AN ABSTRACT OF THE THESIS OF

Claudia McLaughlin Troxel for the degree of Doctor of Philosophy in Toxicology presented on October 3, 1996. Title: Aflatoxin B_1 Metabolism, CYP1A Induction, and the Influence of CYP1A Induction on Aflatoxin B_1 Metabolism in Zebrafish.

Abstract approved by: _____
George S. Bailey

While small fish models are assuming prominence as alternative models for chemical carcinogenesis studies, little is known about the actual metabolism of carcinogens in these models. This thesis investigated the metabolism of the potent hepatocarcinogen aflatoxin B_1 (AFB_1) and its modulation by CYP1A induction in the zebrafish.

HPLC analysis showed that the major metabolites excreted into water following intraperitoneal injection of [^{3}H]AFB_1 in zebrafish were aflatoxicol, aflatoxicol-glucuronide and unreacted AFB_1. Fish intraperitoneally injected with 50-400 μg [^{3}H]AFB_1/kg body weight displayed a linear dose response for hepatic DNA-adduction at 24 hours. AFB_1-DNA adduct levels showed no statistical difference from 1 to 21 days after injection, suggesting poor adduct repair in this species. DNA binding in female fish was 1.7-fold higher than in males. An *in vitro* AFB_1 metabolism assay verified that zebrafish liver supports AFB_1 oxidation to the 8,9-epoxide proximate electrophile.

Investigations were conducted assessing CYP1A response in zebrafish. Intraperitoneal injection of 75-150 mg Aroclor 1254/kg body weight or dietary exposure to 500 ppm failed to induce CYP1A protein or associated 7-ethoxyresorufin-*O*-deethylase (EROD) activity. However, dietary β-naphthoflavone (βNF) at 500 ppm induced CYP1A approximately 3-fold above controls. A single intraperitoneal injection of 150 mg βNF/kg body weight showed maximum CYP1A protein levels between 24 to 36 hours, while EROD activity peaked before 48 hours. CYP1A showed dose-responsiveness in fish intraperitoneally injected with 25-150 mg βNF/kg body weight. Dietary exposure to 0.75 ppm 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) significantly induced CYP1A.

The effect of TCDD on the metabolism of AFB_1 in zebrafish was examined. In addition to aflatoxicol, aflatoxicol-glucuronide, and parent AFB_1 excreted by the control AFB_1 group, the TCDD-pretreated group also produced unresolved aflatoxin M_1/aflatoxicol-M_1 and aflatoxicol-M_1-glucuronide. Hepatic AFB_1-DNA adduction was approximately 4-fold higher in the TCDD treated group compared to controls. This significant difference could not be explained by increased capacity for bioactivation of AFB_1 as measured by an *in vitro* AFB_1 metabolism assay. However, it was demonstrated that zebrafish have the capacity to bioactivate aflatoxin M_1 to a reactive intermediate, and it is proposed that this intermediate may be responsible for the increased DNA binding.

AFLATOXIN B_1 METABOLISM, CYP1A INDUCTION, AND THE INFLUENCE OF CYP1A INDUCTION ON AFLATOXIN B_1 METABOLISM IN ZEBRAFISH

by

Claudia McLaughlin Troxel

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented October 3, 1996
Commencement June 1997

Doctor of Philosophy thesis of Claudia McLaughlin Troxel presented on October 3, 1996

APPROVED:

Redacted for Privacy

Major Professor, representing Toxicology

Redacted for Privacy

Chair of Toxicology Program

Redacted for Privacy

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorized release of my thesis to any reader upon request.

Redacted for Privacy

Claudia McLaughlin Troxel, Author

ACKNOWLEDGEMENTS

This thesis project would not have been possible without the contributions of many people. I thank George Bailey for being my advisor, for always making the time to discuss my work, and for his excellent tutelage in the writing of the manuscripts and my thesis. I thank Ashok Reddy for being such a fine mentor, including helping in the design of some of the experiments, teaching me valuable laboratory techniques, and his always helpful comments and discussions. I thank Shelley Su for teaching me the skill of Western Blotting, and for the many probing scientific discussions. I thank Ulrich Harttig, Patricia Loveland, and Patricia O'Neal for their technical assistance, and acknowledge all of the people who assisted in the sampling of the fish for my experiments, including Dan Arbogast, Jean Barnhill, Ashok Reddy, Hsi-Wen Tsai, Tom Miller, Jan Spitsbergen, Chance McDonald, Patricia O'Neal, and Jerry Hendricks. I also thank Tom Miller for his excellent care and maintenance of the zebrafish. I thank Cliff Pereira for his help in the statistical analysis of the data, and appreciate all the help from the support staff in the Marine/Freshwater Biomedical Sciences Center and in the Department of Food Science and Technology. I thank Dr. Buhler, Dr. Reed, Dr. Rossignol, Dr. Ryan, and Dr. Williams for serving on my committee.

I can't even begin to thank my husband, Patrick, and our special daughter, Savannah, for always being there for the good and bad days, and for not letting me lose sight of the most important things in life. I would also like to acknowledge my parents, Jim and Claudia McLaughlin, for all of their support and encouragement, and for teaching me that anything is possible. Claudia

CONTRIBUTIONS OF AUTHORS

Dr. Ashok Reddy and Patricia O'Neal were involved in the experimental design used in Chapter 2. Dr. Reddy also assisted in the analytical methods employed. Dr. Jerry Hendricks was the principal investigator for the grant supporting this research. Dr. Buhler's lab provided technical assistance in the EROD assay, and supplied the primary antibody used for CYP1A quantification (Chapter 3).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

AFLATOXIN B_1 METABOLISM, CYP1A INDUCTION, AND THE INFLUENCE OF CYP1A INDUCTION ON AFLATOXIN B_1 METABOLISM IN ZEBRAFISH

CHAPTER 1

INTRODUCTION

Fish are becoming an increasingly important model in science. While the first studies investigating metabolism in fish suggested low or no biotransformation capabilities, subsequent investigations have shown that is not the case. Not only do fish possess many of the metabolizing enzymes that mammals do, but they also are susceptible to the carcinogenic process. Fish were found to be responsive to carcinogens when an epizootic outbreak of liver tumors developed in hatchery-reared trout. The carcinogen responsible for the tumors was identified as the potent mycotoxin aflatoxin B_1 (AFB_1), which was a contaminant of the diet. The rainbow trout has since been shown to be the most sensitive species to aflatoxin's carcinogenic effects (Bailey *et al.*, 1996). Tumors have also been found in feral fish populations, particularly bottom feeding fish. For many of these cancer epizootics, correlations have been made between the presence of neoplasia and environmental contamination, especially in bottom-feeding fish (Moore and Myers, 1994; Hawkins *et al.*, 1995). For example, polycyclic aromatic hydrocarbons (PAHs) have been linked to hepatic lesions in English sole from Puget Sound and brown bullhead in the Black River (Hawkins *et al.*, 1995, and references therein). It was also discovered that when fish are exposed to some of

these compounds, such as the PAHs and polychlorinated biphenyls (PCBs), a particular enzyme, cytochrome P4501A, becomes induced, and can serve as a marker of recent exposure.

Many species of small fish are currently being developed as models for carcinogenesis. Rainbow trout have certainly proven to be a valuable model for carcinogenesis studies, but trout are limited by several factors including an annual spawning cycle, delayed maturity until approximately 2 years of age, and the requirement of cold water temperatures such that they are unsuitable for many *in situ* exposure protocols. Most aquarium fish, on the other hand, spawn regularly, reach maturity within months, and can withstand a wide range of temperatures. The guppy, Japanese medaka, and fathead minnow are only a few of the small fish species being investigated. While numerous studies have been and continue to be conducted investigating the susceptibility of these various species of fish to carcinogens, surprisingly few studies have explored the mechanistic basis behind the results of these exposures.

My thesis work was part of a U.S. ARMY grant assessing the suitability of zebrafish as a model for carcinogenesis. The Japanese medaka is currently the small fish of choice for these studies, but no one species is sensitive to all carcinogens. Therefore, zebrafish are being investigated for their suitability as a complimentary species. Zebrafish have the added advantage of being a popular model for the study of genetics and developmental biology. Chapter two explores *in vivo* aflatoxin B_1 metabolism and hepatic DNA-adduction after intraperitoneal administration. The experiments described therein investigate the capacity of the zebrafish for bioactivation

and detoxification of this carcinogen. It has been shown by other studies conducted in our laboratory that zebrafish are resistant to the carcinogenic effects of AFB_1 when this mycotoxin is administered in the diet.

Environmental exposures are seldom limited to just one chemical or carcinogen as they are under the carefully controlled laboratory settings. Coexposures can have a variety of effects, ranging from protection to enhancement of the toxicity or carcinogenicity of a compound. As mentioned earlier, CYP1A is an enzyme commonly elevated when an animal has been exposed to any number of 3-methylcholanthrene-type inducers. The consequence of such induction in the normal biology of the animal is not certain. Chapter 3 characterizes the induction of CYP1A in zebrafish following exposure to three different inducers; the commercial PCB mixture Aroclor 1254, the synthetic flavone β-naphthoflavone (βNF), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent inducer known. The consequence of induction of CYP1A and the other associated enzymes following dietary administration of TCDD on AFB_1 metabolism and hepatic DNA adduction is then addressed.

Chemical Carcinogenesis

One of the first documentations that an environmental agent is capable of causing cancer was presented by Sir Percival Pott in the late 1700's. He linked the high incidence of scrotal cancer in chimney sweeps to exposure to soot and coal tar. It was not until the early 1900s that Pott's hypothesis of the carcinogenic potential of the tar was tested and confirmed in a laboratory setting by Yamagiwa and Ichikawa, who found dermal application of coal tar on rabbits caused cancer (reviewed in Phillips, 1983).

Today, the American Cancer Society estimates that one in four individuals in America will develop cancer over the course of their lifetime. Doll and Peto (1981) proposed that approximately 80% of all human cancer cases in the United States are caused by dietary factors, tobacco smoke, and other environmental exposures. Because of the prominence and severity of this disease, much attention has been focused on cancer research.

While evidence of carcinogenicity in humans obtained by epidemiological studies is the most desirable, these data are not often available. Therefore, scientists have turned to laboratory animals for testing the potential of an agent to cause cancer. Rodent models have been preferred for these studies, but they have several limitations. For example, these experiments are time consuming and expensive, and therefore only a limited number of compounds can be tested per year. In addition, the mandatory use of the maximum tolerated dose in these studies calls into question the validity of the results (Ames and Gold, 1991; Ames *et al.*, 1993). Furthermore, many granting agencies are encouraging the supplementation of mammalian models with alternative models and approaches.

For the past three decades, the rainbow trout model has been investigated and developed as a nonmammalian alternative model for carcinogenesis studies (reviewed by Bailey *et al.*, 1996). The first demonstration of the susceptibility of rainbow trout to carcinogens occurred in the late 1950's and early 1960's, during an epizootic outbreak of liver tumors in hatchery-reared trout. The cause was traced to a contaminant in cottonseed oil used in the diet, which was identified as the potent mycotoxin, AFB_1 (Sinnhuber *et al.*, 1968; Ayres, *et al.*, 1971). Since then, rainbow

trout have proven to be susceptible to a number of other various carcinogens as well, including dimethylnitrosamine (Ashley and Halver, 1968; Grieco *et al.*, 1978), diethylnitrosamine (DEN) (Shelton *et al.*, 1984; Hendricks, *et al.*, 1994) and benzo[*a*]pyrene (Hendricks *et al.*, 1985).

Rainbow trout are not the only fish species susceptible to the carcinogenic process. In a hallmark study in 1965, Stanton demonstrated that DEN was capable of inducing hepatic tumors in the zebrafish. Further studies confirmed the responsiveness of zebrafish to DEN and also to nitrosomorpholine, although a water exposure to aflatoxin B_1 failed to elicit tumors (Pliss and Khudoley, 1975; Khudoley, 1984; Bauer *et al.* 1972). Surprisingly, the research on carcinogenesis in zebrafish ended with these few studies. Information on the sensitivity of many other small fish models is on the increase, however (reviewed in Hawkins *et al.*, 1995).

Carcinogenic Process

Cancer is generally believed to be a multistage process involving initiation, promotion, and progression, although much debate surrounds the details associated with these stages. The following is only a brief summary of some of the theories concerning the possible mechanisms of carcinogenesis. Most of the basic principles can be found in Williams and Weisburger (1991), Weinberg (1989a), Pitot and Dragen (1991), and Harris (1991).

Initiation

The first step of the carcinogenic process is initiation, and usually involves electrophilic interaction of a compound with DNA. There are two classifications of initiators: direct-acting carcinogens and procarcinogens. Direct-acting carcinogens are reactive electrophiles requiring no further activation, such as many cytostatic agents used in chemotherapy. Mechanisms of action include alkylation and acylation of DNA. Procarcinogens, on the other hand, require metabolic activation to a reactive intermediate, or proximate carcinogen (reviewed by Guengerich, 1992). It is this electrophilic intermediate which can then bind to macromolecules including DNA (Miller and Miller, 1981). The DNA adduct resulting from the electrophile binding to DNA may be repaired, may lead to cell death if the damage is extensive, or may become a permanent DNA mutation following replication.

The consequence of mutagenesis depends on numerous factors, including the type and position of the mutation. Point mutations occur when a single base is substituted. When a purine is substituted for another purine, or a pyrimidine for a pyrimidine, the substitution is a transition; conversely, a transversion occurs when a purine is replaced by a pyrimidine (or visa versa). Consequences of point mutations range from having virtually no effect on gene function to completely inactivating a gene product. Frameshift mutations arise when a base is inserted or deleted, resulting in a shift in the reading frame of a gene. Usually the gene product is nonfunctional. Not only is the type of mutation important, but the location of the mutation on the DNA is also crucial. If it occurs in a non-critical portion of the gene, such as on a non-transcribed region, there may be no consequence. Mutations leading to the activation of oncogenes or the

inactivation of tumor suppressor genes, on the other hand, can have a substantial impact, which will be discussed in the following section. Once a gene containing a mutagenic DNA lesion undergoes a round of replication without repair, the mutation becomes fixed and the change is irreversible.

Promotion

The initiated cell can remain dormant until appropriate factors, such as hormones, viruses, or chemicals, stimulate cellular proliferation. This reversible process is called promotion, and permits the clonal expansion of cells to a preneoplastic lesion. Increased cellular proliferation also decreases the available amount of time for repairing DNA, possibly leading to genomic instability (Swenberg, 1993). Activation of oncogenes and/or inactivation of tumor suppressor genes (or antioncogenes) is believed to play a role in the transformation to neoplasia.

Proto-oncogenes are normal constituents of the cell that are important for cellular function, but when activated, appear to play a central role in the carcinogenesis process. Proto-oncogenes can be activated to oncogenes by mutations, gene amplification, or chromosomal translocations (reviewed in Ladik and Förner, 1994). Such activation can lead to a loss of growth regulation and differentiation of the cell (reviewed in Smith *et al.*, 1993). There is speculation that activation of at least two oncogenes is required for the full neoplastic conversion of a cell, although not all the evidence supports this model (Weinberg, 1989b). A family of oncogenes known as *ras* oncogenes are commonly mutated at codons 12, 13, and 61 in human tumors, but the percentage of *ras* mutations varies greatly depending on the tumor type (Bos, 1989). Importantly, *ras* mutations are

not limited to just human tumors, but have been found in tumors in many other species including fish (Chang *et al.*, 1991; Van Beneden and Ostrander, 1994).

Tumor suppressor genes or antioncogenes are also essential for normal cellular function, and their numerous functions include regulating apoptosis and growth, DNA synthesis and repair, involvement in intercellular communication, and protease inhibition (Harris, 1991; Greenblatt *et al.*, 1994). In contrast to the oncogenes, inactivation of the tumor suppressor gene is required for cellular transformation, and requires the loss of function of both alleles. The *p53* suppressor gene is currently one of the most commonly mutated genes detected in human tumors, and unlike the *ras* mutations, *p53* mutations have been discovered in over 30 codons (Purchase, 1994; for review see Greenblatt *et al.*, 1994). However, there has often been a rather poor correlation between species in type and frequency of *ras* mutations and, in particular, *p53* mutations (McMahon, 1994; Eaton and Gallagher, 1994).

