane:ethanol, 19:1, dried over anhydrous sodium sulfate, and evaporated to dryness under nitrogen. Extracts were re-dissolved in 78% methanol:water, filtered through a 0.45 μm nylon membrane and injected (50 μl) onto a C18 reverse-phase HPLC column. Gradient separation was performed using 78% methanol: 22% water (20 min), 100 % methanol (5 min), and 78% methanol: 22% water (5 min), at a 1 ml/min flow rate. Detection used UV (262 nm) and radiochemical means (INUS detector). TCB metabolites were tentatively identified by co-elution with standards. Baseline separation of known TCB metabolites showed elution of 2-OH-3,4,3',4'-TCB at 17.7
min, 4-OH-3,3',4',5-TCB at 18.2 min, 5-OH-3,4,3',4'-TCB at 18.7 min, 6-OH-3,4,3',4'-TCB at 19.6 min, and parent 3,4,3',4'-TCB at 22.6 min.

**HISTOLOGICAL STUDIES**

In a separate study, catfish were assessed for morphological damage in the intestine following dietary BNF administration. Animals (n = 18) were divided into 3 groups (n = 6) and received either control, or BNF treated diet at 10 and 50 mg BNF/kg diet. Following 10 days of dietary exposure, intestine was harvested, profusely rinsed with ice cold 0.9% saline, and samples of proximal and distal intestine collected. Samples were fixed in 10% formalin for at least 24 h, and 4 μm sections stained with hematoxylin-eosin following standard techniques.

**STATISTICAL ANALYSIS**

The statistical software SigmaStat for Windows (Version 1.0, Jandal Corporation, San Rafael, CA) was used for data analysis. One way ANOVA and Student’s t-test were used to determine significant differences at \( p < 0.05 \). Whenever ANOVA showed significant differences, multiple comparison tests were used (Student-Newman-Keuls).

**RESULTS**

\( [14C] \)-TCB concentrations on a per-unit basis were highest in the postinfusate, and sequentially lower in the mucosa and the blood, for all treatments examined (Table 3.1). Postinfusate \( [14C] \)-TCB levels were approximately 1.3, 2.6, and 1.9-fold greater than mucosa concentrations for control catfish, and those animals preexposed to 10 mg and 50 mg BNF/kg diets, respectively. Mucosa concentrations were
Table 3.1 - $[^{14}\text{C}]-3,4,3',4'$-Tetrachlorobiphenyl (TCB) concentration gradients between postinfusate and mucosa, and mucosa and blood interfaces following *in situ* perfusion, for control catfish and animals preexposed to 10 and 50 mg BNF/kg diet for 10 days. Gradients were calculated as fold differences of $[^{14}\text{C}]-$TCB molar equivalents concentrations on a per-unit basis (postinfusate > mucosa > blood).

<table>
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<tr>
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<th>Postinfusate to mucosa</th>
<th>Mucosa to blood</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.3</td>
<td>21.7</td>
</tr>
<tr>
<td>BNF 10 mg/kg</td>
<td>2.6</td>
<td>23.3</td>
</tr>
<tr>
<td>BNF 50 mg/kg</td>
<td>1.9</td>
<td>23.6</td>
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approximately 21.7, 23.3, and 23.6-fold higher than levels in blood for corresponding treatments. Across all treatments examined, the gradient differences were greatest between the blood and mucosa, as compared to differences seen between mucosa and postinfusate.

Blood $[^{14}\text{C}]-\text{TCB}$ molar equivalent (Meq) (combination of parent$[^{14}\text{C}]-\text{TCB}$ and its metabolites) concentrations were 751.2 ± 262.6 pmol/ml, 645.5 ± 79.6 and 612.4 ± 40.6 pmol/ml for control and animals exposed to 10 and 50 mg BNF/kg diet, respectively (Figure 3.1). The systemic uptake of $[^{14}\text{C}]-\text{TCB}$ during the 60-min perfusion corresponded to 2.6, 2.1 and 1.9% of the total $[^{14}\text{C}]$ dose administered, for the same respective treatments. Dietary treatment with BNF, at both doses, resulted in negligible effects upon $[^{14}\text{C}]-\text{TCB}$ systemic bioavailability in comparison to control animals, with no significant dose or interdose effects (ANOVA: $p < 0.05$).

$[^{14}\text{C}]-\text{TCB}$ concentrations in intestinal mucosa, following in situ perfusion, remained constant with increasing doses of BNF preexposure. $[^{14}\text{C}]-\text{TCB}$ mucosal concentrations of 16.3 ± 5.6, 15.0 ± 4.6, and 14.5 ± 1.5 nmol/g tissue were detected for control, 10 mg and 50 mg BNF/kg diet, respectively (Figure 3.1). Postinfusate levels of $[^{14}\text{C}]-\text{TCB}$ were less consistent across treatments, with values of 21.4 ± 6.1, 39.0 ± 16.2, and 28.0 ± 8.6 pmol/ml, for the same treatments (Figure 3.1). There were no significant dose or interdose effects of BNF pretreatment upon subsequent $[^{14}\text{C}]-\text{TCB}$ concentrations in both mucosa and postinfusate of the in situ preparation (ANOVA: $p < 0.05$).
Figure 3.1 - Concentration of $[^{14}C]$-3,4,3',4'-tetrachlorobiphenyl in the blood, mucosa and postinfusate after 60-min incubation in in situ isolated perfused intestine of (a) vehicle-treated catfish, and (b) following β-naphthoflavone (BNF) exposure to 10 mg and 50 mg BNF/kg diets for 10 days.

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The effect of BNF pretreatment upon intestinal metabolism in the catfish was examined through determination of total P450 content, and AHH activity. Total P450 concentrations were 0.08 ± 0.02, 0.07 ± 0.03, and 0.10 ± 0.02 nmol/mg for control, and BNF-treated animals at 10 and 50 mg BNF/kg diet, respectively (Figure 3.2). There were no significant changes in total P450 content following dietary treatment with BNF for either dose tested (ANOVA: $p < 0.05$). As compared to controls (1.24 ± 1.08 pmol/min/mg protein), there was a significant 9.2- and 12.5-fold increase in AHH activities, following exposure to 10 mg and 50 mg BNF/kg diet, respectively (Figure 3.3). Interdose differences in AHH activity were not significant (ANOVA, Student-Newman-Keuls: $p < 0.05$).

[14C]-TCB was biotransformed to a small degree in intestinal mucosa (0.54 to 1.27%) during the 60-min in situ preparation (Table 3.2). [14C]-TCB metabolites were also detected in the postinfusate (0.24 to 0.37%) and in the blood (1.00 to 1.58%). Along with parent compound, four other peaks eluted in the analysis of sample extracts. Two of the peaks were tentatively identified as 2-OH-3,4,3',4'-TCB and 4-OH-3,5,3',4'-TCB by co-elution with standards. Equivalent amounts of the two identified metabolites were found in blood, mucosa and postinfusate of nearly all treatments groups. The only exception were significantly lower blood levels of 4-OH-3,5,3',4'-TCB in BNF-treated animals, in comparison to controls (ANOVA: $p < 0.05$) (Table 3.2). There were no other significant effects of BNF treatment upon the extent and profile of metabolism of [14C]-TCB in the catfish intestine (ANOVA: $p < 0.05$).
Figure 3.2 - Total cytochrome P450 content in intestinal mucosa of controls and catfish treated with β-naphthoflavone (BNF) at 10 and 50 mg BNF/kg diet for 10 days.
Figure 3.3 - The effect of dietary treatment with β-naphthoflavone (BNF) at 10 and 50 mg BNF/kg diet for 10 days upon intestinal mucosa aryl hydrocarbon hydroxylase (AHH) activity. * indicates statistical significance ($p < 0.05$).
Table 3.2 - Metabolites of [\(^{14}\)C]-3,4,3',4'-tetrachlorobiphenyl (TCB) detected in blood, mucosa and postinfusate following in situ perfusion, for control, and catfish exposed to 10 and 50 mg BNF/kg diet for 10 days prior to preparation.

<table>
<thead>
<tr>
<th></th>
<th>% of total metabolites[^a]</th>
<th>% of 2-OH-3,4,3',4'-TCB[^b]</th>
<th>% of 4-OH-3,5,3',4'-TCB[^b]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.58 ± 0.80</td>
<td>N.D. to 0.65</td>
<td>0.27 to 0.70</td>
</tr>
<tr>
<td>10 mg BNF/kg diet</td>
<td>1.00 ± 0.25</td>
<td>0.22 to 0.37</td>
<td>N.D.</td>
</tr>
<tr>
<td>50 mg BNF/kg diet</td>
<td>1.00 ± 0.13</td>
<td>N.D. to 0.25</td>
<td>N.D. to 0.18</td>
</tr>
<tr>
<td><strong>Mucosa</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.57 ± 0.36</td>
<td>N.D. to 0.21</td>
<td>N.D. to 0.20</td>
</tr>
<tr>
<td>10 mg BNF/kg diet</td>
<td>1.27 ± 0.40</td>
<td>N.D. to 0.43</td>
<td>N.D. to 0.08</td>
</tr>
<tr>
<td>50 mg BNF/kg diet</td>
<td>0.54 ± 0.48</td>
<td>N.D. to 0.09</td>
<td>N.D. to 0.17</td>
</tr>
<tr>
<td><strong>Postinfusate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.24 ± 0.08</td>
<td>N.D.</td>
<td>N.D. to 0.05</td>
</tr>
<tr>
<td>10 mg BNF/kg diet</td>
<td>0.37 ± 0.09</td>
<td>N.D. to 0.10</td>
<td>N.D. to 0.03</td>
</tr>
<tr>
<td>50 mg BNF/kg diet</td>
<td>0.33 ± 0.05</td>
<td>N.D. to 0.12</td>
<td>N.D. to 0.03</td>
</tr>
</tbody>
</table>

Note: Peaks eluted before parent compound were presumed to be metabolites. N.D., not detected, < 0.005% for mucosa and postinfusate, < 0.05% for blood.