Progression

The irreversible transformation of benign, preneoplastic lesions to malignant neoplasms is termed progression. During this final stage of carcinogenesis, the neoplastic cells frequently invade surrounding tissues and organs and enter the lymphatic or vascular system to be carried to other sites in the body for implantation (metastasis). Malignant neoplasms are associated with karyotypic changes, lack of cellular differentiation, metastasis, increased growth rate, and a heterogenous cell population (Weinberg, 1989a; Robbins and Kumar, 1987). Activation and/or inactivation of critical genes is believed to play an important role in this final stage of carcinogenesis

as well, but there is still much to learn about which genes are actually involved. The future holds much promise in further elucidating more of the many important factors associated with this disease.

Biotransformation in Fish

Although it had been originally hypothesized that aquatic organisms were incapable of metabolizing xenobiotics, numerous studies have since proven the contrary. Indeed, biotransformation of xenobiotics is just as significant in aquatic species as it is in mammals.

Phase I Metabolism

Biotransformation is usually divided into two phases: phase I and phase II metabolism. Phase I metabolism serves to add or expose functional groups, thereby increasing the compound's polarity. While the increased polarity may allow the compound to be directly excreted, phase I metabolism typically primes the xenobiotic for conjugation by a phase II enzyme. The reactions involved in phase I metabolism include oxidation, reduction, and hydrolysis, and utilize a multitude of different enzymes (Sipes and Gandolfi, 1991; Hodgson and Levi, 1994).

Organic lipophilic compounds are metabolized primarily by oxidation via the mixed function oxidases (MFOs) and flavin monooxygenase (FMO). Although these enzyme systems are more thoroughly characterized in mammalian systems, research in aquatic species is growing. FMO metabolizes primary, secondary, and tertiary amines,

and sulfur-containing compounds (Sipes and Gandolfi, 1991; Ziegler, 1993). This membrane bound enzyme requires the electron donor NADPH and the prosthetic group FAD. In fish, FMOs are important in N-oxidation reactions, such as the oxidation of the endogenous substrate trimethylamine to trimethylamine oxide (Goldstein and Dewitt-Harley, 1973; Agustsson and Strom, 1981; Stegeman and Hahn, 1994). FMO-like activity has been detected in rainbow trout (Schlenk and Buhler, 1991a; 1991b; 1993), cod (Agustsson and Strom, 1981), striped bass (Cashman *et al.*, 1990), and nurse shark (Goldstein and Dewitt, 1973), although no FMO activity was detected in channel catfish (Schlenk *et al.*, 1993).

The cytochrome P450s, a superfamily of heme proteins, are the most prominent class of phase I enzymes. These enzymes are important in the metabolism of both endogenous and exogenous substrates, including fatty acids, steroids, prostaglandins, drugs, and carcinogens. The MFO system requires the utilization of two enzymes: the heme protein, cytochrome P450, and NADPH-cytochrome P450 reductase, a flavoprotein responsible for shuttling electrons to the P450 enzyme and requiring NADPH as a cofactor. Although only one NADPH cytochrome P450 reductase enzyme has been isolated, Nebert and Nelson (1991) predict that "each mammalian species might easily have at least 60 and perhaps more than 200 individual P450 genes". Currently, P450s have been found in every phylum investigated.

In fish livers, total amounts of P450s vary from 0.1 nmol/mg to 2.0 nmol/mg protein, although average hepatic levels in untreated fish range from 0.2 to 0.5 nmol/mg (Stegeman, 1989). Several P450s have been purified or cloned from various fish species. P450s identified in rainbow trout include members of families 1A, 2K, and a

number of P450s involved in steroid metabolism such as 11, 17, and 19 (reviewed in Stegeman and Hahn, 1994). One of the catalytic functions of CYP2K1 is the bioactivation of aflatoxin B_1 to the reactive intermediate, which is capable of binding to protein, DNA, and RNA (Williams and Buhler, 1983). Topminnows appear to have a P450 with properties similar to CYP2E1, including the ability to bioactivate the procarcinogen nitrosodiethylamine (Kaplan *et al.*, 1991). Scup appear to possess CYP1A, CYP2B and a CYP3A-like isozyme (Stegeman and Hahn, 1994; Husoy *et al.*, 1994), and studies in zebrafish show expression of CYP1A- and CYP2K1-like proteins (Buchmann *et al.*, 1993; Collodi *et al.*, 1994). CYP1A has also been detected in induced cod, perch, plaice, little skate, and medaka (Stegeman and Hahn, 1994; Schell *et al.*, 1987). Immunoblots from eight fish species show that the apparent molecular weight of hepatic CYP1A in fish ranges from 54,000-59,000 daltons (Goksoyr *et al.*, 1991; Collodi *et al.*, 1994).

CYP1A is readily induced by a number of compounds including PAHs (benzo[*a*]pyrene, benzanthracenes), many flavones and indoles, 3-methylcholanthrene, and halogenated aromatic hydrocarbons (biphenyls, dibenzo-*p*-dioxins and dibenzofurans). CYP1A response in fish is currently being developed as a biomarker for certain environmental exposures (Goksoyr, 1995; Stegeman and Lech, 1991; Wirgin *et al.*, 1994). Levels of CYP1A can be assessed by measuring catalytic activity, antibody detection of protein, and cDNA probes for CYP1A mRNA. Because this P450 plays a critical role in the metabolism of aromatic hydrocarbon procarcinogens, much research is now devoted to unraveling the mechanism of induction of CYP1A and

understanding the many factors, such as temperature and reproductive status, which influence this response.

In mammals, the induction of CYP1A1 is regulated at the gene transcription level via the Ah (aryl hydrocarbon) receptor (AhR), a helix-loop-helix DNA-binding protein. In the unliganded state, the Ah receptor is found in the cytosol of the cell associated with two 90 kDa proteins (at least one is heat shock protein 90 (Hsp90)) and possibly a 46 kDa protein. Upon diffusion into the cell, the ligand binds to the Ah receptor. After ligand binding, the proteins dissociate from the complex, the receptor complex translocates to the nucleus, and the ARNT protein (Ah receptor nuclear translocator protein) binds to the transformed AhR. The order of these events and the cellular location of ARNT are currently unclear, and proteins other than Hsp90 and ARNT are believed to play a role in the process, such as proteins involved in phosphorylation/ dephosphorylation of ARNT and the Ah receptor. Once in the nucleus, the receptor binds certain regions in DNA, including the dioxin- or xenobiotic-responsive regulatory elements, (DREs, XREs) upstream of CYP1A1 in DNA, increasing the rate of transcription of this gene product (Ah receptor regulation reviewed in Hankinson, 1995; Whitlock, 1993; Okey *et al.*, 1994). It is important to note that in mammals the Ah receptor is also responsible for upregulating other enzymes (such as glucuronosyl transferase, quinone-oxido-reductase, glutathione-S-transferase, CYP1A2, and CYP2B1) and for inducing proteins involved in cell growth and differentiation (Hankinson, 1995). Evidence from studies in fish show consistency of CYP1A induction with a receptor-mediated response, and there has been a correlation between induction and the presence of the Ah receptor in fish (Stegeman, 1993; Stegeman and Hahn, 1994).

Phase II Metabolism

Phase II enzymes typically conjugate phase I metabolites with an endogenous molecule, such as a sugar moiety, a sulfate group, an amino acid, or glutathione. The addition of this conjugate increases the water solubility of the metabolite even further, commonly leading to excretion via bile or the kidneys. While there is evidence of some of the other phase II reactions, such as sulfation, occurring in fish, the most prominent piscine phase II reactions identified and studied are glucuronidation and glutathione conjugation.

Glucuronidation is just as important in fish as in mammals in the metabolism of endogenous compounds, such as sex steroids and bilirubin, and xenobiotics containing an appropriate functional group (James, 1987; reviewed in Clarke *et al.*, 1991; George, 1994). In general, uridine diphosphate glucuronosyltransferases (UDPGT) catalyze the transfer of glucuronic acid from the high energy cofactor UDP-glucuronic acid to the substrate to form *O*-glucuronides (phenols, alcohols, and carboxylic acids), *N*-glucuronides (carbamates, sulfonamides, and aromatic amines), and *S*- or *C*-glucuronides (thiols, thiocarbamic acids, some drugs) (Dauterman, 1994; Gibson and Skett, 1986). In fish, *O*-glucuronides are the most common conjugates, followed by *N*-glucuronides. As in mammals, UDPGT activity is microsomal. While primarily located in the liver, UDPGT activity is also found in the kidney, gill and intestines (George, 1994). Benzo[*a*]pyrene, aflatoxin B_1, benzene, and tetrachlorobiphenyl are just a few of the xenobiotics that can be glucuronidated by fish (Clarke *et al.*, 1991).

Glutathione-S-transferases (GSTs) mediate the conjugation of the endogenous tripeptide, glutathione, with an endogenous or exogenous electrophilic metabolite. These ubiquitous, primarily cytosolic, enzymes exist as homo- or heterodimers. In mammals, the GSTs are currently grouped into four classes, and all but one class exhibit activity for the substrate 1-chloro-2,4-dinitrobenzene (CDNB) (George, 1994; Dauterman, 1994). Following conjugation with glutathione, the metabolite may be directly excreted, or more typically, further metabolized to a mercapturic acid and then excreted. All fish examined so far demonstrate activity for CDNB, and GSTs have been purified in several fish species (reviewed in George, 1994). It appears from these studies that fish also have multiple isoforms consisting of two subunits. Tissue-specific expression of the various GSTs appears to depend on both the specific isoform and fish species, but the GSTs are predominately found in liver, kidney, gills, and intestine (George, 1994).

Aflatoxin B_1 Metabolism and DNA Adduction

Aflatoxins are mycotoxins produced by the fungal molds *Aspergillus flavus* and *Aspergillus paraciticus*. These mycotoxins frequently contaminate improperly stored foodstuffs, such as corn and peanuts. Aflatoxin B_1 (AFB_1) is the most potent metabolite produced, and has recently been classified as a Group I carcinogen in humans (IARC, 1993). Species response to this carcinogen is varied. Mice, coho salmon, and channel catfish are fairly resistant whereas rainbow trout, ducks and rats are susceptible to AFB_1-induced carcinogenesis. Investigations have shown that such species differences

in carcinogenic response can generally be explained by differences in biotransformation of this carcinogen (Eaton *et al.*, 1994; Eaton and Gallagher, 1994).

Aflatoxin B_1 Metabolism

AFB_1 requires metabolic bioactivation to the reactive intermediate, the AFB_1-8,9-epoxide (AFBO), to exert its biological effects (Essigman *et al.*, 1982; Swenson *et al.*, 1977). It is this unstable metabolite that is capable of binding to RNA, DNA, and other cellular macromolecules. The bioactivation of AFB_1 is typically a P450-mediated reaction, although alternative pathways, such as prostaglandin H synthetase- and lipooxygenase- mediated pathways, do exist. The primary P450s responsible for bioactivation of AFB_1 in humans are CYP1A2 and CYP3A4 (Gallagher *et al.*, 1994; Shimada and Guengerich, 1989; Eaton and Gallagher, 1994). CYP2K1 catalyzes AFB_1 activation in rainbow trout, and this P450 displays higher activity towards AFBO formation than rat P450s (Williams and Buhler, 1983).

Differences in the production of the epoxide can sometimes explain the variations in sensitivity between species, such as rainbow trout compared to rat and Coho salmon (Williams and Buhler, 1983; Bailey *et al.*, 1988). However, phase II detoxification reactions also can play a crucial role. Although mice actually have a higher microsomal activity for AFBO production compared to rats, mice are resistant to AFB_1 carcinogenicity due to a high activity of glutathione-*S*-transferase toward the epoxide (Degen and Neuman, 1981; Eaton *et al.*, 1994; Eaton and Ramsdell, 1992). Glutathione conjugation of AFBO does not appear to be a significant reaction in fish (Gallagher and Eaton, 1995; Valsta *et al.*, 1988).

Other metabolic pathways include additional phase I and phase II reactions. Reduction of AFB_1 by a cytosolic reductase generates aflatoxicol (AFL), a metabolite identified in rats, rabbits, birds, and fish (Eaton *et al.*, 1994; Gallagher and Eaton, 1995; Toledo *et al.*, 1987). In mammals, O-demethylation or hydroxylation produces the metabolites aflatoxin P_1 (AFP_1), aflatoxin Q_1 (AFQ_1), and aflatoxin M_1 (AFM_1). Trout can also form AFM_1 and can further reduce it to aflatoxicol M_1 ($AFL\text{-}M_1$) (Loveland *et al.*, 1979; 1983). AFL and $AFL\text{-}M_1$ can be further metabolized by glucuronidation, a particularly important reaction in fish (Loveland *et al.*, 1984; Toledo *et al.*, 1987). While hydroxylated metabolites of AFB_1 are generally considered to be detoxification products, the metabolites AFM_1 and AFL are still carcinogenic (Eaton *et al.*, 1994; Shoenhard *et al.*, 1981; Nixon *et al.*, 1981; Bailey *et al.*, 1994). In addition, in some species, AFL serves as a reservoir for AFB_1, in that it can be oxidized back to AFB_1 for subsequent bioactivation (Salhab and Edwards, 1977; Loveland *et al.*, 1977). A summary of the AFB_1 biotransformation pathways in rainbow trout is found in Figure 1.1.

Significance of aflatoxin B_1 DNA adducts

Aflatoxin-8,9-epoxide, the reactive intermediate produced by bioactivation of AFB_1, can bind to cellular DNA, primarily forming the *trans*-8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1 adduct (Swenson *et al.*, 1977; Eaton and Gallagher, 1994; Bailey, 1994). This adduct can undergo rearrangement to a ring opened formamidopyrimidine (FAPY) derivative, which is very persistent over time. Despite its persistence, however, evidence points to the initial adduct formed being the most important in

tumorigenesis (Bailey, 1994). Molecular dosimetry studies in both rats and trout demonstrate that DNA adduct formation highly correlates with tumor response, as does AFB_1 dose with the number of DNA adducts generated. These correlations are even more powerful because they display a strong linear relationship, even at low doses, suggesting that there is not a threshold for AFB_1 genotoxicity (Bechtel, 1989; Appleton *et al.*, 1982; Choy, 1993; Dashwood *et al.*, 1992; Bailey, 1994).

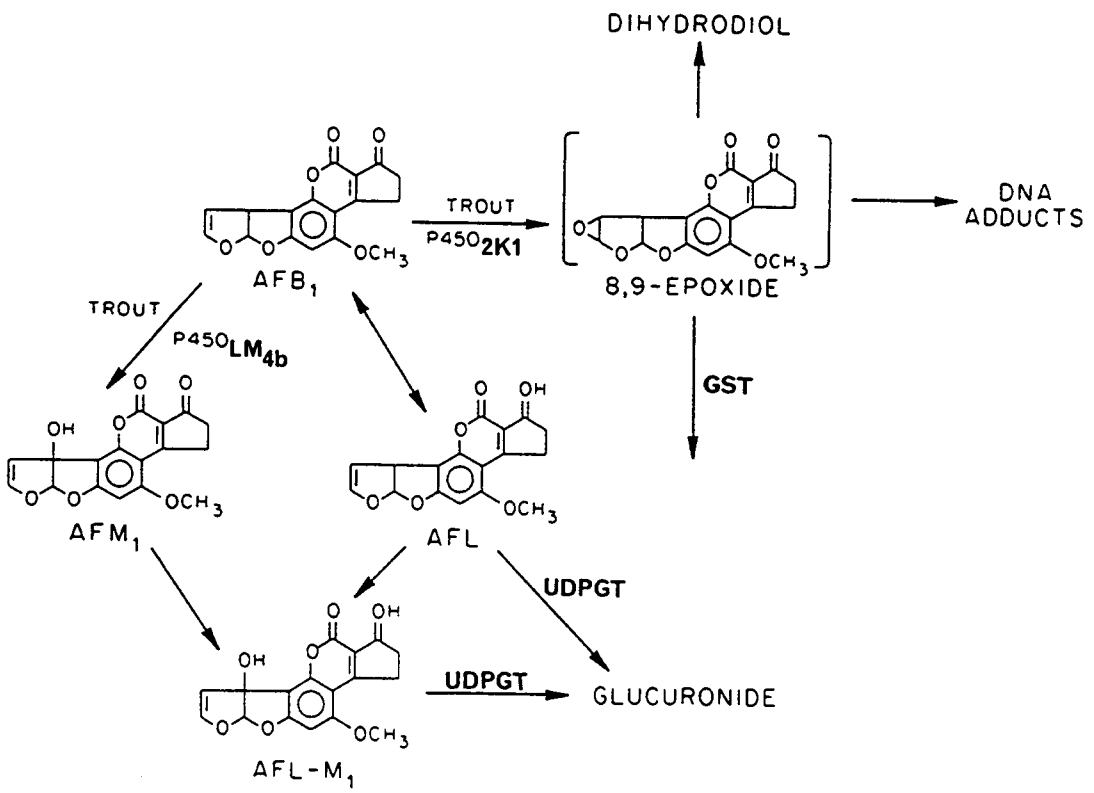

Figure 1.1 Biotransformation pathways of AFB_1 in rainbow trout. Abbreviations used: AFM_1, aflatoxin M_1; AFL-M_1, aflatoxin M_1; AFL, aflatoxicol; UDPGT, UDP glucuronosyltransferase; GST, glutathione-*S*-transferase (from Breinholt, 1994).