[^a]: Total of all extracted radioactivity that eluted before the parent compound.
[^b]: Peaks tentatively identified by co-elution with standards.
Dietary BNF at either dose resulted in morphological changes in the intestine of catfish. Thinning of the submucosa with submucosal separation were perceptible, as well as increase in Goblet cell numbers (Figure 3.4). Morphological changes were more evident with the higher BNF treatment (50 mg BNF/kg diet).

**DISCUSSION**

These studies demonstrated that 1.9 to 2.6 % of the total $[^{14}\text{C}]-\text{TCB}$ dose was absorbed in the *in situ* intestinal preparations during the 60-min perfusion. Taking into consideration the short duration of exposure and limited intestinal surface area (only a fraction of the intestine was used in the *in situ* perfusion), the amount of $[^{14}\text{C}]-\text{TCB}$ which reached the systemic circulation was appreciable. Under *in vivo* conditions, dietary xenobiotic exposures need to be examined in context with digestive processes, since xenobiotics are usually ingested in association with food (James and Kleinow, 1994). Typically, shorter transit times of foodstuffs in the intestine result in decreased xenobiotic bioavailability (Watkins, III and Klaassen, 1997). Gastrointestinal transit times longer than one hour are likely to be seen under *in vivo* conditions, suggesting that chemical uptake would be much higher than seen in the present study. Studies by Lane and Jackson (1969) have estimated that 24 to 36 hours (at 12°C) are needed for catfish to completely empty their digestive tracts after a meal.

Similar amounts of $[^{14}\text{C}]-\text{TCB}$ were absorbed for all treatments, suggesting that BNF exposure at either dose did not influence TCB transfer from the lumen to the systemic circulation. Likewise, similarity in TCB gradients across the postinfusate to mucosa and mucosa to blood interfaces further support this contention. The greatest
Figure 3.4 - Proximal intestine of (A) control catfish (160×) and (B) catfish exposed to 50 mg BNF/kg diet for 10 days (63×). Intestine of catfish treated with BNF display thinning and separation of submucosa (SM), and increase in number of goblet cells (G).
TCB Meq concentration gradients seen were between mucosa and blood (21.7 to 23.6x), and were consistent across treatments. Concentration gradients between postinfusate and mucosa (1.3 to 2.6x), although less consistent for all treatments, were overall much smaller. This pattern of TCB distribution in the compartments of the *in situ* preparations were previously attributed to the relative lipid content of the different compartments (Doi et al., 2000). These results would suggest that TCB transfer from the mucosa to the blood is the rate limiting factor in the overall systemic uptake of TCB from the intestinal lumen. It is possible that the TCB transfer through the mucosa is dependent on cytosolic transporters, which would limit chemical transfer rate to the blood. Cytosolic transporters of lipophilic xenobiotics have been identified, and include glutathione-S-transferase (Dixit et al., 1982), lipoproteins (Vetter et al., 1985), and fatty acid binding proteins (Larsen et al., 1991). The contribution of these mechanisms to the systemic bioavailability of TCB is only speculative at this time.

Previous studies in catfish by James et al. (1996), utilizing *in situ* conditions identical to the present study, have shown that BNF pretreatment (10 mg BNF/kg diet for at least 2 weeks) did not affect either the biotransformation or bioavailability of dietary BaP metabolites, including 3-OH-BaP and two conjugated compounds (BaP-9-sulfate and 9-benzo(a)pyrenyl-β-d-glucopyranosiduronic acid). In both controls and BNF-treated animals, the two conjugated BaP metabolites were absorbed as administered (without prior metabolism), while 3-OH-BaP was metabolized prior to absorption. Contrasting results were seen in similar *in situ* preparations examining the uptake and metabolism of parent BaP (Kleinow et al., 1998). In the latter study, BNF
treatment altered the metabolite profile of BaP, and increased the amounts of parent BaP and unconjugated metabolites that were transferred to the systemic circulation. Systemic bioavailability of BaP Meq was shown to increase 2.6 to 5.5-fold with BNF exposure, but metabolites accounted for only 10.2 to 23.1% of the total increase. These results suggested that BNF preexposure modulated the bioavailability of parent BaP through effects that included, but were not restricted to BaP metabolism. Although not investigated, the possibility that BNF exposure could have some effect on the intestinal permeability of xenobiotics through non-specific toxicity was discussed by the authors (Kleinow et al., 1998).

BNF dietary exposure has been formerly shown to cause both structural (reduction in body weight, epithelial hyperplasia in the gills and fins) and biochemical (increase in hepatic microsomal phospholipid) alterations in fish (Harris et al., 1988; Grady et al., 1992). The present study demonstrated that dietary BNF administration at 10 mg, but more markedly at 50 mg BNF/kg diet, resulted in morphological changes in the intestine, including thinning and separation of the submucosal layer, as well as an increase in the number of Goblet cells. The morphological alterations described herein held remarkable similarity to the structural changes related to TCB exposure seen in previous studies (Doi et al., 2000), indicating that these may not be chemical-specific. The lack of effect of BNF exposure on the bioavailability of [14C]-TCB in the present study, despite the observed morphological effects, suggest that these structural changes are not likely to influence the transfer of xenobiotics across the intestine.
In earlier studies in our laboratory, TCB preexposure at both 0.5 and 5 mg TCB/kg diet resulted in significant decreases in the systemic bioavailability of a subsequent and similar $[^{14}\text{C}]$-TCB dose (Doi et al., 2000). Changes in $[^{14}\text{C}]$-TCB bioavailability did not correlate to metabolism, as TCB pretreatment, in a similar manner to BNF exposure in the present study, had no significant effect on $[^{14}\text{C}]$-TCB metabolism during the 60-min perfusion. A couple of factors associated to TCB exposure were forwarded as potential modulators of the intestinal permeability of $[^{14}\text{C}]$-TCB, including decreases in the diffusion gradient (by increases in tissue loads), and non-specific toxicity (morphological changes similar to those described herein were seen in catfish given 0.5, but mostly 5 mg TCB/kg diet for 10 days) (Doi et al., 2000). It is probable that structural changes caused by TCB preexposure had a minor role in the modulation of $[^{14}\text{C}]$-TCB uptake in previous studies, considering that like alterations in the present study had no effect on the bioavailability of $[^{14}\text{C}]$-TCB. These conclusions reinforce previously discussed assumptions that the effect of TCB preexposure on the subsequent bioavailability of $[^{14}\text{C}]$-TCB is more likely to be diffusion-related (Doi et al., 2000). Decreased dietary PCB uptake following previous dietary and dermal (contaminated sediments) exposures have been demonstrated experimentally (Hansen et al., 1976; Dabrowska et al., 1996), and modeled by Barber et al. (1991) and Clark et al. (1990).

Intestinal AHH activity was significantly increased 9.2- and 12.5-fold following pretreatment with 10 and 50 mg BNF/kg diet, respectively. AHH activity has been shown to correlate in a linear fashion to CYP1A content in intestinal
microsomes of catfish (Doi et al., 2000), however, total CYP content did not correlate to AHH with BNF treatment. These results corroborate the previous assumption that CYP1A probably corresponds to a small fraction of total CYP content in intestinal microsomes of catfish (James et al., 1997; Doi et al., 2000). A comparison between catfish and a number of other fish species have shown that intestinal CYP1A activities are considerably lower in catfish (Van Veld et al., 1988a; Van Veld et al., 1988b; Van Veld et al., 1991; James et al., 1997; Kleinow et al., 1998). These differences appear to be not only species-related, but also influenced by experimental differences including the composition of the diet (Rosenberg, 1991; James et al., 1997). Intestinal AHH activity for control animals fed a semi-synthetic diet reported herein (1.24 ± 1.08 pmol/min/mg protein) were comparable to previous reports (3.9 ± 1.9 pmol/min/mg protein) (James et al., 1997). Increased AHH activities had been reported for animals fed a commercial chow (approximately 6-fold), or a commercial chow with BNF (approximately 18-fold) (James et al., 1997). The relative AHH induction in animals treated with 10 mg BNF/kg diet with the semi-synthetic diet (9.2x), as seen in the current study, was higher than observed with commercial chow (3 to 5.6-fold) (James et al., 1997; Kleinow et al., 1998). These results stress the importance of utilizing controlled experimental designs (semi-synthetic diet, depuration of wild-caught animals) for establishment of basal levels of inducible enzymes, as well as for interspecies comparison of relative induction effects of xenobiotics.

\[ ^{14} \text{C} \text{-TCB} \] was metabolized to a small degree in the intestine of catfish over the duration of the \textit{in situ} intestinal preparation. TCB metabolism was highly variable
among individuals, with metabolites representing between 0.54 to 1.27% of total $^{14}$C-TCB Meq recovered in mucosal extracts. There was no effect of BNF pretreatment on the extent and profile of mucosal TCB metabolism in catfish, which consisted of nearly equal amounts of two tentatively identified metabolites, 2-OH-3,4,3',4'-TCB and 4-OH-3,5,3',4'-TCB, for all treatments. Studies in rats have shown that parenteral BNF administration induces hepatic formation of TCB hydroxylated metabolites (4-OH-3,5,3',4', 5-OH-3,4,3',4', and 6-OH-3,4,3',4'-TCB), most likely as a result of CYP1A induction (Morse et al., 1995b). A similar dependence of TCB metabolism upon CYP1A was hypothesized in a study with scup, where changes in TCB metabolite profile (decrease in 5-OH-3,4,3',4'-TCB formation) were shown to be dose-dependent, presumably caused by the inhibitory effects of the high TCB dose on CYP1A activity (White et al., 1997). It is not clear whether or not CYP1A is exclusively involved in the metabolism of TCB in the catfish intestine. Increases in AHH activity following BNF administration were not correlated to significant alterations in TCB metabolism. These findings are consistent with a CYP1A-independent pathway of TCB metabolism in the catfish intestine. Alternatively, the lack of BNF-treatment effect on TCB metabolism could be related to the slow metabolism rate of TCB. White et al. (1997) have shown that the rates of in vitro TCB hepatic metabolism in scup are 2,000 to 4,000 times lower than benzo(a)pyrene metabolism rates in the same species (Stegeman et al., 1981). In addition, even though CYP1A levels were increased in the catfish intestine, it is possible that they were not high enough to influence TCB metabolism in the 60 min course of the in situ
preparation. AHH activities induced by BNF exposure herein were much lower (21 to 63 x) than AHH activities of a number of other fish species, including spot and toadfish (320 to 720 pmol/min/mg protein) following exposure to CYP1A inducers (3-methylcholanthrene and benzo(a)pyrene) (Van Veld et al., 1988a; Van Veld et al., 1988b). It remains to be determined whether the lack of effect of BNF exposure on TCB metabolism is related to CYP1A-independence or to slow rates of CYP1A metabolism.

The percentages of the total [¹⁴C]-TCB Meq recovered as metabolites were not significantly different for the mucosa (0.54 to 1.27%), postinfusate (0.24 to 0.37%), and blood (1.00 to 1.58%). Similar to mucosal extracts, nearly equal amounts of 2-OH-3,4,3',4'-TCB and 4-OH-3,5,3',4'-TCB were found in the postinfusate, for all treatments. Interestingly, significantly lower amounts of 4-OH-3,5,3',4'-TCB were found in the blood of those animals treated with BNF, while no difference was evident for 2-OH-3,4,3',4'-TCB. It appears that the systemic bioavailability of 4-OH-3,5,3',4'-TCB is decreased in BNF-treated animals, but no corresponding increases on the amounts of this metabolite are evident both in the mucosa and postinfusate of corresponding treatments. It is possible that the intestinal permeability of 4-OH-3,5,3',4'-TCB is somehow changed by BNF exposure, but the mechanism of this response will need further investigation. Studies in rodents have shown that 4-OH-3,5,3',4'-TCB is the only metabolite to accumulate in rodent plasma (Brouwer et al., 1990; Morse et al., 1995a; Darnerud et al., 1996), mainly due to 4-OH-3,5,3',4'-TCB competitive binding to transthyretin, a plasma thyroid hormone-binding protein.
Thyroid hormone-binding proteins have been identified in fish species (Larsson et al., 1985; Yamauchi et al., 1999), but it is not known whether or not such proteins are affected by BNF, or if they interact with 4-OH-3,5,3',4'-TCB in fish. The significance of the decreases in blood amounts of 4-OH-3,5,3',4'-TCB following BNF exposure remain to be clarified.

A higher percentage of total TCB metabolites could be seen in intestinal mucosa following BNF preexposure (0.54 to 1.27%) as compared to previous studies in our laboratory where TCB pretreatment resulted in 0.35 to 0.59% of metabolites (Doi et al., 2000). Composite differences in amounts of mucosal TCB metabolites were accompanied by corresponding lower levels in postinfusate total TCB metabolites for BNF (0.33 to 0.37%) in comparison to TCB-pretreated animals (0.51 to 0.59%), and appear to be related mainly to the levels of 4-OH-3,5,3',4'-TCB. Pretreatment with dietary TCB appears to favor the formation of 2-OH-3,4,3',4'-TCB in comparison to 4-OH-3,5,3',4'-TCB (the latter being detected in only 3 of 10 mucosal samples of treated animals) (Doi et al., 2000). The present study suggests that BNF exposure, contrary to TCB, results in formation of nearly equal amounts of both metabolites. 4-OH-3,5,3',4'-TCB is thought to be formed through the rearrangement of a 4,5-epoxide (Klasson Wehler et al., 1989; White et al., 1997), but little is known regarding the pathways of 2-OH-3,4,3',4'-TCB formation. It has been hypothesized that a CYP other than CYP1A may be responsible for the formation of the 4,5-epoxide intermediate (White et al., 1997), and that this pathway would be favored when CYP1A levels decrease. It is possible that TCB preexposure resulted in the induction of a CYP other
than CYP1A (AHH activity levels were not induced at 0.5 mg TCB/kg diet, and showed induction but high variability at 5 mg TCB/kg diet), which would explain the lower levels of 4-OH-3,5,3',4'-TCB following TCB pretreatment. Conversely, BNF exposure resulted in significant increases in AHH activity, previously shown to correlate to increased CYP1A levels in the catfish intestine (Doi et al., 2000), which would in turn favor the 4,5-epoxide pathway resulting in formation of 4-OH-3,5,3',4'-TCB.

In conclusion, these studies have shown that (1) first pass intestinal metabolism appears to be a minor contributor to the total metabolism of dietary TCB, with the formation of small amounts of tentatively identified 2-OH-3,4,3',4'-TCB and 4-OH-3,5,3',4'-TCB, (2) there was no significant correlation between induction of AHH activities by BNF pretreatment and the extent and profile of TCB metabolism, (3) induction of AHH related activities in the intestine following BNF exposure was not correlated to the systemic uptake of a subsequent [14C]-TCB exposure, and (4) dietary BNF caused morphological alterations in the intestine of catfish (especially at the higher BNF dose examined) without affecting subsequent TCB uptake.

REFERENCES


CHAPTER 4: P-GLYCOPROTEIN IN THE CATFISH INTESTINE: INDUCIBILITY BY XENOBIOTICS AND FUNCTIONAL PROPERTIES

INTRODUCTION

P-glycoprotein (pgp) is a membrane bound 170 kDa protein that has been extensively studied in mammals as the mediator for tumor cell multidrug resistance (MDR). Increases in tumor pgp expression confer drug resistance through energy-driven efflux transport, which prevents intracellular accumulation of unrelated cytotoxic drugs, including Vinca alkaloids, paclitaxel (Taxol™), and anthracyclines, to name a few (Dano, 1973; Juliano and Ling, 1976; Bradley et al., 1988; Ford and Hait, 1990). While often overexpressed in tumors, pgp can be found in nontransformed tissues as well. Localized pgp expression has been described in various tissues, including intestinal epithelium, liver bile canaliculi, kidney proximal tubules, adrenal cortex, and brain capillaries (Thiebaut et al., 1987; Cordon-Cardo et al., 1990). A physiological role for pgp is yet to be uncovered, but its distribution in organs with specialized excretory, secretory, and barrier functions is consistent with a postulated protective role against toxic insult (Gottesman and Pastan, 1988; Ford and Hait, 1990; Schinkel, 1997).

A pgp-mediated multixenobiotic resistance (MXR) mechanism has been associated with modulation of xenobiotic accumulation and the ability of aquatic animals to live and thrive in contaminated environments (Kurelec, 1992; Epel, 1998). Studies in fish have shown that the MXR mechanism resembles the mammalian MDR in many ways. Partial cloning of two pgp genes identified in winter flounder have

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shown that fish pgps are homologous to mammalian MDR (Chan et al., 1992). In addition to genetic correlation, cross-reactivity with mammalian pgp antibodies have shown that the distribution of pgp in fish tissues is consistent with patterns seen in mammals, including organs such as the gut, liver, and kidney (Hemmer et al., 1995; Hemmer et al., 1998; Kleinow et al., 2000). Indication of functional similarities have also been provided by studies in killifish, where active transport of the known pgp substrate cyclosporine was shown to occur in renal tubules (Schramm et al., 1995).

The gastrointestinal tract appears to be a major route of contaminant uptake for fish species. There are indications in mammals that pgp may modulate the intestinal bioavailability of dietary drugs and xenobiotics, through efflux activity back into the intestine. Polarized basolateral to apical ATP-dependent pgp-mediated flux in human colonic adenocarcinoma cell lines has been demonstrated for a number of pgp substrates, including vinblastine (Terao et al., 1996) and rhodamine (Yumoto et al., 1999). *In vitro* and *in vivo* studies in mice (van Asperen et al., 1997), rats (Barthe et al., 1998; Yumoto et al., 1999), and humans (Lown et al., 1997) have further demonstrated that the intestinal absorption of drugs, including paclitaxel, cyclosporine and digoxin, may be increased with concurrent administration of inhibitors (e.g. verapamil), which block pgp-mediated transport back into the intestinal lumen.

Common chemical characteristics which determine substrate specificity for pgp are mostly unidentified, with the exception of moderate hydrophobicity (West, 1990; Bain and LeBlanc, 1996; Sharom, 1997). Environmental contaminants identified as potential substrates for pgp in aquatic species include 2-acetylaminoﬂuorene (AAF),
the hydrophobic xenobiotics Aroclor 1254, DDT, DDD, and DDE, and the hydrophobic pesticides daetral, chlorbenside, sulfanate and pentachlorophenol (Kurelec and Pivcevic, 1991; Cornwall et al., 1995; Waldmann et al., 1995; Galgani et al., 1996; Kurelec et al., 1996). Studies in gill tissues of mussels and clams have shown that the pgp inhibitors verapamil and staurosporin modulate AAF accumulation (Kurelec and Pivcevic, 1991; Waldmann et al., 1995; Kurelec et al., 1996), and that hydrophobic xenobiotics and pesticides inhibit the efflux of the pgp substrate rhodamine, in a species-specific manner (Cornwall et al., 1995; Galgani et al., 1996). Evidence suggests that hydrophobic environmental contaminant benzo(a)pyrene (BaP) may be transported by pgp as well. Verapamil-sensitive efflux of BaP in tumor MDR cell lines (Yeh et al., 1992) and in membrane vesicles of human intestinal epithelium (Penny and Campbell, 1994), as well as autoradiographic evidence in the channel catfish intestine, are consistent with a pgp modulatory role in the intestinal uptake of BaP (Kleinow et al., 1996).