The location of AFB_1-induced mutagenesis again depends on the species and gene under investigation. *Ras* mutations, and in particular Ki-*ras* mutations, have been identified in both rat and trout exposed to AFB_1, with hotspots at codons 12 and 13 (Bailey, 1994; Eaton and Gallagher, 1994). Studies in humans have yet to detect activated oncogenes in populations exposed to AFB_1. It appears that inactivation of the *p53* tumor suppressor gene plays a role in humans. An association has been found between mutation in codon 249 in *p53* and liver tumors from individuals from aflatoxin-endemic areas (Harris, 1991). Further refinements in the sensitivity of techniques used in detecting mutations should help to elucidate other molecular events critical in AFB_1-induced carcinogenesis.

References

Agustsson, I., and Strom, A.R. (1981). Biosynthesis and turnover of trimethylamine oxide in the teleost cod, *Gadus morhua*. *J. Bio. Chem.* 256, 8045-8049.

Ames, B.N., and Gold, L.W. (1991). Too many rodent carcinogens: mitogenesis increases mutagenesis. *Science* 249, 970-971.

Ames, B.N., Shigenaga, M.K., and Gold, L.S. (1993). DNA lesions, inducible DNA repair, and cell division: three key factors in mutagenesis and carcinogenesis. *Environ. Health Perspec.* 101(Sup), 35-44.

Appleton, B.S., Goetchius, M.P., and Campell, T.C. (1982). Linear dose-response curve for the hepatic macromolecular binding of aflatoxin B_1 in rats at very low exposures. *Cancer Res.*, 42, 3659-3662.

Ashley, L.M., and Halver, J.E. (1968). Dimethylnitrosamine-induced hepatic cell carcinoma in rainbow trout. *J. Natl. Cancer Inst.* 41, 531-552.

Ayres, J.L., Lee,D.J., Wales, J.H., and Sinnhuber, R.O. (1971). Aflatoxin structure and hepatocarcinogenicity in rainbow trout (*Salmo gairdneri*). *J. Natl. Cancer Inst.* 46, 561-564.

Bailey, G.S. (1994). Role of DNA adducts in the cancer process. In *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance* (D.L. Eaton and J.D. Groopman, Ed.), pp 137-148. Academic Press, New York.

Bailey, G.S., Loveland, P.M., Pereira, C., Pierce, D., Hendricks, J.D., and Groopman, J.D. (1994). Quantitative carcinogenesis and dosimetry in rainbow trout for aflatoxin B_1 and aflatoxicol, two aflatoxins that form the same DNA adduct. *Mut. Res.* 313, 25-38.

Bailey, G.S., Williams, D.E., and Hendricks, J.D. (1996). Fish models for environmental carcinogenesis: the rainbow trout. *Environ. Health Perspec.* 104, 5-21.

Bailey, G.S., Williams, D.E., Wilcox, J.S., Loveland, P.M., Coulombe, R.A., and Hendricks, J.D. (1988). Aflatoxin B_1 carcinogenesis and its relation to DNA adduct formation and adduct persistence in sensitive and resistant salmonid fish. *Carcinogenesis* 9, 1919-1926.

Bauer, L., Tulsan, A.H., and Müller, E. (1972). Ultrastructural changes produced by the carcinogen, aflatoxin B1, in different tissues. *Virchows Arch. Zellpath.* 10, 275-285.

Bechtel, D.H. (1989). Molecular dosimetry of hepatic aflatoxin B_1-DNA adducts: linear correlation with hepatic cancer risk. *Reg. Toxicol. Pharmacol.* 10, 74-81.

Bos, J.L. (1989). *Ras* oncogenes in human cancer: a review. *Cancer Res.* 49, 4682-4689.

Breinholt, V. (1994). Chlorophyllin anticarcinogenesis in the rainbow trout model [Ph.D. dissertation]. Oregon State University, Corvallis, OR.

Buchmann, A., Wannenmacher, R., Kulzer, E., Buhler, D.R., and Bock, K.W. (1993). Immunohistochemical localization of the cytochrome P450 isozymes LMC2 and LM4B (P4501A1) in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-treated zebrafish (*Brachydanio rerio*). *Toxicol. Appl. Pharmacol.* 123, 160-169.

Cashman, J.R., Olsen, L.D., Nishioka, R.S., Gray, E.S., and Bern, H.A. (1990). S-oxygenation of thiobencarb (Bolero) in hepatic preparations from striped bass (*Morone saxatilis*) and mammalian systems. *Chem. Res. Toxicol.* 3, 433-440.

Chang, Y-J., Mathews, C., Mangold, K., Marien, K., Hendricks, J., and Bailey, G.S. (1991). Analysis of *ras* gene mutations in rainbow trout liver tumors initiated by aflatoxin B_1. *Mol. Carcinogen.* 4, 112-119.

Choy, W.N. (1993). A review of the dose-response induction of DNA adducts by aflatoxin B_1 and its implications to quantitative cancer-risk assessment. *Mut. Res.*, 296, 181-198.

Clarke, D.J., George, S.G., and Burchell, B. (1991). Glucuronidation in fish. *Aquat. Toxicol.* 20, 35-56.

Collodi, P., Miranda, C.L., Zhao, X., Buhler, D.R., and Barnes, D.W. (1994). Induction of zebrafish (*Brachydanio rerio*) P450 *in vivo* and in cell culture. *Xenobiotica* 24, 487-493.

Dashwood, R.H., Mariën, K., Loveland, P.M., Williams, D.E., Hendricks, J.D., and Bailey, G.S. (1992). Formation of aflatoxin-DNA adducts in trout and their use as molecular dosimeters for tumor prediction. In *Handbook of Applied Mycology* (Bhatnager, D., Lillehoj, E.B., and Arora, D.K., Eds.), pp. 183-211. Marcel Dekker, New York.

Dauterman, W.C. (1994). Metabolism of toxicants: phase II reactions. In *Introduction to Biochemical Toxicology* (E. Hodgson and P.E. Levi, Ed), pp 113-132. Appleton and Lange, Norwalk, CT.

Degen, G.H., and Neumann H-G. (1981). Differences in AFB_1-susceptibility of rat and mouse are correlated with the capability *in vitro* to inactivate aflatoxin B_1-epoxide. *Carcinogenesis* 2, 299-306.

Doll, R., and Peto, R. (1981). The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J. Natl. Cancer Inst.* 66, 1191-1308.

Eaton D.L., and Gallagher, E.P. (1994). Mechanisms of aflatoxin carcinogenesis. *Annu. Rev. Pharmacol. Toxicol.* 34, 135-172.

Eaton, D.L., and Ramsdell, H.S. (1992). Species- and diet- related differences in aflatoxin biotransformation. In *Handbook of Applied Mycology* (Bhatnager, D., Lillehoj, E.B., and Arora, D.K., Eds.), pp. 157-182. Marcel Dekker, New York.

Eaton, D.L., Ramsdell, H.S., and Neal, G.E. (1994). Biotransformation of aflatoxins. In *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance* (D.L. Eaton and J.D. Groopman, Ed.), pp 45-72. Academic Press, New York.

Essigmann, J.M., Croy, R.G., Bennett, R.A., and Wogan, G.N. (1982). Metabolic activation of aflatoxin B_1: patterns of DNA adduct formation, removal, and excretion in relation to carcinogenesis. *Drug Metab. Rev.* 13, 581-602.

Gallagher, E.P., and Eaton, D.L. (1995). *In Vitro* biotransformation of aflatoxin B_1 in channel catfish liver. *Toxicol. Appl. Pharmacol.* 132, 82-90.

Gallagher, E.P., Stapleton, P.L., Wienkers, L.C., Kunze, K., and Eaton, D.L. (1994). Role of human microsomal and human complementary DNA-expressed cytochromes P4501A2 and P4503A4 in the activation of aflatoxin B_1. *Cancer Res.* 54, 101-108.

George, S.G. (1994). Enzymology and molecular biology of phase II xenobiotic-conjugating enzymes in fish. In *Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives* (D.C. Malins and G.K. Ostrander, Ed), pp 37-85. Lewis Publishers, Boca Raton, FL.

Gibson, G., and Skett, P. (1986). *Introduction to Drug Metabolism*, Chapman and Hall, London.

Goksoyr, A. (1995). Use of cytochrome P450 1A (CYP1A) in fish as a biomarker of aquatic pollution. *Arch. Toxicol.*, Sup. 17, 80-95.

Goksoyr, A., Andersson, T., Buhler, D.R., Stegeman, J.J., Williams, D.E., and Förlin, L. (1991). Immunologic chemical cross-reactivity of β-naphthoflavone-inducible cytochrome P-450 (P450-1A) liver microsomes from different fish species. *Fish Physiol. Biochem.*, 9, 1-13.

Goldstein, L., and Dewitt-Harley, S. (1973). Trimethylamine oxidase of nurse shark liver and its relation to mammalian mixed function amine oxidase. *Comp. Biochem. Physiol.*, **45B**, 895-903.

Greenblatt, M.S., Bennett, W.P., and Harris, C.C. (1994). Mutations in the *p53* tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res.* **54**, 4855-4878.

Grieco, M.P., Hendricks, J.D., Scanlan, R.A., Sinnhuber, R.O., and Pierce, D.A. (1978). Carcinogenicity and acute toxicity of dimethylnitrosamine in rainbow trout (*Salmo gairdneri*). *J. Natl. Cancer Inst.* **60**, 1127-1131.

Guengerich, F.P. (1992). Metabolic activation of carcinogens. *Pharmac. Ther.* **54**, 17-61.

Hankinson, O. (1995). The aryl hydrocarbon receptor complex. *Annu. Rev. Pharmacol. Toxicol.*, **35**, 307-340.

Harris, C.C. (1991). Chemical and physical carcinogenesis: advances and perspectives for the 1990s. *Cancer Res.* **51**, 5023s-5044s.

Hawkins, W.E., Walker, W.W., and Overstreet, R.M. (1995). Carcinogenicity tests using aquarium fish. In *Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment* (G.R. Rand, Ed), pp 421-446. Taylor and Francis, Washington, D.C.

Hendricks, J.D., Meyers, T.R., Shelton, D.W., Casteel, J.L., and Bailey, G.S. (1985). Hepatocarcinogenicity of benzo[*a*]pyrene to rainbow trout by dietary exposure and intraperitoneal injection. *J. Natl. Cancer Inst.* **74**, 839-851.

Hendricks, J.D., Cheng, R., Shelton, D.W., Pereira, C.B., and Bailey, G.S. (1994). Dose-dependent carcinogenicity and frequent Ki-*ras* proto-oncogene dependent activation by dietary N-nitrosodiethylamine in rainbow trout. *Fundam. Appl. Toxicol.* **23**, 53-62.

Hodgson, E., and Levi, P.E. (1994). Metabolism of toxicants: phase I reactions. In *Introduction to Biochemical Toxicology* (E. Hodgson and P.E. Levi, Ed), pp 113-132. Appleton and Lange, Norwalk, CT.

Husoy, A-M, Myers, M.S., Willis, M.L., Collier, T.K., Celander, M., and Goksoyr, A. (1994). Immunohistochemical localization of CYP1A and CYP3A-like isozymes in hepatic and extrahepatic tissues of atlantic cod (*Gadus morhua L.*), a marine fish. *Toxicol. Appl. Pharmacol.* **1994**, 294-308.

International Agency for Research on Cancer (IARC). (1993). IARC monographs on the evaluation of carcinogenic risks to humans. Some naturally occurring

substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. **56**, 245-396.

James, M.O. (1987). Conjugation of organic pollutants in aquatic species. *Environ. Health Perspec.* **71**, 97-103.

Kaplan, L.A.E., Schultz, M.E., Schultz, R.J., and Crivello, J.F. (1991). Nitrosodiethylamine metabolism in the viviparous fish *Poeciliopsis*: evidence for the existence of liver P450pj activity and expression. *Carcinogenesis* **12**, 647-652.

Khudoley, V.V. (1984). Use of aquarium fish, *Danio rerio* and *Poecilia reticulata*, as test species for evaluation of nitrosamine carcinogenicity. *Natl. Cancer Inst. Monogr.* **No. 65**, 65-70.

Ladik, J., and Förner, W. (1994) *The Beginnings of Cancer in the Cell.* Springer-Verlag, Berlin, Germany.

Loveland, P.M., Coulombe, R.A., Libbey, L.M., Pawlowski, N.E., Sinnhuber, R.O., Nixon, J.E., and Bailey, G.S. (1983). Identification and mutagenicity of aflatoxicol-M_1 produced by metabolism of aflatoxin B_1 and aflatoxicol by liver fractions from rainbow trout (*Salmo gairdneri*) fed β-naphthoflavone. *Food Chem. Toxicol.* **21**, 557-562.

Loveland, P.M., Nixon, J.E., and Bailey, G.S. (1984). Glucuronides in bile of rainbow trout (*Salmo gairdneri*) injected with [^{3}H]aflatoxin B_1 and the effects of dietary B-naphthoflavone. *Comp. Biochem. Physiol.* **578C**, 13-19.

Loveland, P.M., Nixon, J.E., Pawlowski, N.E., Eisele T.A., Libbey, L.M., and Sinnhuber, R.O. (1979). Aflatoxin B_1 and aflatoxicol metabolism in rainbow trout (*Salmo gairdneri*) and the effects of dietary cyclopropene. *J. Environ. Pathol. Toxicol.* **2**, 707-718.

Loveland, P.M., Sinnhuber, R.O., Berggren, K.E., Libbey, L.M., Nixon, J.E., and Pawlowski, N.E. (1977). Formation of aflatoxin B_1 from aflatoxicol by rainbow trout (*Salmo gairdneri*) liver *in vitro*. *Res. Commun. Chem. Pathol. Pharmacol.* **16**, 167-170.

McMahon, G. (1994). The genetics of human cancer: implications for ecotoxicology. *Environ. Health Perspec.* **102**, 75-80.

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

Moore, M.J., and Myers, M.S. (1994). Pathobiology of chemical-associated neoplasia in fish. In *Aquatic Toxicology: Molecular, Biochemical, and Cellular*

Perspectives (D.C. Malins and G.K. Ostrander, Ed), pp 327-386. Lewis Publishers, Boca Raton, FL.

Nebert, D.W., and Nelson, D.R. (1991). P450 gene nomenclature based on evolution. *Methods Enzymol.* **206**, 3-11.

Nixon, J.E., Hendricks, J.D., Pawloswki, N.E., Loveland, P.M., and Sinnhuber, R.O. (1981). Carcinogenicity of aflatoxicol in Fischer 344 rats. *J. Natl. Cancer Inst.* **66**, 1159-1163.