A degree of overlap in pgp and cytochrome P450 inducers and substrate specificity, as well as coincidence in the tissues where they are expressed, has lead to the hypothesis that these two systems may exert complimentary protective functions against xenobiotics (Burt and Thorgeirsson, 1988; Gant et al., 1991; Schuetz et al., 1996). Studies have shown that pgp induction occurs in rat liver cells following exposure to cytochrome P4501A (CYP1A) inducers, including the polycyclic aromatic hydrocarbons BaP and 3-methylcholanthrene (3-MC) (Gant et al., 1991; Chieli et al., 1994; Fardel et al., 1996). Likewise, in vivo studies in rats have shown co-induction of
hepatic pgp and CYP1A following exposure to 3-MC and the carcinogen AAF (Burt and Thorgeirsson, 1988; Gant et al., 1991; Tateishi et al., 1999). The relationship between hepatic pgp and CYP1A has been examined to a limited extent in fish. Blennies exposed for 3 weeks to sediment and food contaminated with crude oil (rich in polycyclic aromatic hydrocarbons), showed increases in hepatic levels of both pgp and CYP1A (Bard et al., 1998). In another study, a 3-day exposure to low aqueous levels of hydrocarbon-rich Diesel-2 oil resulted in no alteration in carp hepatic CYP1A activity. However, when the pgp inhibitor verapamil was added to contaminated water, hepatic CYP1A-related activity was induced following 2 days of exposure, indicating that verapamil inhibited pgp-mediated xenobiotic efflux, increasing the uptake of Diesel-2 oil (Kurelec, 1995).

The purpose of the present study was to characterize the MXR response in the catfish intestine, following dietary exposure to environmental pollutants and prototypic MDR inducers (Figure 4.1). Vincristine (VIN) and β-naphthoflavone (BNF) were selected as prototypic pgp and CYP1A inducers, respectively (Bradley et al., 1988; Licht et al., 1991; James et al., 1997; Kleinow et al., 1998). BaP and 3,4,3',4'-tetrachlorobiphenyl (TCB) were selected according to their environmental relevance, as well as for their CYP1A-inducing abilities (Van Veld et al., 1988a; Van Veld et al., 1988b; Doi et al., 2000) and potential as pgp substrates (BaP) (Yeh et al., 1992; Penny and Campbell, 1994). The specific objectives of these study were (1) to examine the in vivo effect of the prototypic pgp inducer VIN and the CYP1A-inducers BNF, TCB, and BaP upon detectable intestinal C219 immunoreactivity, and (2) to evaluate
Figure 4.1- Compounds associated with p-glycoprotein mediated resistance mechanisms.
functional activity of membrane vesicles obtained from the catfish intestine, using the prototypic pgp substrate vinblastine, and the known pgp inhibitor verapamil.

MATERIALS AND METHODS

CHEMICALS

3,4,3',4'-Tetrachlorobiphenyl was obtained from ChemService Inc., West Chester, PA. Tricane methane sulfonate (MS-222™) was purchased from Argent Chemical Company, Redmond, WA. Electrophoresis material including acrylamide, bis N,N'-methylene-bis-acrylamide, 10X TBS, Tween-20, gelatin, nitrocellulose, Kaleidoskope Prestained Standards and the Immun-Blot™ Assay Kit with Goat Anti-mouse IgG Alkaline Phosphatase (GAM-AP) were acquired from Bio-Rad Laboratories, Hercules, CA. The C-219 murine monoclonal antibody was purchased from Signet Laboratories Inc., Dedham, MA. Radiolabeled [3H]-vinblastine was obtained from Amersham Life Sciences, Inc., Arlington Heights, IL. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

EXPERIMENTAL ANIMALS

Channel catfish, Ictalurus punctatus, of both sexes were obtained from the Louisiana State University Aquaculture Research Station, Baton Rouge, LA. All animals were acclimated for at least 4 weeks, under a 12-h light/dark photoperiod, under flow through conditions with dechlorinated tap water (pH 8.0 ± 0.2, temperature 19.4 ± 0.9°C, total hardness 29.2 ± 6.9 mg CaCO₃/l, alkalinity 142.1 ± 15.1 mg/l), and treated in accordance with National Institute of Health guidelines prior and for the duration of the experimental period.
DIETARY TREATMENTS

Three separate experiments were conducted in order to examine the effects of dietary CYP1A inducers and vincristine on pgp-related reactivity using immunoblot analysis. These studies examined varying doses, modes of administration, and types of diet, as follows:

Experiment 1: Dose Response with CYP1A Inducers

In this first experiment, the effects of different doses of dietary BNF, BaP, and TCB were investigated. Fish weighing 342 ± 68 g (n = 42) were divided into groups of six fish. A custom, purified, semi-synthetic diet composed of casein 32%, dextrin 29.8%, cellulose 19%, soybean oil 3%, Menhaden oil 3%, gelatin 8%, salt and vitamin mix 5%, and choline chloride 0.2% (Dyets Inc., Bethlehem, PA) was used as the base diet for all treatments. Semi-synthetic diet was coated with corn oil (1 ml corn oil/100 g diet) with or without chemical additions, and fed ad libitum to animals for 10 days, at a rate of 1% body weight. Groups of catfish were fed corn-oil coated diets containing (1) no additions (controls), (2) BNF at 10 or 50 mg/kg diet, (3) TCB at 0.5 or 5 mg/kg diet, or (4) BaP at 1 or 10 mg/kg diet. All diets were prepared prior to the beginning of the experimental period and kept at -20°C. Diets containing BaP were additionally kept under nitrogen and protected from light to prevent oxidation and photodecomposition.

Experiment 2: CYP1A Inducers Via Indwelling Stomach Tube

To ensure consistency in dose administration so to eliminate variability associated with the amounts of food ingested, fish in the second experiment were outfitted...
with indwelling stomach tubes. Fish were anesthetized with MS-222 buffered with NaHCO₃⁻ (induction and maintenance doses of 106 and 86 mg/l, respectively) 24 to 48 h before diet administration, to facilitate stomach tube installation as described in Kleinow (1991). The stomach tube consisted of 3 pieces: (1) an 8 cm piece of Intramedic™ PE-320 tubing (Becton Dickinson & Co., Parsippany, NJ) angled at approximately 80° which is inserted through the snout, (2) a piece of IV tubing advanced into the stomach at the level of pectoral fins, and (3) a ~53 cm piece of latex tubing (1/8” I.D. x 3/16” wall thickness) attached to the dorsal external extension of the angled PE tubing (Figure 4.2). The latex tubing was sutured to the dorsal fin for stability and plugged distally providing buoyancy when filled with air.

Experimental diets were prepared by evenly distributing the chemicals in a toluene carrier over semi-synthetic diet which was finely ground. Solvent-moist diet was protected from light and dried until the solvent was completely evaporated. Diets were thoroughly rolled and mixed prior to storage at -20°C (BaP diet was additionally kept under nitrogen and protected from light during storage). Immediately prior to gavage administration, diets were mixed with water (1:2.5) producing a slurry compatible with the gavage tube. Four groups of fish (n = 4, 1,502 ± 403 g) were administered the appropriate gavage slurry of the semi-synthetic diet containing (1) control diet without additions, (2) BNF at 200 mg/kg diet, (3) BaP at 40 mg/kg diet, or (4) TCB at 20 mg/kg diet. Fish were treated for 10 days, at a daily rate of 0.25% body weight, divided in two aliquots, administered through the gavage tube twice a day.
Figure 4.2- Schematic representation of stomach tube placement in channel catfish.
Experiment 3: β-Naphthoflavone and Vincristine

This last experiment examined the effect of prototypic CYP1A and pgp inducers on pgp-related expression in the intestine of catfish. Conditions in this section attempted to replicate the dosing protocol of an earlier study which investigated pgp induction in the catfish intestine by the same inducers using immunohistochemistry analysis (Kleinow et al., 2000). Fish weighing 1,453 ± 366 g (n = 14) were divided into 3 groups of either 4 or 5 fish. Commercial fish feed (Silver Cup trout pellets, Nelson & Sons Inc., Murray UT) was used as the base diet for all treatments. Control and BNF diets were prepared by coating the commercial chow with corn oil (1 ml corn oil/100g diet). Control diets were prepared with pure corn oil. BNF diets were coated with corn oil at 10 mg BNF/kg diet. VIN diets (20 µg/kg diet) were prepared by spraying an aqueous VIN solution over the diet, which was dried at room temperature before feeding. Animals were given the appropriate experimental diet ad libitum for 16 days, at a rate of 3% body weight.

MEMBRANE VESICLE PREPARATION

Following termination of exposure, fish were fasted for 24 h prior to tissue collection. Fish were sacrificed, and the whole intestine was harvested. Intestine was divided in two sections, and the most proximal discarded, based on earlier studies, which indicated that pgp-related levels and responsiveness to dietary inducers are greater in the distal intestine of catfish (Kleinow et al., 2000). Distal intestine was promptly rinsed with cold modified Sack’s preservation buffer (5 mM KH₂PO₄, 14 mM NaHCO₃, 54 mM K₂HPO₄, 5.9 mM KHCO₃, and 205 mM mannitol). The
intestinal section was then cut open, thoroughly rinsed with cold Sack's buffer, and blotted dry. Intestinal mucosa was scraped using the edge of a glass slide. Intestinal mucosa of randomly paired animals from like treatment groups were pooled for vesicle preparation to ensure adequate sample size in experiment 1. Intestinal mucosa of individual animals were used for the remaining experiments. Aliquots of liver tissue (~2 g) were harvested following removal of intestine, and processed similarly to mucosa, when collected. Because protocols for immunoblot detection of pgp in fish tissues are available only for liver (Cooper et al., 1996), hepatic vesicles were used in the present study as validation for the method developed for intestinal tissues. Tissues were homogenized in plasma membrane buffer (5 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 0.2 mM magnesium chloride) containing 0.1 μM PMSF and 1 mM EDTA. For experiments 2 and 3, PMSF was substituted by 50 μl protease inhibitor cocktail/g tissue (Protease inhibitor cocktail, Sigma Chemical Co., St. Louis, MO), which contained 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), pepstatin A, transepoxy succinyl-L-leucylamido(4-guanidino)butane (E-64), bestatin, leupeptin and aprotinin. The homogenate was centrifuged at 1,000 x g for 10 min at 4°C to sediment nuclei and intact cells. The supernatant was saved, and the pellet was resuspended in plasma membrane buffer and re-centrifuged. Supernatants from both runs were combined, and centrifuged at 15,000 x g for 20 min at 4°C to sediment mitochondria and lysosomes. Resulting supernatants were centrifuged at 100,000 x g for 1 h at 4°C to sediment plasma membrane vesicles and endoplasmic reticulum. Final pellets were resuspended in plasma membrane buffer and stored at -80°C until analysis.
WESTERN BLOT ANALYSIS

Mixed membrane vesicles were subjected to SDS-PAGE, electrophoretic transfer to nitrocellulose and immunoblotting procedures for the detection of pgp. Membrane vesicles quantitated according to Lowry (Lowry et al., 1951) were solubilized (1:5) in sample buffer consisting of either (1) 62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromophenol blue (heated at 95°C for 4 min) (experiment 1), or (2) 62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, and 0.001% bromophenol blue (room temperature for 20 min) (experiment 2 and 3). After solubilization, vesicles (16 µg or 30 µg) were loaded on a 7.5% acrylamide/bis-acrylamide gel and electrophoresis performed at a constant voltage of 200V on a Bio-Rad Mini-protean™ II Dual Slab Cell, using a 25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 0.1% SDS running buffer. Following electrophoresis, proteins were transferred to a 0.45 µm nitrocellulose membrane using a Bio-Rad Mini Trans-Blot™ Electrophoretic Transfer Cell. Transfer was performed at 100V for 2 h, using a 25 mM Tris, pH 8.3, 192 mM glycine, 20% v/v methanol transfer buffer. After transfer, immunoassay was performed using the mammalian C-219 monoclonal antibody and the Bio-Rad Immun-Blot™ Assay Kit with Goat Anti-mouse IgG Alkaline Phosphatase (GAM-AP). The nitrocellulose membrane was washed twice in Tris-buffered saline (TBS) (20 mM Tris, pH 7.5, 500 mM NaCl) for 5 min. Washes were followed by 1-h blocking with a 3% gelatin in TBS solution and a 5-min TTBS (0.05% Tween-20 in TBS) wash. Membranes were then incubated overnight with C-219 (0.2 µg/ml) in antibody buffer (1% gelatin in TTBS), followed by two washes in

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TTBS for 5 min. Following overnight incubation with C219, a 90-min conjugate binding with a 33% solution of GAM-AP in antibody buffer was performed. Five-min washes were performed twice with TTBS and once with TBS. Color developer used to detect immunoreactivity consisted of a mixture of two reagents in water, one containing nitroblue tetrazolium in aqueous dimethylformamide with magnesium and another consisting of 5-bromo-4-chloro-3-indoyl phosphate. Prestained Standards were used for molecular weight determination, and contained the dye bound molecular weight markers calibrated for each lot: myosin, β-galactosidase, and bovine serum albumin. Quantitation of the signal was performed using computerized densitometry of scanned images (ScanJet 4C, Hewlett-Packard, Greeley, CO). Relative density of the immunoreactive band was measured using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). Correction of inter-gel variability was performed by measurement of a standard sample loaded in all gels.

The mammalian C219 monoclonal antibody was selected for these studies for its specificity. This antibody recognizes highly conserved cytoplasmic amino acid sequences that have been identified in MDR proteins (Kartner et al., 1985). Successful detection of pgp-related proteins with C219 have been described in mussels (Cornwall et al., 1995; Galgani et al., 1996), clams (Waldmann et al., 1995), fish (Hemmer et al., 1995; Hemmer et al., 1998), as well as in a wide variety of mammalian species (Cordon-Cardo et al., 1990; Georges et al., 1990; Beaulieu et al., 1997).
Transport assays were performed with membrane vesicles from animals treated with BNF (50 mg/kg diet) or VIN (20 μg/kg diet), and respective controls. Experiments were performed using the prototypic pgp substrate $[^3H]$-vinblastine (VBL), and the known inhibitor verapamil (VP), following a previously described method developed for multidrug resistant cell lines (Horio et al., 1988).

Vesicles of control (22 or 50 μg), BNF-treated (13 μg) or VIN-treated (50 μg) animals were incubated with 8 nM $[^3H]$-VBL (specific activity 12.5 Ci/mmol, 13.7 mCi/mg) solution in transport buffer (10 mM Tris-HCl, pH 7.5, 0.25 M sucrose) in a final volume of 100 μl at 25°C in a shaking incubator for 20 min. The incubation was carried out with one of the following additions: (1) Transport buffer containing MgCl$_2$ (10 mM), (2) Transport buffer containing MgCl$_2$, ATP (3 mM) or ATP (1mM) with an ATP regenerating system that included creatine phosphate (CP) (3 mM) and creatine kinase (CK) (30 μl/ml), or (3) Transport buffer containing MgCl$_2$, ATP (or ATP with the regenerating system), and verapamil (VP) (5 μg/ml). After a 20-min incubation, the reaction was stopped by addition of 1 ml of ice-cold transport buffer. This solution was filtered under vacuum, through 0.2 μm nylon filters (Alltech Associates Inc., Deerfield, IL) presoaked in 10% fetal calf serum to decrease non-specific binding. Filters were washed twice with 4 ml of ice-cold transport buffer, and oven dried at 50°C for 10 min. Following addition of 10 ml of Ultima Gold LSC-Cocktail (Packard, Meriden, CT) to the filters, samples were dark adapted overnight and radioactivity (of vesicles trapped in the filter) was measured by liquid scintillation counting.
Incubations of 30 sec duration, under the same conditions, were used as a determinant of non-specific binding to the filters. Each treatment was carried out in triplicate, using pooled samples obtained from 3 different animals.

STATISTICAL ANALYSIS

Data analysis for the immunoblot sections was performed using the statistical software SigmaStat for Windows, version 1.0, Jandal Corporation, San Rafael, CA. Analysis were performed with \( p < 0.05 \), using one-way ANOVA.

RESULTS

WESTERN BLOT ANALYSIS

Experiment 1: Dose Response with CYP1A Inducers

A ~ 80 kDa protein that crossreacted with C219 was detected in mixed membrane vesicles of all chemical treatments, with control animals showing traces of detectable protein (Figure 4.3). Large individual variability was observed, with standard deviations often equal to the means. Staining intensity of the immunoreactive protein was highly increased in select animals following exposure to BNF, TCB and BaP at both dosage levels for each compound (Figure 4.3). Relative densities ranged from 0.04 to 0.97 for controls, 0.95 to 21.42 for animals exposed to BNF, 0 to 47.62 for those treated with TCB, and 0.59 to 20.93 following BaP administration (Figure 4.4). Increases in immunoreactivity levels with treatments were not significant due to the high variability in response (ANOVA: \( p < 0.05 \)), and did not appear to follow a dose-response relationship.
Figure 4.3- A ~ 80 kDa immunoreactive protein was detected by Western Blot analysis using P-glycoprotein C219 monoclonal antibody (mAb) in intestinal mucosa from controls and catfish treated with β-naphthoflavone (BNF) at 10 and 50 mg/kg diet, 3,4,3',4'-tetrachlorobiphenyl (TCB) at 0.5 and 5 mg/kg diet, and benzo(a)pyrene (BaP) at 1 and 10 mg/kg diet. Experimental diets were administered for 10 days, at a rate of 1% body weight. Standard markers are myosin (201 kDa), β-galactosidase (116 kDa) and bovine serum albumin (80 kDa). Each gel represents pooled mixed membrane vesicles (16 µg total protein per lane) from 2 animals for each treatment. A, B, C. Immunoassay detection using C219 mAb shows cross-reactivity with a protein of ~ 80 kDa (arrow). D, E, F. Respective blank controls (without C219) show non-specific immunoreactivity.
Figure 4.4- P-glycoprotein C-219 monoclonal antibody immunoreactive protein levels in membrane vesicles from catfish intestine, following a 10-day dietary exposure with β-naphthoflavone (BNF) at 10 mg and 50 mg; 3,4,3',4', tetrachlorobiphenyl (TCB) at 0.5 mg and 5 mg; and benzo(a)pyrene (BaP) at 1 mg and 10 mg (mg/kg of diet). Each column represents mean ± SD (n = 6, every two animals randomly pooled). Relative density was a measure of the staining intensity of C-219 immunoreactivity corrected for inter-gel variability.
Experiment 2: CYP1A Inducers Via Indwelling Stomach Tube

The methodological modifications instituted in this section were based on results displayed in Figure 4.5, which demonstrated that alterations in sample buffer (removal of β-mercaptoethanol and boiling) resulted in the detection of a ~170 kDa protein in addition to the ~80 kDa protein. The use of a protease inhibitor cocktail in addition to changes in sample buffer resulted in the exclusive detection of a ~170 kDa C-219 immunoreactive protein in intestinal and hepatic membrane vesicles of all treatments (Figure 4.6). Immunoreactive protein levels were higher in liver membrane vesicles compared to intestine. Staining intensity of the immunoreactive protein was consistent across treatments in vesicles from distal intestine following exposure to BNF, TCB and BaP versus controls (Figure 4.7A) (ANOVA: \( p < 0.05 \)). Likewise, no significant alterations in immunoreactivity were observed in hepatic samples following BNF, BaP or TCB exposure (Figure 4.7B) (ANOVA: \( p < 0.05 \)).