Okey, A.B., Riddick, D.S., and Harper, P.A. (1994). The Ah receptor: Mediator of the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds. *Toxicol. Let.* **70**, 1-22.

Phillips, D.H. (1983). Fifty years of benzo(*a*)pyrene. *Nature* **303**, 468-472.

Pitot, H.C., and Dragan, Y.P. (1991). Facts and theories concerning the mechanisms of carcinogenesis. *FASEB J.* **5**, 2280-2286.

Pliss, G.B., and Khudoley, V.V. (1975). Tumor induction by carcinogenic agents in aquarium fish. *J. Natl. Cancer Inst.* **55**, 129-136.

Purchase, I.F.H. (1994). Current knowledge of mechanisms of carcinogenicity: genotoxins versus non-genotoxins. *Human and Exper. Toxicol.* 13, 17-28.

Robbins, S.L., and Kumar, V. (1987). *Basic Pathology.* pp 182-213. W.B. Saunders Co., Philadelphia, PA.

Salhab, A.S., and Edwards, G.S. (1977). Comparative *in vitro* metabolism of aflatoxicol by liver preparations from animals and humans. *Cancer Res.* **37**, 1016-1021.

Schell, J.D., Cooper, K.O., and Cooper, K.R. (1987). Hepatic microsomal mixed-function oxidase activity in the Japanese medaka (*Oryzias latipes*). *Environ. Toxicol. Chem.* **6**, 717-721.

Schlenk, D., and Buhler, D.R. (1991a). Flavin-containing monoxygenase activity in liver microsomes from the rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* **20**, 13-24.

Schlenk, D. and Buhler, D.R. (1991b). Role of flavin-containing monooxygenase in the *in vitro* biotransformation of aldicarb in rainbow trout (*Oncorhynchus mykiss*). *Xenobiotica* **21**, 1583-1589.

Schlenk, D., and Buhler, D.R. (1993). Immunological characterization of flavin-containing monooxygenases from the liver of rainbow trout (*Oncorhynchus*

mykiss): sexual- and age- dependent differences and the effect of trimethylamine on enzyme regulation. *Biochim. Biophys. Acta* 1156, 103-106.

Schlenk, D., Ronis, M.J.J., Miranda, C.L., and Buhler, D.R. (1993). Channel catfish liver monooxygenases: immunological characterization of constitutive cytochromes P450 and the absence of active flavin-containing monooxygenases. *Biochem. Pharmacol.* 45, 217-221.

Schoenhard, G.L., Hendricks, J.D., Nixon, J.E., Lee, D.J., Wales, J.H., Sinnhuber, R.O., and Pawlowski, N.E. (1981). Aflatoxicol-induced hepatocellular carcinoma in rainbow trout (*Salmo gairdneri*) and the synergistic effects of cyclopropenoid fatty acids. *Cancer Res.* 41, 1011-1014.

Shelton, D.W., Hendricks, J.D., and Bailey, G.S. (1984). The heaptocarcinogenicity of diethylnitrosamine to rainbow trout and its enhancement by Aroclors 1254 and 1254. *Toxicol. Letters* 22, 27-31.

Shimada, T., and Guengerich, F.P. (1989). Evidence for cytochrome P-450$_{NF}$, the nifedipine oxidase, being the principle enzyme involved in the bioactivation of aflatoxins in human liver. *Proc. Natl. Acad. Science USA* 86, 462-465.

Sinnhuber, R.O., Wales, J.H., Ayres, J.L., Engebrecht, R.H., Amend, D.L. (1968). Dietary factors and hepatoma in rainbow trout (*Salmo gairdneri*). 1. Aflatoxins in vegetable protein feedstuffs. *J. Natl. Cancer Inst.* 41, 711-718.

Sipes, I.G., and Gandolfi, A.J. (1991). Biotransformation of toxicants. In *Casarett and Doull's Toxicology: The Basic Science of Poisons* (M.O. Amdur, J. Doull, and C.D. Klaassen, Ed.), pp 127-200. Pergamon Press, Maxwell House, New York.

Smith, M.R., Mathews, N.T., Jones, K.A., and Kung, H-F. (1993). Biological actions of oncogenes. *Pharmac. Ther.* 58, 211-236.

Stanton, M.F. (1965). Diethylnitrosamine-induced hepatic degeneration and neoplasia in the aquarium fish, *Brachydanio rerio*. *J. Natl. Cancer Inst.* 34, 117-130.

Stegeman, J.J. (1989). Cytochrome P450 forms in fish: catalytic, immunological and sequence similarities. *Xenobiotica*, 19, 1093-1110.

Stegeman, J.J. (1993). Cytochrome P450 forms in fish. *Handb. Exp. Pharm.* 105, 279-288.

Stegeman, J.J., and Hahn, M.E. (1994). Biochemistry and molecular biology of monooxygenases: current perspectives on forms, functions, and regulation of cytochrome P450s in aquatic species. In *Aquatic Toxicology: Molecular,*

Biochemical, and Cellular Perspectives (D.C. Malins and G.K. Ostrander, Ed), pp 87-206. Lewis Publishers, Boca Raton, FL.

Stegeman, J.J., and Lech, J.J. (1991). Cytochrome P-450 monooxygenase systems in aquatic species: carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. *Environ. Health Perspec.* **90**, 101-109.

Swenberg, J.A. (1993). Cell proliferation and chemical carcinogenesis: conference summary and future directions. *Environ. Health Perspec.* **101**, 153-158.

Swenson, D.H., Lin, J.-K., Miller, E.C., and Miller, J.A. (1977). Aflatoxin B_1-2,3-oxide as a probable intermediate in the covalent binding of aflatoxins B_1 and B_2 to rat liver DNA and ribosomal RNA *in vivo*. *Cancer Res.* **37**, 172-181.

Toledo, C., Hendricks, J., Loveland, P., Wilcox, J., and Bailey, G. (1987). Metabolism and DNA-binding *in vivo* of aflatoxin B_1 in medaka (*Oryzias latipes*). *Comp. Biochem. Physiol.* **87C**, 275-281.

Valsta, L.M., Hendricks, J.D., and Bailey, G.S. (1988). The significance of glutathione conjugation for aflatoxin B_1 metabolism in rainbow trout and coho salmon. *Food Chem. Toxicol.* **26**, 129-135.

Van Beneden, R.J., and Ostrander, G.K. (1994). Expression of oncogenes and tumor suppressor genes in teleost fishes. In *Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives* (D.C. Malins and G.K. Ostrander, Ed.), pp 295-325. Lewis Publishers, Boca Raton, FL.

Weinberg, R.A. (1989a). Oncogenes and multistep carcinogenesis. In *Oncogenes and the Molecular Origin of Cancer.* (R. A. Weinberg, Ed.), pp 307-326. Cold Spring Harbor Laboratory Press, New York.

Weinberg, R.A. (1989b). Oncogenes, antioncogenes, and the molecular basis of multistep carcinogenesis. *Cancer Res.* **49**, 3713-3721.

Whitlock, J.P. (1993). Mechanistic aspects of dioxin action. *Chem. Res. Toxicol.* **6**, 754-763.

Williams, D.E., and Buhler, D.R. (1983). Purified form of cytochrome P-450 from rainbow trout with high activity toward conversion of aflatoxin B_1 to aflatoxin B_1-2,3-epoxide. *Cancer Res.* **43**, 4752-4756.

Williams, G.M., and Weisburger, J.H. (1991). Chemical carcinogenesis. In *Casarett and Doull's Toxicology: The Basic Science of Poisons* (M.O. Amdur, J. Doull, and C.D. Klaassen, Ed.), pp 127-200. Pergamon Press, Maxwell House, New York.

Wirgin, I.I., Grunwald, C., Courtenay, S., Kreamer, G.-T., Reichert, W.L., and Stein, J.E. (1994). A biomarker approach to assessing xenobiotic exposure in Atlantic tomcod from the North American Atlantic coast. *Environ. Health Perspec.* 102, 764-770.

Ziegler, D.M. (1993). Recent studies on the structure and function of multisubstrate flavin-containing monooxygenases. *Annu. Rev. Pharmacol. Toxicol.* 33, 179-199.

CHAPTER 2

IN VIVO AFLATOXIN B_1 METABOLISM AND HEPATIC DNA ADDUCTION IN ZEBRAFISH (*Danio rerio*)

Claudia M. Troxel,

Ashok P. Reddy, Patricia E. O'Neal,

Jerry D. Hendricks, and George S. Bailey.

Toxicology Program

Oregon State University

Corvallis, OR 97331

Abstract

The zebrafish (*Danio rerio*) is assuming prominence in developmental genetics research. By comparison, little is known of tumorigenesis and nothing is known of carcinogen metabolism in this species. This study evaluated the ability of zebrafish to metabolize a well-characterized human carcinogen, aflatoxin B_1 (AFB_1), to phase I and phase II metabolites and assessed hepatic AFB_1-DNA adduction *in vivo*. Fish i.p. injected with 50-400 μg [^{3}H]AFB_1/kg body weight displayed a linear dose-response for hepatic DNA binding at 24 hours. AFB_1-DNA adduct levels among treatments showed no statistical difference over the period from 1 to 21 days after injection, suggesting poor adduct repair in this species. DNA binding in female fish was 1.7-fold higher than in males ($p < 0.01$). An *in vitro* AFB_1 metabolism assay verified that zebrafish liver extracts oxidize AFB_1 to the 8,9-epoxide proximate electrophile (Km = 79.0 $\pm$ 16.4 μM, Vmax = 11.7 $\pm$ 1.4 pmol/min/mg protein at 28°C). The excretion of AFB_1 and its metabolites was also examined by HPLC. As is typical of other fish studied, major metabolites excreted were aflatoxicol (AFL) and aflatoxicol-glucuronide (AFL-g), followed by unreacted AFB_1. AFL appeared as early as 5 minutes after injection, whereas AFL-g was a significant metabolite after 18 hours. This study shows that *in vivo* administration of AFB_1 in zebrafish results in moderate adduction of the carcinogen to liver DNA, and that zebrafish have the capacity for both phase I and phase II metabolism of AFB_1. The approximate 4-fold difference between rainbow trout and zebrafish AFB1-DNA covalent binding index appears insufficient to explain the relative resistance of zebrafish to dietary AFB1 hepatocarcinogenicity.

Introduction

Aquarium fish are gaining use as alternative models for chemical carcinogenesis studies. Many contributing factors include short life span, short time to tumor response, ability to spawn regularly, reduced husbandry costs, the ability to include the whole fish sagittal-section on one microscope slide for histology, and a low spontaneous tumor incidence (Hawkins *et al.*, 1988). The zebrafish was the first aquarium species in which chemically-induced tumors were demonstrated (Stanton, 1965). Later studies confirmed their responsiveness to diethylnitrosamine and also to nitrosomorpholine (Khudoley, 1984; Pliss and Khudoley, 1975). However, subsequent investigations have focused on life stage effects and toxicity upon exposure to various chemicals and heavy metals (Braunbeck *et al.*,1990a; 1990b; Nagel *et al.*, 1991, Kalsch *et al.*, 1991; Bresch *et al.*, 1990; Nielson *et al.*, 1990; Dave and Xiu, 1991), with surprisingly little work on the response of zebrafish to carcinogens.

Aflatoxins are secondary metabolites produced by the fungus *Aspergillis flavus*, with aflatoxin B_1 identified as the most toxic metabolite. AFB_1 is a potent hepatocarcinogen and hepatotoxin in some species, such as the rat, duck, and rainbow trout, whereas other species, such as the mouse, seem to be fairly resistant to its effects. AFB_1 has recently been classified as a Group 1 carcinogen in humans (IARC, 1993). Aflatoxin exposure may occur via contaminated food, such as moldy corn and peanuts. Research has indicated that interspecies differences in sensitivity to this hepatocarcinogen can often be explained by variations in the biotransformation of AFB_1 (Eaton and Gallagher, 1994; Bechtel, 1989; Bailey *et al.*, 1996). The activation of AFB_1 to the *exo*-AFB_1-8,9-epoxide is thought to be responsible for its carcinogenic

effects because this unstable, highly reactive intermediate can bind to cellular macromolecules including DNA (Essigman *et al.*, 1982; Swenson *et al.*, 1977). Recent studies have shown that the *endo*-AFB_1 epoxide stereoisomer can also be produced, and although this epoxide is the more stable stereoisomer, it does not appear to be as efficient in forming DNA adducts (Raney *et al.*, 1992a; 1992b; Eaton and Gallagher, 1994). The rate and ratio of formation of the *exo*- and *endo*-AFB_1 epoxide stereoisomers depends on the species under investigation. Formation of this intermediate alone is not enough to account for carcinogenesis, since detoxification is possible via a glutathione-S-transferase mediated reaction. Successful scavenging of the *exo*-AFB_1 epoxide by this pathway has been shown to be responsible for lower susceptibility to the carcinogenic effects of AFB_1 in mouse compared to rat (Degen and Neumann, 1981; Raney *et al.*, 1992a).

AFB_1 response in fish depends upon the species being investigated. For example, coho salmon and catfish are resistant but rainbow trout are one of the most sensitive species known to the carcinogenic effects of AFB_1 (reviewed in Hendricks, 1994; Bailey *et al.*, 1996). A study in 1968 showed that exposure to microgram quantities of AFB_1 was acutely toxic to zebrafish embryos and larvae (Abedi and McKinley, 1968). However, zebrafish did not develop tumors after 2 ppm water exposure for 3 days (Bauer *et. al.*, 1972). We have recently found AFB_1 to be a relatively weak hepatocarcinogen in this species (unpublished results). The purpose of this study was to characterize the *in vivo* metabolism and hepatic DNA adduction of AFB_1 in the zebrafish as a first step in assessing the mechanisms of AFB_1 resistance in this species.

Materials and Methods

Animals

Adult (sexually mature) male and female zebrafish with an average body weight of 0.5 g were obtained from the Food Toxicology and Nutrition Laboratory at Oregon State University. The zebrafish were maintained in 29 gallon aerated aquariums and a controlled temperature of 26°C (± 1) and a 14 hour light:10 hour dark photoperiod. During the week, fish were fed twice daily with TetraMin Staple Food (flakes) each morning, and a combination of Oregon Test Diet (OTD) (Sinnhuber *et al.*, 1977) and brine shrimp in the afternoon on Monday, Wednesday, and Friday, and TetraMin Staple Food and brine shrimp on Tuesday and Thursday afternoons. On weekends, fish received only one feeding per day consisting of OTD and brine shrimp. Fingerling rainbow trout (*Oncorhynchus mykiss*) were reared in the Food Toxicology and Nutrition Laboratory as described (Sinnhuber *et al.*, 1977).

Chemicals

AFB_1 was purchased from Sigma Chemical Company (St. Louis, MO), and was assessed for purity by thin layer chromatography (TLC); [^{3}H]AFB_1 was from Moravek Biochemicals (Brea, CA) and the chemical and radiopurity was checked by TLC followed by radioscanning; Proteinase K, and RNase, DNase free were from Boehringer Mannheim Biochemicals (Indianapolis, IN); HPLC grade acetonitrile, methanol, tetrahydrofuran, and J.T. Baker C_{18} Empore extraction disks were from VWR (Seattle,

WA); Hoechst #33258 from Calbiochem-Behring Corp. (La Jolla, CA); aflatoxin HPLC standards aflatoxicol (AFL), aflatoxicol-glucuronide (AFL-g), aflatoxin-M_1 (AFM_1), and aflatoxicol-M_1 (AFL-M_1) were prepared in our laboratory by previously published methods (Loveland *et. al.*, 1983; 1984); all other chemicals were purchased from Sigma (St. Louis, MO) or Aldrich Chemical Company (Milwaukee, WI).

Metabolism of [^{3}H]AFB_1

Adult female zebrafish, fasted for 24 hours, were intraperitoneally (i.p.) injected with 400 μg/kg body weight of [^{3}H]AFB_1 (11.6 Ci/mmol) in 50% ethanol. The fish were rinsed with 2 ml of water after injection to account to remove residual traces of radioactivity, and placed in the dark in individual beakers containing 50 ml water. Water samples were collected and counted at various time points up to 24 hours for metabolite analysis. AFB_1 and metabolites were extracted from the aqueous samples using C_{18} Empore Extraction Disks. The disks were washed with 45 ml of the elution solvent (10 mM potassium acetate (KOAc), pH 5.0, adjusted to 60% methanol (MeOH)) in 15 ml increments, and then conditioned with 45 ml of 100% MeOH, 45 ml of 50% MeOH, and 45 ml of 10% MeOH. Pooled water samples from each time point (approximately 150 ml) were adjusted to 10% methanol and were loaded onto the conditioned disks. Prior to elution, the disks were washed with 10 ml of 10 mM KOAc, pH 5.0, adjusted to 10% MeOH, to wash off proteins, salts, and exchanged tritium. AFB_1 and metabolites were eluted with 15 ml of 10 mM KOAc, pH 5.0, adjusted to 60% methanol. Total recovery rates (comprised of all fractions and the disk, which was cut up and counted) were greater than 85%, and the amount of

radioactivity associated with the 60% methanol eluant represented 60-88% of the total radioactivity. The methanol eluates, containing the aflatoxin metabolites, were concentrated using a rotary evaporator and redissolved in 15% acetonitrile: methanol: tetrahydrofuran (AMT; 15:20:6 v:v:v) and 85% 0.02 M KOAc, pH 5.0. Reverse phase high pressure liquid chromatography was performed with a Beckman Model 334 instrument using a 4.6 x 150 mm, 5 micron Phenomenex C_{18} column. The solvent system consisted of AMT (15:20:6 v:v:v) and 0.02 M KOAc, pH 5.0, with a 15 minute linear gradient from 15% AMT to 46% AMT. The flow rate was 1 ml/min, and UV detection was at 345 nm. Effluent samples of 30 drops (0.3 ml) were collected for liquid scintillation counting using a Beckman LS 7500 scintillation counter.

Hepatic aflatoxin-DNA adduction

To evaluate the dose-response of [^{3}H]AFB_1-DNA binding, adult male and female zebrafish fasted for 24 hours were i.p. injected with 0, 50, 100, 200, or 400 μg/kg body weight of [^{3}H]AFB_1 (11.6 Ci/mmol) in 50% EtOH and were rinsed with 2 ml of water to remove any residual skin contamination. Twenty-four hours after injection, livers were sampled with the gall bladders carefully removed, with 3 pools of 3 livers per dose. After sampling, the livers were not rinsed but immediately frozen in liquid nitrogen. DNA isolation and purification was based on the procedure by Strauss (1991), with the following modifications. Liver pieces were homogenized with digestion buffer. The samples were first extracted with equal volumes of phenol saturated with Tris-HCl buffer, pH 8.0., and then extracted twice with equal volumes of chloroform: isoamyl alcohol (24:1). The DNA was precipitated using 0.1 volume of 3 M sodium acetate and

2 volumes of cold 100% EtOH. After centrifugation, the EtOH was removed and the DNA allowed to dry. The DNA was dissolved in 0.5 ml of purified water by heating the samples at 38°C for 30 minutes, and then DNase-free RNase was added to the sample (final concentration 5μg/ml), and the incubation continued for another 30 minutes. The samples were extracted twice with equal volumes of chloroform: isoamyl alcohol (24:1), and the DNA was again precipitated. After centrifugation, the EtOH was removed, and the DNA was washed with 70% ice-cold EtOH. DNA samples were stored at -20°C after dissolving the DNA in Tris-ethylenediaminetetraacetic acid (EDTA) (TE) buffer, pH 8.0. DNA concentration was quantified using the microfluorometric procedure described by Cesarone *et al.* (1979), with a Hoefer TKO100 DNA fluorometer. To determine the amount of [^{3}H]AFB$_1$ bound to the DNA, samples were hydrolyzed by mixing with equal volumes of 1.0 M perchloric acid and heating at 70°C for 20 minutes. The hydrolyzed samples were then counted with a scintillation counter. Control liver samples spiked with [^{3}H]AFB$_1$ during the DNA isolation process revealed that there was negligible carry-over of non-bound tritium through the isolation process.

To verify the sex-related difference in DNA binding observed in the dose-response study, an experiment was conducted with 9 groups of 3 females, and 7 groups of 3 males. The fish were i.p. injected with 400 μg/kg body weight of [^{3}H]AFB$_1$ (13.0 Ci/mmol), the highest dose used in the dose-response experiment. Livers were sampled 24 hours later, and the amount of hepatic DNA binding was determined.

To evaluate the time-course of AFB$_1$-DNA binding, adult male and female zebrafish were i.p. injected with 400 μg/kg body weight of [^{3}H]AFB$_1$ (15.4 Ci/mmol),

and livers were sampled 1, 2, 4, 7, 14, and 21 days after injection. Livers were immediately frozen in liquid nitrogen. DNA binding was determined as described above.

In vitro AFB_1-metabolism assay

The *in vitro* metabolism of AFB_1 was carried out with juvenile trout (n=10), or adult female (n=20) or male (n=6) zebrafish liver homogenates. The fish were fasted for 24 hours before sampling. Female zebrafish and juvenile trout livers were excised and immediately frozen in liquid nitrogen and stored at -80°C. Male zebrafish livers were immediately placed into homogenization buffer and homogenized and then frozen due to difficulties with protease release during liver excision. Livers were homogenized in a buffer containing 0.1 M potassium phosphate (pH 7.25), 20% glycerol, 1 mM EDTA, 0.1 mM butylated hydroxytoluene, 1 mM dithiolthreitol, and 0.1 mM phenylmethylsulfonylfluoride on ice. Samples were centrifuged at 1,000x g for 10 minutes, and the supernatant decanted and stored at -80°C. Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

The *in vitro* AFB_1-metabolism assay was based on the method of Monroe and Eaton (1987), as modified by Takahashi *et al.* (1996). Samples were preincubated for 2 minutes at room temperature before initiating the reaction with NADPH and reduced glutathione. Incubations were carried out for 45 minutes. Michaelis-Menton kinetics were determined with trout homogenates at both 13°C and 28°C using substrate (AFB_1) concentrations of 5, 10, 20 and 40 μM AFB_1, and with female zebrafish homogenates

at 28°C at substrate concentrations of 10, 20, 40 and 80 μM AFB_1. The initial rates of epoxide formation were linear with substrate concentration. Male zebrafish homogenates were assayed using 80 μM AFB_1, the highest substrate concentration, for comparison to female zebrafish. All assays were conducted in duplicate. Incubations without homogenates were used to correct for the low inherent epoxidation activity of butylated hydroxyanisole (BHA)-induced mouse cytosolic protein included in the assay mixture.

Statistical analysis

Statistical analysis was performed with SAS, version 6.8 (SAS Institute Inc., 1989). Analysis was performed by either a one-way or two-way analysis of variance. A p value of less than 0.05 was considered significant.

Results

In vivo metabolism of [^{3}H]AFB_1

Early excretion kinetics of [^{3}H]AFB_1 were evaluated by measuring the amount of radioactivity recovered in water within a 24 hour time period after i.p. injection (Figure 2.1). By 24 hours, 47% of the radioactivity administered to the zebrafish was recovered in the water. Excretion occurred rapidly with 25% of the administered radioactivity recovered by 45 minutes. This amount represented more than half of the total radioactivity recovered in the 24 hour time period. The remaining 53% of the

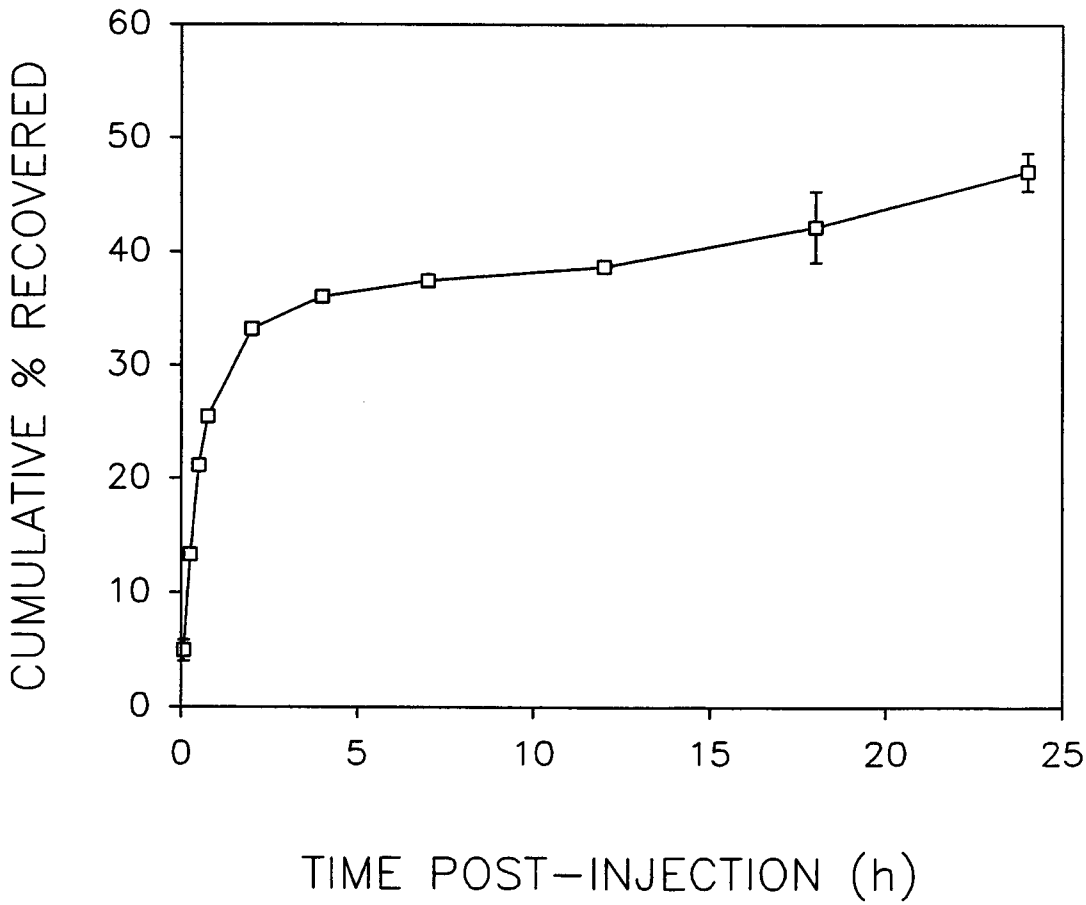

Figure 2.1. The cumulative percentage of radioactivity excreted into water within 24 hours after i.p. injection of [^{3}H]AFB$_1$. (n=3 females, error bars are ± SEM).

administered dose is presumed to represent material found bound strongly to macromolecules and material eventually eliminated via fecal excretion.

The major metabolites of AFB_1 recovered in the water at various time points after i.p. injection were identified as aflatoxicol (AFL), aflatoxicol-glucuronide (AFL-g), and unreacted parent AFB_1 (Figure 2.2). Identification of these peaks was based on the retention times of known aflatoxin standards generated in our lab. Formation and excretion of AFL occurred rapidly in the zebrafish and was present in the water at 5 minutes, the earliest time point examined. Over the 24 hour time-period, AFL was the predominant metabolite, accounting for 17.3% of the original dose of AFB_1 administered. AFL-g and unreacted AFB_1 accounted for 4.1% and 3.6%, respectively.

Figure 2.3 shows the proportion of AFB_1 and metabolites recovered in the HPLC profile at each time point investigated, with values normalized to 100. AFL was the major metabolite recovered in the water at each time point up to 18 hours, accounting for 60 to 80% of the metabolites recovered. After 18 hours, AFL-g was the major metabolite, and represented over 80% of metabolites recovered at the 24 hour time point. An unidentified polar peak accounting for only a minor percentage of the metabolites was occasionally detected, being most prominent at 5 minutes. While this peak could represent a more polar metabolite, it did not co-elute with authentic AFB_1-glutathione conjugate, and was not reproducible in another experiment conducted investigating aflatoxin metabolism (Chapter 3, this thesis). The role of sulfate conjugation in AFB_1 metabolism by zebrafish investigated in another experiment by aryl sulfatase treatment indicated non-detectable levels of this conjugate (Chapter 3, this thesis). Unresolved aflatoxicol-M_1 (AFL-M_1) plus aflatoxin-M_1 (AFM_1) represented

only a very minor fraction of the metabolites, comprising less than 2% of the metabolites recovered at any one time point.

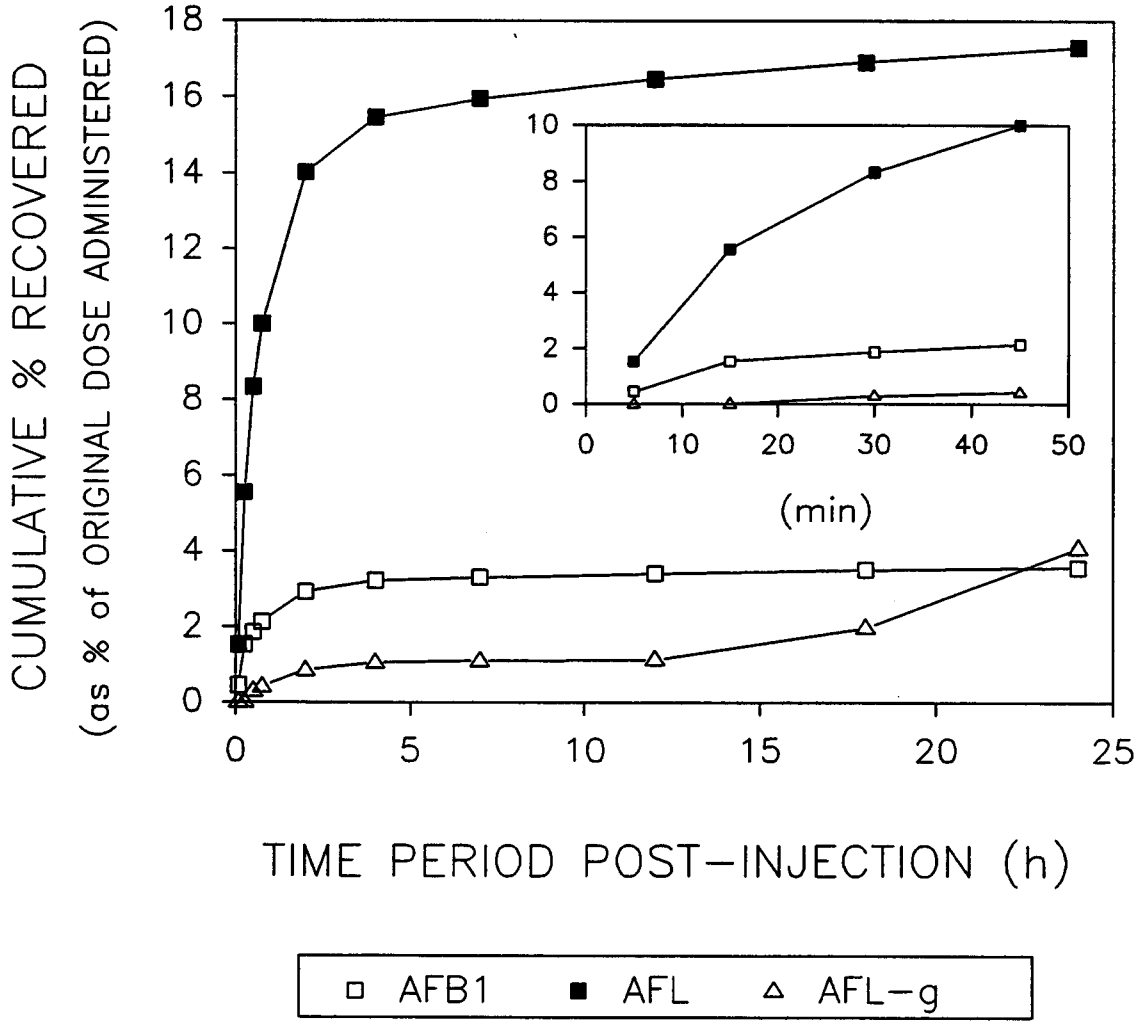

Figure 2.2. The cumulative percentage of AFB_1 and metabolites excreted into water as determined by HPLC as a percentage of the original dose administered. Each data point represents the pooled water samples from 3 females.