Experiment 3: β-Naphthoflavone and Vincristine

A ~170 kDa C-219 immunoreactive protein was detected in membrane vesicles of all treatments (Figure 4.8). The average staining intensity of immunologically related protein in the intestine following exposure to VIN and BNF was not significantly altered in comparison to controls, due to high interindividual variability (Figure 4.9) (ANOVA: \( p < 0.05 \)).

FUNCTIONAL STUDIES

The first \textit{in vitro} transport experiment demonstrated that the dynamics of VBL accumulation in membrane vesicles was affected by the addition of ATP as an energy
Figure 4.5- A) Alterations in sample preparation prior to loading resulted in the detection of a ~ 170 kDa protein (full arrow) in addition to the ~ 80 kDa previously detected (dotted arrow) in mixed membrane vesicles of distal intestine of catfish using C219 monoclonal antibody (mAb). B) Control gel (no C219 mAb) shows non-specific binding. Experimental diets were prepared using semi-synthetic diet and were given for 10 days at a rate of 1% body weight. Control diet consisted of 10 ml corn oil/kg of diet, BNF diet consisted of 50 mg BNF/10 ml corn oil/kg of diet. 1. Standard markers are myosin (201 kDa), β-galactosidase (116 kDa) and bovine serum albumin (80 kDa). 2. Control diet (n = 1), sample buffer with β-mercaptoethanol (β-merc.), boiled (95°C for 4 min). 3. Control diet, sample buffer with β-merc., room temperature (23°C for 20 min). 4. BNF diet (pooled sample from 2 animals), sample buffer with β-merc., boiled. 5. BNF diet, sample buffer with β-merc., room temperature. 6. BNF diet, sample buffer without β-merc., boiled. 7. BNF diet, sample buffer without β-merc., room temp.
Figure 4.6- Western Blot analysis using P-glycoprotein C219 monoclonal antibody (mAb) in mucosa from distal intestine and liver of channel catfish detected a ~ 170 kDa protein in all treatments. Mixed membrane vesicles were obtained from controls (CON) and animals exposed to β-naphthoflavone (BNF) at 200 mg/kg diet, 3,4,3',4'-tetrachlorobiphenyl (TCB) at 20 mg/kg diet, and benzo(a)pyrene (BaP) at 40 mg/kg diet for 10 days by gavage, at a rate of 0.25% body weight. Standard markers are myosin (218 kDa), β-galactosidase (125 kDa) and bovine serum albumin (78 kDa). Each gel represents samples (16 µg total protein per lane) from individual animals for each treatment. A, B, C, D. Immunoassay detection using C219 mAb shows cross-reactivity with a protein of approximately 170 kDa (arrow). E, F, G, H. Respective blank controls (without C219) show non-specific immunoreactivity.
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**Intestine**

**Liver**

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(A) Image of Intestine protein bands

(B) Image of Liver protein bands

(C) Image of Intestine protein bands

(D) Image of Liver protein bands

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Figure 4.7- P-glycoprotein C-219 immunoreactive protein levels in membrane vesicles from catfish intestine (A) and liver (B), following a 10-day dietary exposure (mg/kg of diet) with β-naphthoflavone (BNF) at 200 mg; 3,4,3′,4′-tetrachlorobiphenyl (TCB) at 20 mg; and benzo(a)pyrene (BaP) at 40 mg. Each column represents mean ± SD. Relative density was a measure of the staining intensity of C-219 immunoreactivity corrected for inter-gel variability.
Figure 4.8- Immunoblot analysis using P-glycoprotein C219 monoclonal antibody (mAb) in mucosa from distal intestine of channel catfish detected a ~ 170 kDa protein in all treatments. Mixed membrane vesicles were obtained from controls (CON) and animals exposed to β-naphthoflavone (BNF) at 10 mg/kg diet or vincristine (VIN) 20 μg/kg diet for 16 days, at a rate of 3% body weight. Standard markers are myosin (218 kDa), β-galactosidase (125 kDa) and bovine serum albumin (78 kDa). Each gel represents samples (16 μg total protein per lane) from individual animals for each treatment. A, B, C. Immunoassay detection using C219 mAb shows cross-reactivity with a protein of approximately 170 kDa (arrow). D, E, F. Respective blank controls (without C219) show non-specific immunoreactivity.

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Figure 4.9- P-glycoprotein C-219 immunoreactive protein levels in membrane vesicles from catfish intestine of control animals, or following a 16-day dietary exposure of β-naphthoflavone (BNF) at 10 mg/kg of diet and vincristine (VIN) at 20 μg/kg of diet. Each column represents mean ± SD. Relative density was a measure of the staining intensity of C-219 immunoreactivity corrected for inter-gel variability.
source, as well as by the inhibitor VP. This study utilized membrane vesicles collected from control and BNF-treated animals (50 mg/kg diet) from experiment 1, where treated animals displayed C219 crossreactivity with ~ 80 kDa protein. In control animals, there was a composite 7.8-fold increase in the amount of VBL accumulated in the vesicles following the addition of ATP and the ATP regenerating system (1.56 ± 0.32 pmol VBL/mg protein), and a 58% decrease with VP addition (0.66 ± 0.22 pmol VBL/mg protein) (Figure 4.10). A similar profile was seen with vesicles from BNF-treated animals. A composite 1.9-fold increase in the amount of VBL accumulation was detected following the addition of ATP and the ATP regenerating system (1.39 ± 0.44 pmol VBL/mg protein), and a 81% decrease was observed with the addition of the inhibitor VP (0.27 ± 0.17 pmol [3H]-VBL/mg protein) (Figure 4.10).

In the second in vitro transport experiment, different from the previous transport study, there was essentially no transport of VBL occurring in either treatment group. Membrane vesicles used in this experiment were collected from control and VIN-treated catfish (20 µg/kg diet) from experiment 3, which displayed C219 crossreactivity with ~ 170 kDa protein. Minimal VBL accumulation was detected in both treatments without and with the addition of ATP. For both control and VIN-exposed animals, no VBL accumulation beyond background levels was observed (Figure 4.11).

**DISCUSSION**

These studies have identified in the catfish intestine a 170 kDa protein immunochemically related to the mammalian pgp. Previous immunohistochemistry studies have shown localized C219 reactivity in the intestinal epithelium of a number of fish...
Figure 4.10- Characterization of [$^3$H]-vinblastine (VBL) accumulation in mixed membrane vesicles from distal intestine mucosa collected from controls and catfish exposed to β-naphthoflavone (BNF) at 50 mg/kg of diet for 10 days (semi-synthetic diet at 1% body weight, collected with PMSF). Pooled membrane vesicle protein from 3 animals was incubated for 20 min with: a) VBL (8 nM [$^3$H]-VBL), b) VBL + ATP (1 mM ATP, 3 mM creatine phosphate, 30 μg/ml creatine kinase), and c) VBL + ATP + VP (5 μg/ml verapamil). Values are means of triplicate measurements. Data was corrected for non-specific binding (30 sec incubations).
Figure 4.11- Characterization of $[^3]$H-vinblastine (VBL) accumulation in mixed membrane vesicles from distal intestine mucosa of catfish collected from controls and animals exposed to vincristine (VIN) at 20 μg/mg of diet for 16 days (commercial trout chow at 3% body weight, collected with protease inhibitor cocktail). Pooled membrane vesicle protein from 3 animals was incubated for 20 min with: a) VBL (8 nM $[^3]$H-VBL), b) VBL + ATP (3 mM ATP), and c) VBL + ATP + VP (5 μg/ml verapamil). Values are means of triplicate measurements. Data was corrected for non-specific binding (30 sec incubations).
species (Hemmer et al., 1995; Hemmer et al., 1998; Kleinow et al., 2000), in a
distribution pattern very similar to mammalian species (Thiebaut et al., 1987; Thiebaut
et al., 1989; Cordon-Cardo et al., 1990). The catfish protein described in experiments 2
and 3, is within the molecular weight range for C219 reactive proteins described both
in aquatic (135 to 240 kDa) (Minier et al., 1993; Cornwall et al., 1995; Waldmann et
al., 1995; Galgani et al., 1996) and mammalian (130 to 170 kDa) species (Kartner et
al., 1985). The detection of a single band in the expected molecular weight, and the
specificity of C219, which recognizes highly conserved pgp epitopes (Georges et al.,
1990), support previous evidence from immunohistochemistry studies that indicate
that a pgp-mediated mechanism is present in the intestine of catfish (Kleinow et al.,
2000).

Initial immunoblot studies described herein have shown C219 reactivity with a
protein of approximate molecular weight of 80 kDa. A wide number of proteins
distinct from the 170 kDa, but related to the MDR phenotype have been described
(Vendrik et al., 1992), and C219 crossreactivity with some of these proteins, usually of
lower molecular weight, have been demonstrated (Kawai et al., 1994). There have also
been reports of expression of half-MDR proteins assembling as heterodimers, and
being implicated in peptide transport (Bahram et al., 1991). It is likely that the ~ 80
kDa protein detected in the present studies is a degradation product of the 170 kDa,
considering that methodological modifications (addition of protease inhibitor cocktail
during sample collection and changes in sample preparation) resulted in the
disappearance of the low molecular weight band. Fragmentation of full-length pgp has
been shown to occur in human pgp-containing membranes following treatment with trypsin, which resulted in the formation of several pgp fragments in the 30 to 60 kDa range (Nuti et al., 2000). The consistency and specificity of the ~ 80 kDa band detection in the intestinal mucosa of catfish suggest that breakdown may be occurring on a specific site of the protein, which could be more susceptible in catfish than other species. One potential site is the linkage region of pgp, which reportedly divides the protein in two mirror halves, and is one of the least conserved regions across species (Gros et al., 1986; Ueda et al., 1987; Gros et al., 1988; van der Bliek et al., 1988; Gottesman and Pastan, 1993). These assumptions, however, are only speculative at this time, and further studies are needed to clarify the significance of these findings.