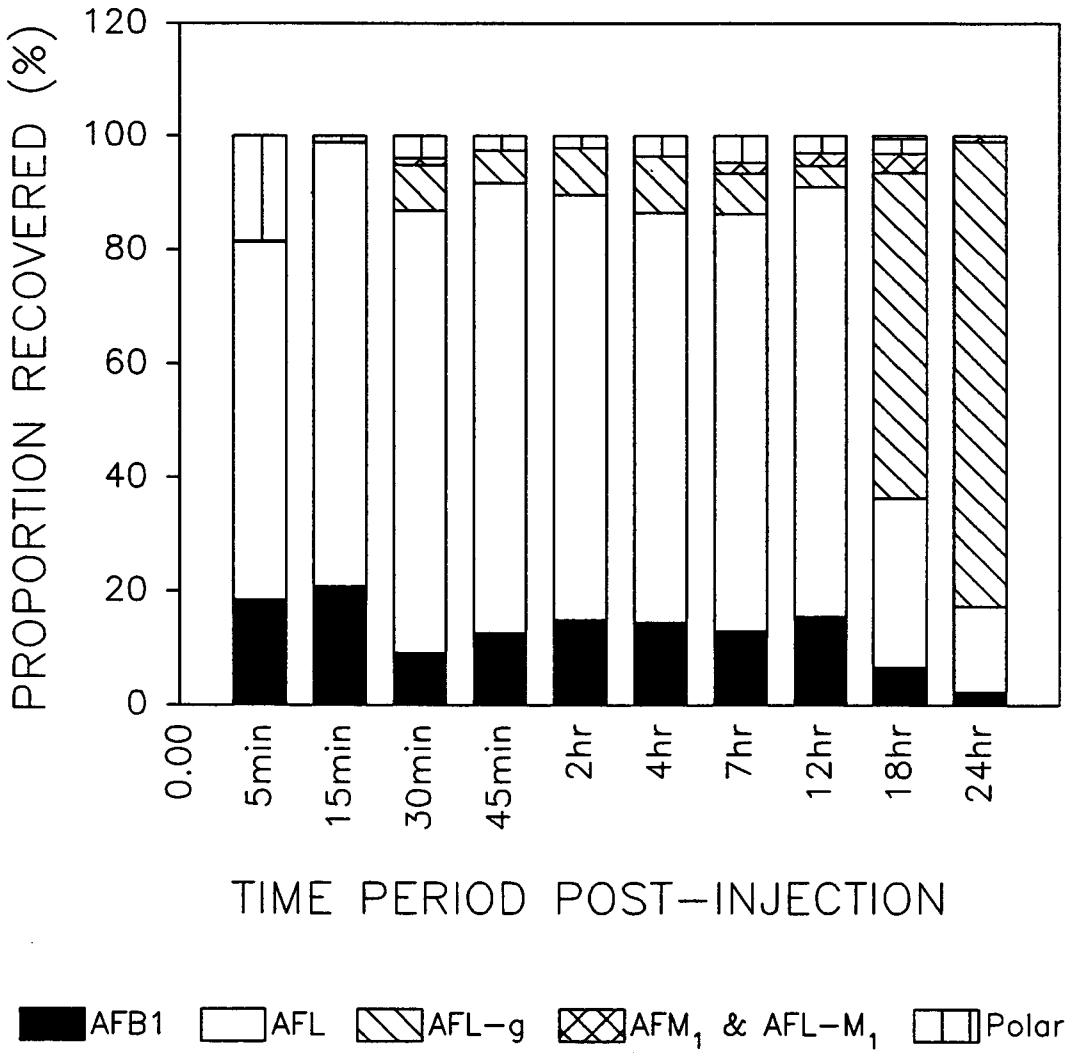

Figure 2.3. The proportion of AFB_1 and metabolites excreted into water at various time points over 24 hours as determined by HPLC. Values are normalized to 100.

In vivo AFB_1-DNA adduction in liver

The *in vivo* dose-response for AFB_1-DNA adduct formation was linear from 50-400 μg AFB_1/kg body weight (Figure 2.4). Females had a higher level of DNA binding than the males at all doses tested. The covalent binding index (CBI), where CBI = μmol chemical bound/mol DNA/mmol chemical administered/kg body weight, was 32,700 $\pm$ 21,800 for males, and 56,000 $\pm$ 15,500 for females. Females had a DNA binding index which was significantly higher (1.7 fold higher) than males ($p < 0.01$). A follow-up study investigating sex-related difference in AFB_1-DNA adduction verified the results found in the dose-response experiment. The binding index in the second experiment was 68,000 $\pm$ 31,400 for females and 29,100 $\pm$ 12,600 for males, which represents a 2.3 fold higher binding index in females compared to males ($p < 0.05$).

An investigation of the time-course of [^{3}H]AFB_1-DNA binding was conducted to assess when maximum DNA adduct formation occurred, and to investigate the persistence of DNA adducts in this species (Figure 2.5). Although there was some trend toward highest adduction at day 7, the time-course experiment failed to show any statistical difference in binding levels from 1 to 21 days. Therefore, the time at which maximal adduct formation occurs could not be calculated. The half-life of the DNA adducts was also not evident from this experiment. However, a significant difference was again noted between male and female AFB_1-DNA binding levels, with the females exhibiting a binding index on average 42,000 units higher than the males ($p < 0.001$ when comparing the average of females to the average of males).

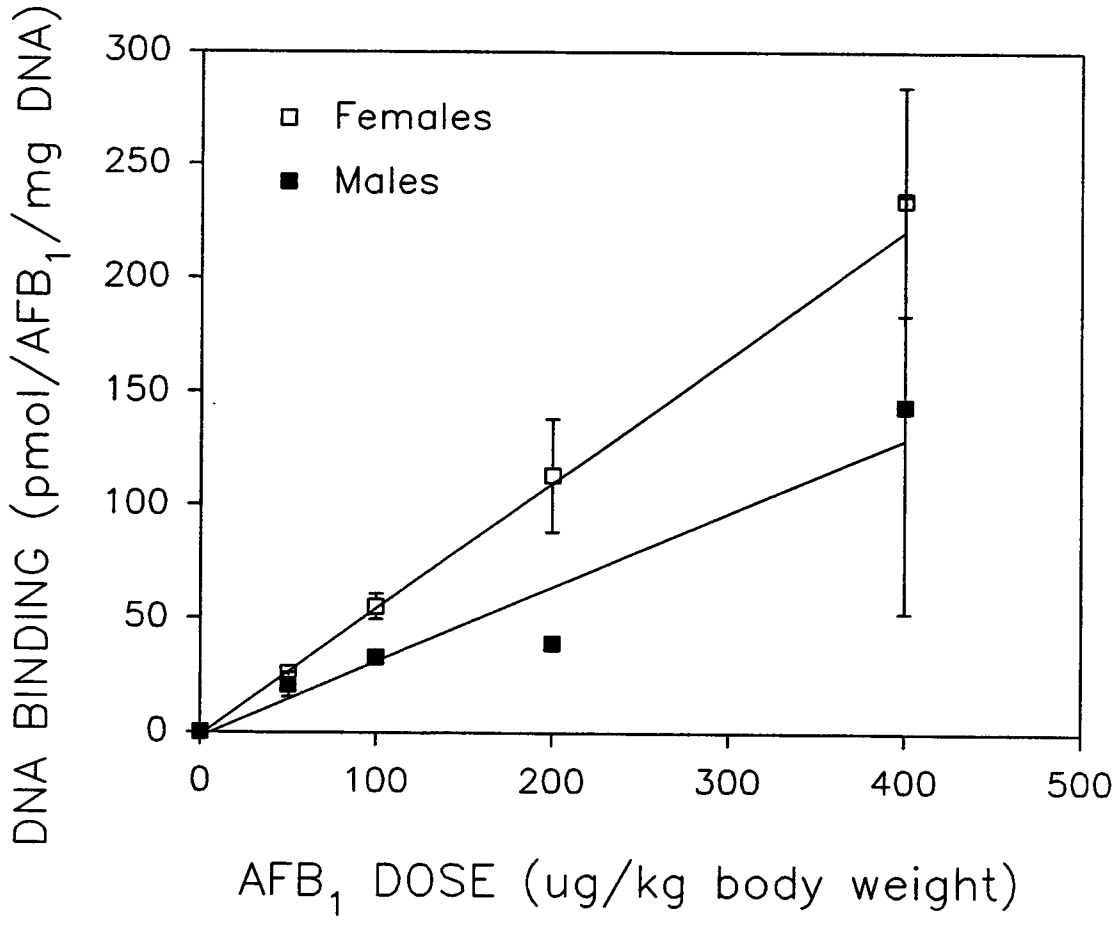

Figure 2.4. The *in vivo* dose response of hepatic aflatoxin-DNA adduct formation at 24 hours. Fish were i.p. injected with 50-400 μg [^{3}H]AFB$_1$/kg body weight (data are means $\pm$ SEM from 3 pools of 3 fish each.)

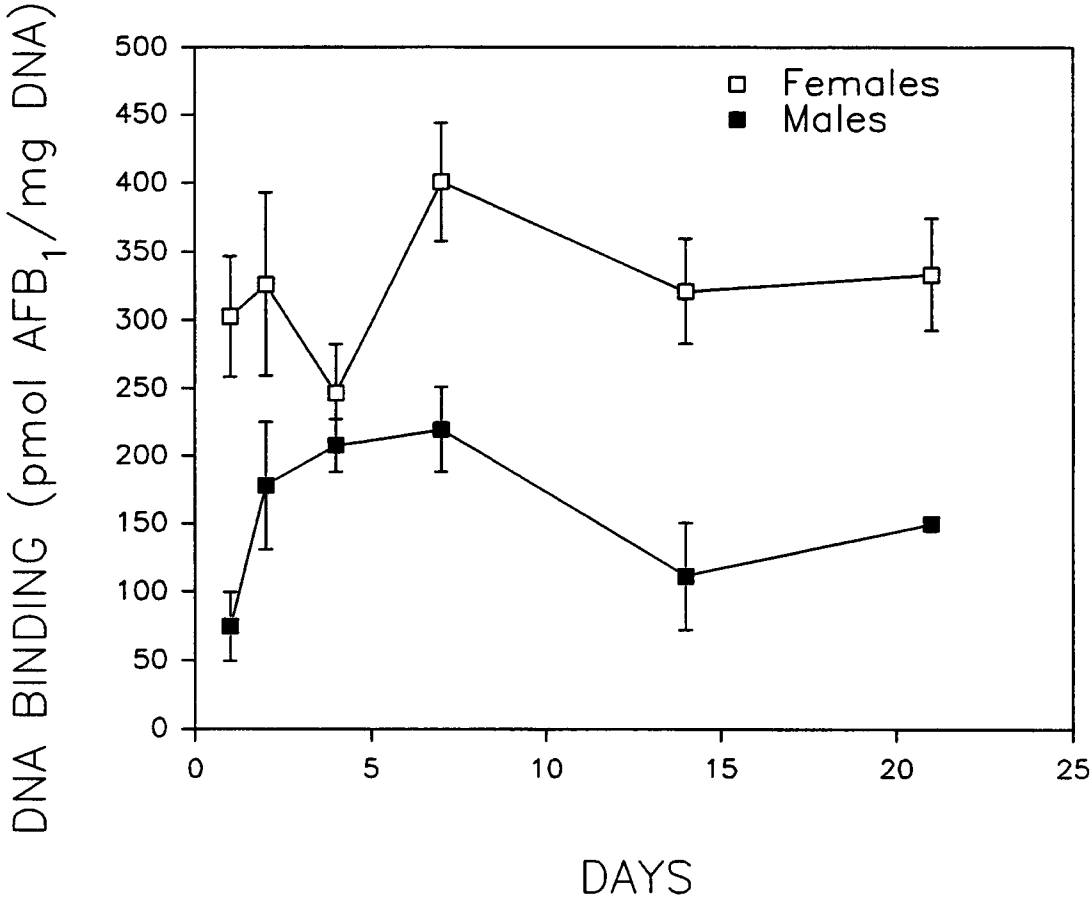

Figure 2.5. Aflatoxin-DNA adduct formation from 1-21 days. (Data are means ± SEM from 3 pools of 3 fish each; data for males at Day 21 are mean ± SEM from 2 pools of 3 fish each).

In vitro AFB_1-metabolism assay

Trout liver homogenates exhibited a higher Vmax for oxidation of AFB_1 to the 8,9-epoxide at both 13°C and 28°C compared to the female zebrafish. As was expected, trout Vmax decreased at the lower temperature. However, the values at 13°C are probably more representative of the physiological response in the trout, since this is their normal environmental temperature. At 13°C, the trout liver homogenates had a 3.1 fold higher Vmax and a similar Km when compared to female zebrafish homogenates at 28°C (Table 2.1) in this experiment. The 7-fold lower Km for AFL production suggests that the reaction would predominate over AFB_1-8-9-epoxide formation at low AFB_1 concentrations *in vivo* in zebrafish, and is consistent with the rapid excretion of AFL within 5 minutes of AFB_1 treatment.

Not surprising was the relatively high amount of aflatoxicol which was formed in the zebrafish liver homogenates. At 80 μM AFB_1, male and female liver homogenates exhibited comparable activities for aflatoxicol production, with the female zebrafish liver homogenates possessing an activity of 8.17 $\pm$ 0.09 pmol/min/mg protein, and male zebrafish an activity of 8.70 $\pm$ 0.04 pmol/min/mg protein. Male zebrafish did appear to have a lower activity towards formation of the AFB_1-epoxide than females (4.10 $\pm$ 0.08 pmol/min/mg protein compared to 5.90 $\pm$ 0.10 pmol/min/mg protein, $p < 0.01$).

Table 2.1. Michaelis-Menton kinetics of AFB_1-epoxide formation in rainbow trout and zebrafish liver homogenates and aflatoxicol formation in zebrafish liver homogenates with the substrate AFB_1[a].

Species	Temperature	Km (μM)	Vmax (pmol/min/mg)
	Production of AFB_1-8,9-epoxide:		
Rainbow trout	13°C	81.2 ± 12.8	35.9 ± 4.1
Rainbow trout	28°C	109.3 ± 15.0	66.6 ± 7.1
Zebrafish (female)	28°C	79.0 ± 16.4	11.7 ± 1.4
	Production of AFL:		
Zebrafish (female)	28°C	11.2 ± 1.3	9.1 ± 0.3

[a] n=10 for trout, and n=20 for zebrafish; all assays were run in duplicate, with nonenzymatic background activity subtracted; errors are given as asymptotic standard error. Only female zebrafish were used for this comparison against juvenile rainbow trout, which do not show sexual differences in AFB_1 metabolism prior to maturity.

Discussion

The results demonstrate that zebrafish have the capacity for both Phase I and Phase II metabolism of AFB_1. The major *in vivo* metabolites excreted into water were AFL and AFL-g, followed by unreacted AFB_1. This metabolic profile is similar to that seen in other fish species, such as the Japanese medaka, rainbow trout, and channel catfish (Toledo *et al.*, 1987; Gallagher and Eaton, 1995; Loveland *et al.*, 1984). AFL, produced by a cytosolic reductase reaction, was recovered in the water at 5 minutes, the earliest time point examined. This has also been observed in the Japanese medaka (Toledo *et al.*, 1987). It is important to note that while AFL is a polar metabolite of AFB_1, it has been shown to be 70% as mutagenic and just as carcinogenic as AFB_1 in rainbow trout, and is also a potent carcinogen in Fischer 344 rats (Coulombe *et al.*, 1982; Schoenhard *et al.*, 1981; Nixon *et al.*, 1981; Bailey *et al.*, 1994). The major conjugation (Phase II) reaction was glucuronidation, which is an important reaction in eliminating hydroxylated compounds in fish (Clark *et al.*, 1991). No evidence of glutathione conjugation was present, as shown by co-chromatography with a glutathione conjugate standard. Formation and excretion of the aflatoxin metabolites occurred rapidly in the zebrafish after i.p. injection.