A noteworthy finding was that protein degradation, resulting in the detection of the ~ 80 kDa band was seen in samples from catfish treated with BNF, TCB, and BaP, but not in controls. Apparent increases in crossreactivity with the ~ 80 kDa protein in treated animals may have been a result of a nearly equal cleavage of the 170 kDa protein. In control animals, gel electrophoresis of samples not subjected to boiling resulted in the detection of the 170 kDa. Studies with mammalian MDR cells have shown that variations in electrophoretic mobility and quantitation of pgp may occur with variations in sample preparation and electrophoretic conditions (Greenberger et al., 1988). Increases in molecular mass (Greenberger et al., 1988) and formation of aggregates which cannot migrate in the gel (Yoshimura et al., 1989; Staats et al., 1990) are some of the changes that have been associated with boiling the sample prior to loading. For those animals that were treated in vivo with dietary BNF, TCB and BaP, a
combination of methodological alterations other than removal of the boiling step prior
to electrophoresis (exclusion of β-mercaptoethanol from sample buffer, and use of a
protease inhibitor cocktail during sample collection) were required for elimination of
the ~ 80 kDa band. The mechanism by which in vivo exposure to the dietary
xenobiotics examined herein altered the pgp-related protein and increased the
susceptibility to breakdown is unknown at this time. Because breakdown was observed
with all treatments examined, it is possible that xenobiotic exposure resulted in non­
specific intestinal toxicity, which in turn may have released additional intestinal
proteases or potentiated their effect on intestinal tissues. Non-specific intestinal
toxicity resulting from dietary exposure to TCB (Doi et al., 2000) and BNF (see
Chapter 3) have been observed in the catfish intestine, but how or whether or not the
extent of such exposures results in alterations at the subcellular level remains to be
investigated.

Vincristine, an anti-cancer Vinca alkaloid, has been shown to be a substrate for
pgp in MDR cell lines (Fojo et al., 1985; Naito and Tsuruo, 1989; Sehested et al.,
1989). In addition, exposures to stepwise concentrations of VIN have been used in the
development of cell lines displaying varying levels of MDR (Licht et al., 1991). In
vivo studies in rats have shown that intraperitoneal administration of VIN (1 mg/kg)
had no effect on intestinal or hepatic MDR mRNA levels (Vollrath et al., 1994). The
absence of significant effect of dietary VIN on pgp-related reactivity in the catfish
intestine, in the present study, would suggest that this compound is not associated with
intestinal pgp induction in this fish species. Early studies, however, have shown an
apparent induction of intestinal C219 reactivity in catfish, detected by immunohistochemistry and image analysis, following a comparable dietary VIN exposure (Kleinow et al., 2000). Differences in sensitivity and specificity between methods of detection could account for the observed disparity in pgp-related responses in catfish intestine. Immunohistochemical detection at the single-cell level has been shown to be a more sensitive method when compared to immunoblot, especially at low levels of protein expression (van der Heyden et al., 1995; Beck and Grogan, 1997). Another possibility is that C219 non-specific reactivity concealed pgp-related differences in immunoblot detection. Pgp-related immunoreactivity in the intestine is not exclusively restricted to the intestinal epithelium brush border where transport activity is thought to occur. The collection of intestinal mucosa by scraping, as used in the present study may have included elements of the submucosa and muscular layers. Dispersed immunoreactivity to C219, apart from the epithelium, has been shown to occur in the catfish intestine (Kleinow et al., 2000), and studies in mammals have shown that C219 reacts with the heavy chain in myosin (Terao et al., 1996). Relative variation in myosin content in the samples, in turn, could have contributed to interanimal variability. The reported molecular weight of mammalian muscle proteins that cross-react with C219 is in the 200 kDa range (Thiebaut et al., 1989), but non-specific cross-reactivity with C219 in the catfish intestine has not been investigated. More information on cross-reactivity with C219 in fish tissues are needed for full evaluation of its significance on the findings here described.
Pgp has been previously associated with a generalized stress response. Increases in MDR mRNA in human carcinoma cell lines were observed following exposure to thermal and chemical insults (e.g., sodium arsenite), a response which was attributed to a heat-shock responsive element in the MDR promoter region (Chin et al., 1990b; Kioka et al., 1992). Similar observations have been made in mussels, where pgp-related expression (C219 immunoreactivity) and transport (verapamil-sensitive rhodamine accumulation) in gill tissues were modulated by exposure to thermal and chemical (e.g. sodium arsenite) stressors (Eufemia and Epel, 2000). It is possible that the pgp-related responses in the catfish intestine here described differ from earlier studies due to stress. While there is no evidence for such a change, animal lots may have been exposed to different environmental stressors, which in turn may have influenced the pgp-related response to subsequent xenobiotic exposures.

A great deal of effort has been directed in the identification of specific agents that induce pgp expression. The results obtained have showed that the regulation of pgp induction is apparently very complex, and dependent on numerous factors, including type and length of drug exposure, species, tissues, and experimental model. For example, studies with rodent cell lines derived from both tumors and normal tissues (kidney, ileum, liver) have shown that increases in MDR mRNA occurred following exposure to the cytotoxic drugs adriamycin and daunomycin, but not colchicine (Chin et al., 1990a). MDR expression in human tissues examined in this same experiment were unresponsive to exposure to all cytotoxic drugs examined. In contrast, in vivo studies in rats have shown that parenteral exposure to colchicine, resulted in increases
in MDR mRNA expression in liver (Vollrath et al., 1994). These induction related effects appear to be restricted to the liver, as similar findings were not observed in other organs (e.g. kidney, adrenal gland). The regulation of pgp induction by xenobiotics appears to be as complex as seen with cytotoxic drugs. BNF administration to rats (40 mg/kg/day i.p., for 3 consecutive days) resulted in no response in MDR expression in enterocytes, at the mRNA and protein levels (Lown et al., 1996). In contrast, apparent induction of intestinal pgp-related reactivity was demonstrated in catfish following dietary BNF exposure (10 mg/kg for 16 days) (Kleinow et al., 2000). Administration of a chargrilled meat diet, rich in polycyclic aromatic hydrocarbons (PAHs), including BaP, had no effect on pgp expression in human intestine (Fontana et al., 1999), while BaP resulted in increased MDR mRNA levels in rat liver epithelial cells (Fardel et al., 1996). Likewise, modulation of transport of pgp substrates by Diesel-2 oil (rich in PAHs) have been shown to occur in various aquatic organisms, including snails (Kurelec et al., 1995), clams (Kurelec et al., 1996), and fish (Kurelec, 1995). The interaction between BaP and pgp is confounding not only in regards to induction, but also in the ability of pgp to transport BaP. Studies in humans have shown BaP to be a substrate for pgp in breast cancer cells (Yeh et al., 1992) and membrane vesicles from intestinal epithelium (Penny and Campbell, 1994). Contrasting results were obtained both in vitro (using pig kidney epithelial cell lines and their derivatives which express MDR genes) and in vivo (using wild-type and MDR knockout mouse) where BaP intracellular accumulation was not affected by pgp (Schuetz et al., 1998). Inconsistent results were also obtained with in vitro studies in mussels examining Aroclor 1254 (a
commercial PCB mixture which contains TCB) as a substrate for pgp transport. These studies, utilizing a fluorescent dye assay, have showed that Aroclor 1254 does not appear to influence pgp-related transport in one species of mussel (*Mytilus californianus*), but acts as a substrate or inhibitor in another species (*Mytilus galloprovincialis*) (Cornwall et al., 1995; Galgani et al., 1996). It remains to be clarified whether such disagreement result from species, tissues, dosage, or other experimental differences.

With the exception of VIN, a known substrate for pgp, all other xenobiotics examined (BNF, BaP, and TCB) have been shown to induce CYP1A in the intestine of a number of fish species (Van Veld et al., 1988a; Van Veld et al., 1988b; Kleinow et al., 1998; Doi et al., 2000). The lack of detectable pgp-related induction in the catfish intestine by those CYP1A inducers examined is consistent with a mechanism of pgp regulation distinguishable from the Ah receptor pathway, as hypothesized for mammalian species (Gant et al., 1991; Schuetz et al., 1995). Because the response of pgp to CYP1A inducers have been shown to be inconsistent, more information is needed before generalizations regarding the interaction between this two systems can be made.

Transport assays utilizing vinblastine (VBL) as a substrate have demonstrated contrasting results. The first transport assay demonstrated that intestinal membrane vesicles of control and BNF-treated catfish are capable of VBL transport with classic functional MDR characteristics of ATP-dependency and verapamil-sensitivity (Horio et al., 1988; Doige and Sharom, 1992). Trends of increased transport following ATP
addition to the system, as well as decreased transport following VP introduction were comparable in both controls and BNF-treated animals. Similar ATP dependency of VBL transport was shown to occur in intestinal brush border membrane vesicles of adult frogs, which was also inhibited by the addition of verapamil (Castillo et al., 1995). Likewise, functional transport of pgp substrates, reversible by addition of pgp inhibitors, have been previously described in gill tissues of aquatic species, including snails, mussels, and clams (Cornwall et al., 1995; Kurelec et al., 1995; Waldmann et al., 1995; Galgani et al., 1996; Kurelec et al., 1996). Conversely, transport assays conducted with a second set of controls and VIN-treated catfish showed no indication of VBL transport. Two major methodological differences introduced in the second set of experiments may have diminished VBL transport in vesicles. First, it is possible that protease inhibitor cocktail used for sample collection may have competitively inhibited VBL transport. Evidence has been presented that newly developed protease inhibitors are substrates for pgp (Kim et al., 1998; Zhang et al., 1998). Additional studies examining individual constituents of the protease inhibitor cocktail used herein are needed to investigate these assumptions. Another possibility is that the commercial chow, fed to the animals used in the second transport assays may have interfered with pgp-related transport. In vitro studies in rats have shown that transport of the pgp substrate etoposide was increased in everted gut sacs pretreated with a natural rodent diet, compared to an artificial diet (Lo and Huang, 1999). These results were attributed to a possible inhibition of pgp efflux by flavonoid-related dietary components present in natural diets. It is possible that residues of constituents of commercial fish feed that
interfere with pgp-related transport may have affected the functionality of the in vitro system used in the present study, but this assumption needs to be further investigated.

The controversy regarding the response of pgp to xenobiotics suggests that protection against xenobiotic toxicity may not be the major or only function of this protein. It is likely, however, that pgp is somehow associated to survival, due to its highly conserved nature and broad distribution across a variety of species. A physiological function that has been associated with this transporter, which would have relevance for aquatic species, is a role in cell volume regulation, through the activation of a chloride channel (Valverde et al., 1992). It appears that cells expressing MDR have a faster compensatory response for decreases in cell volume, resulting from changes in tonicity, through activation of cell-swelling chloride channels. Another pertinent physiological role of pgp is related to peptide transport. There have been reports of expression of half-MDR proteins, assembling as heterodimers, that function as a peptide transporter (Bahram et al., 1991). These peptide transporters belong in the multidrug-resistance family and share approximately a 30% amino acid sequence identity with human MDR1. It is possible that pgp-related transporters in fish may play a role in protein absorption. Studies with various teleost species (Noaillac-Depeyre and Gas, 1979; Stroband and Kroon, 1981; Lied and Solbakken, 1984) have shown that protein absorption by endocytosis occurs in the distal intestine, by a highly developed vacuolar system in the epithelial cells. Future investigations of these associations would begin to clarify the physiological role of intestinal pgp in aquatic species.
In summary, immunoblot studies contained herein demonstrate that a protein immunochemically related to the mammalian pgp transporter is present in the catfish intestine. The intestinal pgp of catfish has an approximate molecular weight of 170 kDa and is very susceptible to degradation. Pgp-related immunoreactivity, as detected by immunoblotting, was not apparently altered by the in vivo dietary administration of the pgp substrate VIN, or the CYP1A inducers BNF, BaP and TCB. These findings have produced conflicting results with previous immunohistochemistry studies, suggesting that method sensitivity and pgp localization may be at issue. Functional analysis, showing classic MDR characteristics such as energy-dependent and VP-sensitive ability of membrane vesicles to transport VBL, suggest that the immunoreactive protein identified in the catfish intestine is not only immunologically, but also functionally related to the mammalian MDR. Furthermore, these studies indicate that components in the protease inhibitor cocktail or the commercial diet used herein, may inhibit pgp-related transport in intestinal vesicles of catfish.

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CHAPTER 5: SUMMARY AND CONCLUSIONS

Fish inhabiting polluted environments are exposed to a variety of contaminants in the diet, including the ubiquitous polychlorinated biphenyls (PCBs). The extent of systemic toxic and biochemical effects of dietary PCBs are directly related to the amount of chemical that is taken up by the consumer. Therefore, the understanding of factors that influence the systemic bioavailability of PCBs from the gastrointestinal tract becomes essential in the determination of the impact of dietary exposures. The studies described here examined various factors which would modulate the bioavailability of the coplanar PCB, 3,4,3',4'-tetrachlorobiphenyl (TCB) in the intestine of the catfish, including lipid micelle composition, contaminant load, nonspecific toxicity, and intestinal biotransformation, as well as the effect of TCB upon the vectorial transporter p-glycoprotein.

Initial in vitro and in situ experiments demonstrated that micelle fatty acid composition affected both the solubility of [14C]-TCB in lipid micelles, as well as the bioavailability of [14C]-TCB from the intestine. These studies indicate that variations in fatty acid composition in the intestinal lumen, which will vary according to the fat content of the diet, may greatly affect the extent of lipophilic contaminants uptake from the intestine. These factors are especially relevant for fish species, as seasonal variations in temperature and diet have shown to affect constituent fatty acids chain length, and even degree of saturation.

Sequential in situ studies demonstrated that a 10-day dietary exposure to TCB at either 0.5 or 5 mg/kg diet resulted in equivalent decreases in the systemic
bioavailability of a subsequent $[^{14}\text{C}]$-TCB exposure. These alterations in $[^{14}\text{C}]$-TCB bioavailability appeared to occur from the combined effect of decreased transport of $[^{14}\text{C}]$-TCB to the blood and increased retention in the lumen. One sensible explanation for this event would be that tissue accumulation of unlabeled TCB could have altered the concentration gradients of $[^{14}\text{C}]$-TCB in comparison to controls, resulting in decreased lumen to blood gradient. One interesting finding which may be involved with altered transport of $[^{14}\text{C}]$-TCB to the systemic circulation, is the significant lower intestinal mucosa cytosolic $[^{14}\text{C}]$-TCB molar equivalent (Meq) values (composite of parent $[^{14}\text{C}]$-TCB and metabolites). This observation is consistent with the saturation of unidentified xenobiotic transporters by unlabeled TCB which, in turn, competitively decreased the transfer of $[^{14}\text{C}]$-TCB to the systemic circulation.

Alterations in systemic bioavailability of $[^{14}\text{C}]$-TCB following TCB pre-exposure did not strictly correlate with alterations in metabolic activities, as cytochrome P4501A content and aryl hydrocarbon hydroxylase (AHH) activities were induced, but highly variable, only in animals fed the 5 mg TCB/kg dose in vivo. A noteworthy finding was that cytochrome P4501A (CYP1A) content correlated in a linear fashion to AHH activities, suggesting that the differential CYP1A induction seen in the higher TCB dose group was most likely related to differences in the amounts and bioavailability of ingested TCB, rather than inhibitory effects, which have been reported to occur in other fish species treated with high TCB doses. Another interesting feature of $[^{14}\text{C}]$-TCB intestinal biotransformation was that even though single pass metabolism through the intestinal mucosa appeared to be a minor event,
the predominant metabolites formed (2-OH-3,4,3',4'-TCB and 4-OH-3,5,3',4'-TCB) differed from profiles described for mammals and the liver of scup, a fish species. These findings indicate that the pathways of [\(^{14}\)C]-TCB metabolism may be different in catfish, which would be consistent with previous assumptions that CYP1A probably corresponds to a small fraction of total P450 content in the intestine of catfish.

Additional *in situ* studies utilizing the prototypic CYP1A inducer β-naphthoflavone (BNF) at either 10 or 50 mg BNF/kg diet for 10 days resulted in no significant effect on the subsequent systemic bioavailability of a [\(^{14}\)C]-TCB dose from the intestine. These studies further confirmed that metabolism nor histological effect played a major role in the modulation of the *in situ* systemic bioavailability of [\(^{14}\)C]-TCB, as seen with previous TCB preexposures. Additionally, the similarity in the histological effects of dietary BNF to previously seen with TCB exposures indicated that this changes were not chemical-specific, and unlikely to have influenced the subsequent systemic bioavailability of [\(^{14}\)C]-TCB. These findings collectively supported the assumption that decreases in [\(^{14}\)C]-TCB bioavailability following TCB preexposure were diffusion-related.

Studies examining the vectorial transporter p-glycoprotein have showed that pgp in the catfish intestine holds structural and functional similarities with pgps described in other species. C219 monoclonal antibody crossreactivity with a ~170 kDa protein was seen in immunoblot analysis utilizing membrane vesicles of intestinal mucosa of catfish. Detection of this protein was complicated by its apparent susceptibility to degradation, as to preclude the use of standard Western Blot
methodology. In addition, this protein did not appear to be induced by \textit{in vivo} dietary exposures to the pgp inducer vincristine, or the CYP1A inducers TCB, BNF or benzo(a)pyrene. These results conflict with previous reports that demonstrated, using immunohistochemistry and image analysis, intestinal pgp induction in catfish by some of these same compounds. Once again, methodological differences may be at issue. Functional analysis indicated that this catfish protein had classic pgp transport characteristics, such as ability to accumulate the pgp substrate vinblastine in an ATP-dependent, verapamil-sensitive manner. However, transport may be affected by protease inhibitors used during tissue processing, or by unidentified compounds in commercial fish diets.

In summary, these studies have shown that micelle lipid composition and \textit{in vivo} dietary exposure to TCB played a role in the modulation of the systemic bioavailability of \textsuperscript{14}C-TCB. \textsuperscript{14}C-TCB systemic uptake appears to be affected by a prior TCB exposure in a dose-independent fashion, which appears to be unrelated to metabolism or CYP1A-related induction. First pass intestinal biotransformation was evident, with several hydroxylated metabolites being produced, however, metabolism was unrelated to CYP1A induction and was a quantitatively minor event. Prior exposure to the CYP1A inducer BNF confirmed that systemic bioavailability of \textsuperscript{14}C-TCB is apparently unrelated to metabolism, or to non-specific toxic effects in the intestine. These latter findings support the diffusional character of the modulation of \textsuperscript{14}C-TCB uptake by prior exposure to TCB. Studies with the catfish pgp indicate that
this protein may be functional in the catfish intestine, however, pgp was not responsive to dietary TCB, BaP, or BNF under the conditions examined.
Dear Ms. Willis,

I am a graduate student at Louisiana State University finishing my doctoral program. One of my manuscripts was accepted for publication in Toxicological Sciences (Effect of micelle fatty acid composition and 3,4,3',4'-tetrachlorobiphenyl (TCB) exposure on intestinal [14C]-TCB bioavailability and biotransformation in channel catfish in situ preparations. ToxSci #99-266), and I am trying to obtain written copyright permission from Oxford University Press to include it in my dissertation (a requirement of my Graduate School).

I would appreciate it if you could assist me in this matter. Please contact me if you have any question.

Thank you for your assistance,

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Adriana Maria Doi

Major Field: Veterinary Medical Sciences

Title of Dissertation: Intestinal Bioavailability and Biotransformation of 3,4,3',4'-Tetrachlorobiphenyl in the Channel Catfish, Ictalurus punctatus

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