The *in vitro* assay assessing AFB_1 metabolism again demonstrated the proficiency of AFL formation in zebrafish. This assay also evaluated the ability for AFB_1 epoxidation to the reactive intermediate by trapping the epoxide as the aflatoxin-glutathione adduct formed. The results from this assay revealed that zebrafish possess the enzymes necessary for bioactivation of this carcinogen. When compared to trout liver homogenates assayed at 13°C, zebrafish appear to have a comparable Km and only

a 3-fold lower Vmax, which suggests that zebrafish should be a fairly sensitive species for AFB_1 carcinogenesis. Buchmann *et al.* (1993) conducted immunohistochemical analysis to detect the presence of the cytochrome P450 isozyme LMC2 (CYP2K1) in zebrafish. This isozyme is believed to be primarily responsible for the bioactivation of AFB_1 to the epoxide in rainbow trout (Williams and Buhler, 1983). The immunohistochemical analysis of zebrafish using antibodies directed against trout CYP2K1 showed that this isozyme was constituitively expressed in the liver, kidney, skin, and oral mucosa of zebrafish, with lower expression in gills, pseudobranch, intestines, and ovaries. It is possible that this or a similar P450 may be responsible for the observed bioactivation of AFB_1 in zebrafish.

A significant difference in DNA adduct formation between male and female zebrafish was observed in all DNA binding experiments performed. An *in vitro* AFB_1 metabolism assay also confirmed the difference between males and females in their ability to bioactivate AFB_1 to the *exo*-AFB_1 epoxide, which is trapped with high selectivity by the mouse cytosolic glutathione transferase in this assay (Rany *et al.*, 1992b). The *in vitro* assay did not measure differences in the abilities of males and females to produce the *endo*-epoxide, which is not efficiently trapped by the mouse enzyme. It might be that there is a different ratio of *endo*- and *exo*-epoxide production in males and females. Differences between sexes in their ability to metabolize xenobiotics are not unusual. A difference in xenobiotic biotransformation between male and female zebrafish was previously noted by Buchmann *et al.* (1993), where males tended to have higher 7-ethoxyresorufin-*O*-deethylase activity. This activity is usually associated with the presence of cytochrome P4501A1. The *in vivo* dose-response of

hepatic aflatoxin-DNA adduct formation was linear from 50-400 μg AFB_1/kg body weight, which is consistent with experiments examining hepatic DNA binding in other fish species following AFB_1 administration (Dashwood *et al.*, 1988; Toledo *et al.*, 1987; and Witham *et al.*, 1982). Using the highest dose, the time-course of aflatoxin-DNA binding showed no statistical difference in binding levels from 1 to 21 days, suggesting slow DNA repair in zebrafish liver. A useful method for comparing DNA binding between species is the CBI (cumulative binding index, units of μmol chemical bound/mol DNA/mmol chemical administered/kg body weight). A composite CBI calculated from the three DNA-binding experiments is approximately 70,600 in female zebrafish and 35,000 for males. For comparison, the CBI for rat and Japanese medaka is approximately 10,000 and 13,000, respectively, and is 240,000 for trout (Toledo *et al.*, 1987, and references therein). From these data, zebrafish exhibit approximately a 4-fold lower capacity for DNA adduct formation compared to trout, and a 5-fold higher capacity than rat and medaka.

The results from both the investigation of *in vivo* DNA binding after i.p. administration of [^{3}H]AFB_1 and the *in vitro* AFB_1 metabolism assay suggest that zebrafish should be a fairly sensitive model for AFB_1 carcinogenesis, if indeed the amount of DNA adducts present in this species truly correlates with tumor incidence as demonstrated in the trout model (Dashwood, *et al.*, 1992). Not only are zebrafish capable of bioactivating AFB_1 to its active epoxide form, but the DNA adducts that result seem to be persistent over time. However, a study conducted in our laboratory investigating dietary exposure to AFB_1 is providing evidence to the contrary. Zebrafish appear to be quite resistant to the carcinogenic effects of AFB_1 when administered by

the dietary route (Tsai, 1996). A preliminary investigation of DNA binding after dietary administration of [^{3}H]AFB_1 showed only a low level of adduct formation (unpublished results). This dramatic difference is suggestive of a difference in absorption. It appears that if one could get the carcinogen to the target organ, zebrafish might prove quite sensitive. Studies assessing carcinogenicity of i.p. administered AFB_1 have yet to be conducted. It may also be that, while there is evidence for persistence of DNA adduct formation after i.p. administration, these adducts simply do not go on to form initiated cells with any great efficiency in this species. Finally, even if initiation occurs, there could be a lack of promotion/progression of these cells in zebrafish liver. Further studies will be required to test these hypotheses.

Other studies conducted in our laboratory with dietary administration of high amounts of diethylnitrosamine, dimethylnitrosamine, or dimethylbenz[*a*]anthracene for 6 months have shown limited or no tumor response as well (Tsai, 1996; unpublished results). However, high doses of dietary methylazoxymethanol acetate did manage to elicit almost a 30% tumor response (Tsai, 1996). Detailed metabolism studies have not been conducted after administration of these various carcinogens, so it is uncertain if these low tumor responses are the product of low capacity for bioactivation, high capacity for detoxification and elimination, inability of these compounds when administered in the diet to reach the respective target organs, or some other factor governing the carcinogenic process. Interestingly, zebrafish exposed as embryos or fry to many of these carcinogens are proving to be more sensitive, indicating that zebrafish may be more susceptible to carcinogens at an early stage of development.

In conclusion, adult zebrafish can rapidly metabolize and excrete the potent hepatocarcinogen AFB_1 after intraperitoneal administration. Both *in vitro* and *in vivo* studies verify that this species has the capacity to bioactivate AFB_1 to its reactive intermediate. The DNA adducts that result are suggestive of sensitivity to this carcinogen. These results are in apparent contradiction to the resistance of zebrafish to dietary administration of AFB_1, and suggest a mechanism related to factors other than inherent ability to metabolize and bioactivate this carcinogen.

Acknowledgements

The authors thank Dr. Cliff Pereira and Jim Kolsky for assistance in statistical analysis, Tom Miller for care and maintenance of the zebrafish colony, and Dr. Ulrich Harttig for technical assistance in the *in vitro* AFB_1 metabolism assay. This work was supported by Army grant DAMD17-91-Z-1043 and NIEHS grants 2 T32 ES 07060, ES 00210, and ES 03850. This is technical paper number 11029 from the Oregon State Agricultural Experiment Station.

References

Abedi, Z.H., and McKinley, W.P. (1968). Zebra fish eggs and larvae as aflatoxin bioassay test organisms. *J. Assoc. Off. Anal. Chem.* 51, 902-904.

Bailey, G.S., Loveland, P.M., Pereira, C., Pierce, D., Hendricks, J.D., and Groopman, J.D. (1994). Quantitative carcinogenesis and dosimetry in rainbow trout for aflatoxin B_1 and aflatoxicol, two aflatoxins that form the same DNA adduct. *Mut. Res.* 313, 25-38.

Bailey, G.S., Williams, D.E., and Hendricks, J.D. (1996). Fish models for environmental carcinogenesis: the rainbow trout. *Environ. Health Perspec.* 104, 5-21.

Bauer, L., Tulsan, A.H., and Müller, E. (1972). Ultrastructural changes produced by the carcinogen, aflatoxin B1, in different tissues. *Virchows Arch. Zellpath.* 10, 275-285.

Bechtel, D.H. (1989). Molecular dosimetry of hepatic aflatoxin B_1-DNA adducts: linear correlation with hepatic cancer risk. *Reg. Toxicol. Pharmacol.* 10, 74-81.

Braunbeck, T., Görge, G., Storch, V., and Nagel, R. (1990a). Hepatic steatosis in Zebra Fish (*Brachydanio rerio*) induced by long-term exposure to (gamma)-hexachlorocyclohexane. *Ecotoxicol. Environ. Saf.* 19, 355-374.

Braunbeck, T., Storch, V., and Bresch, H. (1990b). Species-specific reaction of liver ultrastructure in zebrafish (*Brachydanio rerio*) and trout (*Salmo gairdneri*) after prolonged exposure to 4-chloroaniline. *Arch. Environ. Contam. Toxicol.* 19, 405-418.

Bresch, H., Beck, H., Ehlermann, D., Schlaszus, H., and Urbanek, M. (1990). A long-term toxicity test comprising reproduction and growth of zebrafish with 4-chloroaniline. *Arch. Environ. Contam. Toxicol.* 19, 419-427.

Buchmann, A., Wannenmacher, R., Kulzer, E., Buhler, D.R., and Bock, K.W. (1993). Immunohistochemical localization of the cytochrome P450 isozymes LMC2 and LM4B (P4501A1) in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-treated zebrafish (*Brachydanio rerio*). *Toxicol. Appl. Pharmacol.* 123, 160-169.

Cesarone, C., Bolognesi, C., and Santi, L. (1979). Improved microfluorometric DNA determination in biological material using 33258 Hoechst. *Anal. Biochem.* 92, 188-197.

Clark, D.J., George, S.G., and Burchell, B. (1991). Glucuronidation in fish. *Aquat. Toxicol.* 20, 35-56.

Coulombe, R.A., Shelton, D.W., Sinnhuber, R.O., and Nixon, J.E. (1982). Comparative mutagenicity of aflatoxins using a *Salmonella*/trout hepatic enzyme activation system. *Carcinogenesis* 3, 1261-1264.

Dashwood, R.H., Arbogast, D.N., Fong, A.T., Hendricks, J.D., and Bailey, G.S. (1988). Mechanisms of anti-carcinogenesis by indole-3-carbinol: detailed *in vivo* DNA binding dose-response studies after dietary administration with aflatoxin B_1. *Carcinogenesis* 9, 427-432.

Dashwood, R.H., Mariën, K., Loveland, P.M., Williams, D.E., Hendricks, J.D., and Bailey, G.S. (1992). Formation of aflatoxin-DNA adducts in trout and their use as molecular dosimeters for tumor prediction. In *Handbook of Applied Mycology* (Bhatnager, D., Lillehoj, E.B., and Arora, D.K., Eds.), pp. 183-211. Marcel Dekker, New York.

Dave, G., and Xiu, R. (1991). Toxicity of mercury, copper, nickel, lead, and cobalt to embryos and larvae of zebrafish, *Brachydanio rerio*. *Arch. Environ. Contam. Toxicol.* 21, 126-134.

Degen, G.H., and Neumann H-G. (1981). Differences in AFB_1-susceptibility of rat and mouse are correlated with the capability *in vitro* to inactivate aflatoxin B_1-epoxide. *Carcinogenesis* 2, 299-306.

Eaton D.L., and Gallagher, E.P. (1994). Mechanisms of aflatoxin carcinogenesis. *Annu. Rev. Pharmacol. Toxicol.* 34, 135-172.

Essigmann, J.M., Croy, R.G., Bennett, R.A., and Wogan, G.N. (1982). Metabolic activation of aflatoxin B_1: patterns of DNA adduct formation, removal, and excretion in relation to carcinogenesis. *Drug Metab. Rev.* 13, 581-602.

Gallagher, E.P., and Eaton, D.L. (1995). *In vitro* biotransformation of aflatoxin B_1 in channel catfish liver. *Toxicol. Appl. Pharmacol.* 132, 82-90.

Hawkins, W.E., Overstreet, R.M., and Walker, W.W. (1988). Carcinogenicity tests with small fish species. *Aquat. Toxicol.* 11, 113-128.

Hendricks, J.D. (1994). Carcinogenicity of aflatoxins in nonmammalian organisms. In *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance* (D.L. Eaton and J.D. Groopman, Ed.), pp 103-136. Academic Press, New York.

International Agency for Research on Cancer (IARC). (1993). IARC monographs on the evaluation of carcinogenic risks to humans. Some naturally occurring

substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. **56**, 245-396.

Kalsch, W., Nagel, R., and Urich, K. (1991). Uptake, elimination, and bioconcentration of ten anilines in zebrafish (*Brachydanio rerio*). *Chemosphere* **22**, 351-363.

Khudoley, V.V. (1984). Use of aquarium fish, *Danio rerio* and *Poecilia reticulata*, as test species for evaluation of nitrosamine carcinogenicity. *Natl. Cancer Inst. Monogr.* **No. 65**, 65-70.

Loveland, P.M., Coulombe, R.A., Libbey, L.M., Pawlowski, N.E., Sinnhuber, R.O., Nixon, J.E., and Bailey, G.S. (1983). Identification and mutagenicity of aflatoxicol-M_1 produced by metabolism of aflatoxin B_1 and aflatoxicol by liver fractions from rainbow trout (*Salmo gairdneri*) fed B-naphthoflavone. *Food Chem. Toxicol.* **21**, 557-562.

Loveland, P.M., Nixon, J.E., and Bailey, G.S. (1984). Glucuronides in bile of rainbow trout (*Salmo gairdneri*) injected with [^{3}H]aflatoxin B_1 and the effects of dietary B-napthoflavone. *Comp. Biochem. Physiol.* **78C**, 13-19.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.

Monroe, D.H., and Eaton, D.L. (1987). Comparative effects of butylated hydroxyanisole on hepatic *in vivo* DNA binding and *in vitro* biotransformation of aflatoxin B_1 in the rat and mouse. *Toxicol. Appl. Pharmacol.* **90**, 401-409.

Nagel, R., Bresch, H., Caspers, N., Hansen, P.D., Markert, M., Munk, R., Scholz, N., and ter Höfte B.B. (1991). Effect of 3,4-dichloroaniline on early life stages of the zebrafish (*Brachydanio rerio*): results of a comparative laboratory study. *Ecotoxicol. Environ. Saf.* **21**, 157-164.

Neilson, A.H., Allard, A-S., Fischer, S., Malmberg, M., and Viktor, T. (1990). Incorporation of a subacute test with zebra fish into a hierarchical system for evaluating the effect of toxicants in the aquatic environment. *Ecotoxicol. Environ. Saf.* **20**, 82-97.

Nixon, J.E., Hendricks, J.D., Pawloswki, N.E., Loveland, P.M., and Sinnhuber, R.O. (1981). Carcinogenicity of aflatoxicol in Fischer 344 rats. *J. Natl. Cancer Inst.* **66**, 1159-1163.

Pliss, G.B., and Khudoley, V.V. (1975). Tumor induction by carcinogenic agents in aquarium fish. *J. Natl. Cancer Inst.* **55**, 129-136.

Raney, K.D., Coles, B., Guengerich, F.P., and Harris, T.M. (1992a). The *endo*-8,9-epoxide of aflatoxin B_1: a new metabolite. *Chem. Res. Toxicol.* 5, 333-335.

Raney, K.D., Meyer, D.J., Ketterer, B., Harris, T.M., and Guengerich, F.P. (1992b). Glutathione conjugation of aflatoxin B_1 *exo*- and *endo*-epoxides by rat and human glutathione-*S*-transferases. *Chem. Res. Toxicol.* 5, 470-478.

SAS Institute Inc., *SAS/STAT User's Guide*, Version 6, Fourth Edition, Cary, NC: SAS Institute Inc, 2, 1-846.

Schoenhard, G.L., Hendricks, J.D., Nixon, J.E., Lee, D.J., Wales, J.H., Sinnhuber, R.O., and Pawlowski, N.E. (1981). Aflatoxicol-induced hepatocellular carcinoma in rainbow trout (*Salmo gairdneri*) and the synergistic effects of cyclopropenoid fatty acids. *Cancer Res.* 41, 1011-1014.

Sinnhuber, R.O., Hendricks, J.D., Wales, J.H., Putnam, G.B. (1977). Neoplasms in rainbow trout, a sensitive animal model for environmental carcinogenesis. *Ann. NY Acad. Sci.* 298, 389-408.

Stanton, M.F. (1965). Diethylnitrosamine-induced hepatic degeneration and neoplasia in the aquarium fish, *Brachydanio rerio*. *J. Natl. Cancer Inst.* 34, 117-130.

Strauss, W.M. (1991). Preparation of genomic DNA from mammalian tissue. In *Current Protocols in Molecular Biology* (Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., Eds.), pp 2.2.1-2.2.2. Greene Publishing Associates and Wiley-Interscience, New York.

Swenson, D.H., Lin, J.-K., Miller, E.C., and Miller, J.A. (1977). Aflatoxin B_1-2,3-oxide as a probable intermediate in the covalent binding of aflatoxins B_1 and B_2 to rat liver DNA and ribosomal RNA *in vivo*. *Cancer Res.* 37, 172-181.

Takahashi, N., Harttig, U., Williams, D.E., and Bailey, G.S. (1996). The model Ah-receptor agonist β-naphthoflavone inhibits aflatoxin B_1-DNA binding *in vivo* in rainbow trout at dietary levels that do not induce CYP1A enzymes. *Carcinogenesis* 17, 79-87.

Toledo, C., Hendricks, J., Loveland, P., Wilcox, J., and Bailey, G. (1987). Metabolism and DNA-binding *in vivo* of aflatoxin B_1 in medaka (*Oryzias latipes*). *Comp. Biochem. Physiol.* 87C, 275-281.

Tsai, H.-W. (1996). Evaluation of zebrafish (*Brachydanio rerio*) as a model for carcinogenesis. [Ph.D. dissertation]. Oregon State University, Corvallis, OR.

Williams, D.E., and Buhler, D.R. (1983). Purified form of cytochrome P-450 form rainbow trout with high activity towards conversion of aflatoxin B_1 to aflatoxin B_1-2,3-epoxide. *Cancer Res.* 43, 4752-4756.

Witham M., Nixon, J.E., and Sinnhuber, R.O. (1982). Liver DNA bound *in vivo* with aflatoxin B_1 as a measure of hepatocarcinoma initiation in rainbow trout. *J. Natl. Cancer Inst.* **68**, 623-628.

CHAPTER 3

CYP1A INDUCTION BY β-NAPHTHOFLAVONE, AROCLOR 1254, AND 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN, AND ITS INFLUENCE ON AFLATOXIN B_1 METABOLISM AND DNA BINDING IN ZEBRAFISH (*Danio rerio*)

Claudia M. Troxel,

Donald R. Buhler, Jerry D. Hendricks,

and George S. Bailey.

Toxicology Program

Oregon State University

Corvallis, OR 97331

Abstract

This study investigated the response of cytochrome P450 1A (CYP1A) in the zebrafish (*Danio rerio*) following exposure to Aroclor 1254, β-naphthoflavone (βNF), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and then investigated TCDD modulation of aflatoxin B_1 (AFB_1) metabolism and hepatic AFB_1-DNA adduction. Aroclor 1254 fed at 500 ppm for 1 to 9 days or intraperitoneal (i.p.) injection of 75 - 200 mg Aroclor 1254/kg body weight failed to induce CYP1A protein or associated 7-ethoxyresorufin-*O*-deethylase (EROD) activity. Dietary βNF at 500 ppm for 3 or 7 days induced CYP1A protein and EROD activity approximately 3-fold above controls. A single i.p. injection of 150 mg βNF showed maximal induction of CYP1A protein and EROD activity by 24 hours, both of which decreased rapidly during the next 6 days. A more refined study showed maximum CYP1A protein levels between 24 and 36 hours, while EROD activity had an observable peak at 16 hours. CYP1A and EROD activity showed dose-responsiveness following single i.p. administration of 25, 50, 100, or 150 mg βNF/kg body weight. Dietary exposure to 0.75 ppm TCDD for 3 days also significantly induced CYP1A. The effect of TCDD on the metabolism of [^{3}H]AFB_1 in zebrafish was then investigated. The major [^{3}H]AFB_1 metabolites excreted in water over 24 hours in the control group were aflatoxicol, aflatoxicol-glucuronide, and parent AFB_1. By contrast, the predominant metabolites in the TCDD-pretreated group were aflatoxicol-M_1-glucuronide, aflatoxicol, aflatoxin M_1 plus aflatoxicol-M_1 unresolved, aflatoxicol-glucuronide, and parent AFB_1. Surprisingly, hepatic AFB_1-DNA adduction was approximately 4-fold higher in the TCDD treated group compared to controls. This significant difference could not be explained by increased capacity for

bioactivation of AFB_1 as measured by an *in vitro* AFB_1 metabolism assay. However, it was demonstrated that zebrafish have the capacity to bioactivate aflatoxin M_1 to a reactive intermediate, and it is proposed that secondary bioactivation of this genotoxic intermediate may be responsible for the increased DNA binding.

Introduction

Fish are becoming important models in many areas in science, including developmental biology, genetics, neurobiology, aquatic toxicology, and carcinogenesis (Powers, 1989). The trout model has been and continues to be extensively characterized as an alternative nonmammalian vertebrate model in carcinogenesis research (for review see Bailey *et al.*, 1996). The use of smaller fish as experimental models is also assuming prominence because they have several advantages over the trout model such as their small size, ability to spawn regularly, reduced husbandry costs, and their overall hardiness (Hawkins *et al.*, 1988). Many small fish species, such as the guppy, Japanese medaka, and zebrafish are proving to be susceptible to numerous carcinogens, including diethyl- and dimethylnitrosamine, 2-acetylaminofluorene, nitrosomorphiline, and methylazoxymethanol acetate (Sato *et al.*, 1973; Khudoley, 1984; Fournie *et al.*, 1987; Nakazawa *et al.*, 1985; Hawkins *et al.*, 1986; Stanton, 1965). Zebrafish are a particularly attractive fish model because they are also being extensively developed in both genetics and developmental biology. Studies in our laboratory investigating the sensitivity of zebrafish to carcinogens are demonstrating that this species is a complex model. Adult zebrafish appear to be somewhat resistant to carcinogens administered in

the diet, but zebrafish are susceptible to carcinogen exposures in the water, particularly when exposed as embryos and fry (Tsai, 1996; unpublished results).

Induction of cytochrome P450 1A (CYP1A) in fish is being investigated as a biomarker of exposure to certain compounds commonly found in the environment such as polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and halogenated dibenzo-*p*-dioxins and dibenzofurans (for review see Goksoyr, 1995). While fish appear to lack the equivalent of inducible phenobarbital-type P450s, most fish species do possess a CYP1A-like P450 that can be effectively induced to a high level (Goksoyr *et al.*, 1991; Stegeman, 1989; Stegeman and Hahn, 1994). CYP1A is not normally constituitively expressed in significant levels. Following induction, it is primarily located in hepatic tissue, although in fish, it can also be found in extrahepatic tissues such as gill, kidneys, gut and heart (Buchmann *et al.*, 1993; Stegeman *et al.*, 1989; Husoy *et al.*, 1994). Induction of CYP1A occurs following exposure to a wide range of compounds, including the classic inducer 3-methylcholanthrene, many flavones and indoles, and the PAHs and PCBs. In mammals, the Ah-receptor is the mediator of induction, and is known to be responsible not only for the induction of CYP1A1, but a host of other enzymes including quinone-oxido-reductase, glucuronsyltransferase, glutathione-S-transferase, CYP1A2, and CYP2B1 (Poland, 1979; Hankinson, 1995).

The enhanced metabolic capabilities following exposure to one of the many CYP1A-inducing agents may result in altered metabolism of other xenobiotics including procarcinogens. One of the most extensively characterized procarcinogens is the potent mycotoxin aflatoxin B_1 (AFB_1), which requires bioactivation to the reactive intermediate, the AFB_1-8-9-epoxide. While both the *endo*- and *exo*-AFB_1 epoxide may be produced,

it is primarily the *exo*-epoxide stereoisomer that is responsible for binding to DNA (Raney *et al.*, 1992a; 1992b; Eaton and Gallagher, 1994). This mycotoxin is carcinogenic in many species, including rat, human, and rainbow trout, the most sensitive species. Numerous investigations have explored the modulation of aflatoxin B_1 metabolism and carcinogenesis by compounds known to act via the Ah receptor. Studies with rainbow trout have shown that pre-exposure to the synthetic flavone β-naphthoflavone (βNF), the PCB mixture Aroclor 1254, and the natural plant constituent indole-3-carbinol reduces AFB_1 carcinogenesis by altering the metabolism of AFB_1 and reducing DNA adduction (Shelton *et al.*, 1986; Goeger *et al.*, 1986; Nixon *et al.*, 1984; Takahashi *et al.*, 1995; 1996). An *in vitro* investigation of βNF-induced channel catfish microsomes showed induction of CYP1A enhanced detoxification of the carcinogen, but did not affect bioactivation of AFB_1 to its reactive intermediate (Gallagher and Eaton, 1995). Other studies investigating βNF-induced rabbit pulmonary and hepatic microsomes and βNF-induced guinea pig liver, kidney, and lung microsomes have also shown increased formation of the less toxic metabolite aflatoxin M_1 and reduced DNA adduction (Daniels and Massey, 1992; Liu, *et al.*, 1993). Although 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent Ah receptor agonist known, surprisingly few studies have investigated the modulation of AFB_1 metabolism and DNA adduction by pre-exposure to TCDD. The purpose of this study was to characterize CYP1A induction in zebrafish by Aroclor 1254, βNF, and TCDD. After characterizing CYP1A induction, further studies were conducted to investigate the *in vivo* modulation of AFB_1 metabolism and hepatic DNA adduction following dietary exposure to TCDD at a concentration known to exert biological effects on metabolizing enzymes.

Materials and Methods

Chemicals

AFB_1 and AFM_1 were purchased from Sigma Chemical Company (St. Louis, MO), and assessed for purity by thin layer chromatography (TLC); [^{3}H]AFB_1 was from Moravek Biochemicals (Brea, CA) and the chemical and radiopurity was checked by TLC followed by radioscanning; βNF was from Fluka Chemical Corp. (Ronkonkoma, NY); Aroclor 1254 was from Monsanto Company (St. Louis, MO); TCDD was from ANALABS (New Haven, CT); 7-ethoxyresorufin and resorufin were from Molecular Probes, Inc. (Eugene, OR); RNase, DNase free were from Boehringer Mannheim Biochemicals (Indianapolis, IN); HPLC grade acetonitrile, methanol, tetrahydrofuran, and J.T. Baker C_{18} Empore extraction disks were from VWR (Seattle, WA); Hoechst #33258 was from Calbiochem-Behring Corp. (La Jolla, CA); aflatoxin HPLC standards aflatoxicol (AFL), aflatoxicol-glucuronide (AFL-g), aflatoxin M_1 (AFM_1), and aflatoxicol-M_1 (AFL-M_1) were prepared in our laboratory by previously published methods (Loveland *et. al.*, 1983; 1984).

Animal care and maintenance

Adult (sexually mature) zebrafish were reared and maintained in the Food Toxicology and Nutrition Laboratory in aerated 29 gallon aquariums with a controlled temperature of 26°C ($\pm$ 1) and a 14 hour light:10 hour dark photoperiod. During the week, fish were fed twice daily with TetraMin Staple Food (flakes) each morning, and

a combination of Oregon Test Diet (OTD) (Sinnhuber *et al.*, 1977) and brine shrimp in the afternoon on Monday, Wednesday, and Friday, and TetraMin Staple Food and brine shrimp on Tuesday and Thursday afternoons. On weekends, fish received only one feeding per day consisting of OTD and brine shrimp. For dietary exposures, fish were fed one feeding of OTD or the test reagent in OTD per day during the course of the treatment. For the Aroclor 1254 dietary exposure only, fish were fed a control diet consisting of the purified casein diet (PC diet) or control diet containing Aroclor 1254 (DeKoven *et al.*, 1992).

Preparation of liver tissue

All fish were fasted for 24 hours before receiving the appropriate treatment. When fish were sampled, livers were immediately excised from the zebrafish, the gall bladders carefully removed, and the unrinsed livers placed on ice until the appropriately sized pools of tissue were obtained. The samples were then flash frozen in liquid nitrogen and stored at -80°C. Before use, livers were homogenized in ice-cold buffer containing 0.1 M potassium phosphate (pH 7.25), 20% glycerol, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiolthreitol, 0.1 mM butylated hydroxytoluene, and 0.1 mM phenylmethylsulfonylfluoride. The samples were centrifuged for 10 minutes at 1,000x g and the supernatant decanted for protein work. When DNA binding was also being investigated, the pellet was saved for DNA isolation and purification.

Investigation of CYP1A induction by Aroclor 1254, βNF, and TCDD

A total of 18 male and 18 female adult zebrafish were fed 500 ppm Aroclor 1254 twice per day and 3 males and 3 females each were sampled on 1, 2, 3, 5, 7, or 9 days. Four males and 4 females were fed the control PC diet twice per day and 1 male and 1 female sampled at days 2, 3, 5 and 7. When dietary exposure failed to show induction of CYP1A, 6 adult, female fish were i.p. injected with 100 or 200 mg Aroclor 1254/kg body weight, 4 were injected with carrier dimethylsulfoxide (DMSO), and all were sampled 24 hours later.

To confirm results from a preliminary investigation which showed CYP1A induction after i.p. exposure to βNF, a time-course study was undertaken. Thirty adult female zebrafish were i.p. injected with 150 mg βNF/kg body weight and sampled at day 1, 2, 3, 4, and 7; 6 controls were injected with DMSO and sampled at day 1, 2, and 3. A concurrent dietary exposure to 500 ppm of βNF was also conducted to investigate whether dietary exposure to this flavone was capable of inducing CYP1A in zebrafish. Sixteen adult female fish were fed 500 ppm βNF and 8 fish fed control diet for 3 or 7 days. To further pinpoint the time of maximum CYP1A induction by βNF, 32 adult female zebrafish were i.p. injected with 150 mg/kg body weight of βNF in DMSO and sampled at 8, 16, 24, or 36 hours after injection. Twenty-four female zebrafish were also injected with 25, 50, or 100 mg/kg body weight of βNF in DMSO and sampled 24 hours later to try to establish a dose response (the 24 hour group in the time-course served as the 150 mg βNF/kg body weight dose). To evaluate if the use of DMSO instead of corn oil as a vehicle influenced the kinetics of induction, 40 females were i.p. injected with 150 mg/kg body weight of βNF in corn oil and sampled

24, 36, 48, 72, and 96 hours after injection. Lastly, to verify previous results demonstrating a lack of detectable CYP1A following exposure to Aroclor 1254, 16 female fish were i.p. injected with 75 or 150 mg Aroclor 1254/kg body weight using DMSO as a carrier. Ten females were i.p. injected with DMSO and 8 females were injected with corn oil and sampled 24 hours later to serve as controls for these experiments.

Finally, to verify that TCDD induces CYP1A in the zebrafish, 16 adult female zebrafish were fed 0.75 ppm TCDD or control diet for 3 days and sampled on the fourth day. This dosage was based on a previous study by Buchmann *et al.* (1993) which also investigated CYP1A in zebrafish.

TCDD modulation of aflatoxin DNA-binding

Sixty-four adult female zebrafish were fasted for 24 hours and then fed control OTD or OTD diet containing 0.75 ppm TCDD for 3 days. The day after feeding was completed, 8 of the control fish and 8 of the TCDD-treated fish were sampled for representative assessment of CYP1A levels and EROD activity at the time of AFB_1 dosage. Then, 12 of the control and 12 of the TCDD-treated fish were i.p. injected with DMSO and the other 12 control and 12 TCDD-treated fish were i.p. injected 400 μg/kg body weight of [^{3}H]AFB_1 (16.6 Ci/mmol) in DMSO. Fish were rinsed with 1 ml of water to account for residual skin contamination before placement in respective 5 gallon buckets containing approximately 16 liters of aerated water. Rinse water accounted for less than 4% of the injected dosage. Livers were sampled 24 hours after injection.

DNA was isolated and purified using a modification of Strauss (1991) (modification found in Troxel *et al.*, Chapter 2 this thesis). DNA was quantified using the microfluorometric procedure of Cesarone *et al.* (1979) with a Hoefer TKO100 DNA fluorometer. The amount of [^{3}H]AFB_1 bound to DNA was determined after hydrolyzing the DNA by heating the samples at 70°C with equal volumes of 1 M perchloric acid for 20 minutes and then counting with a Beckman LS 7500 scintillation counter.

TCDD modulation of AFB_1 metabolism

Twelve adult female zebrafish were fasted for 24 hours and then fed control OTD or OTD diet containing 0.75 ppm TCDD for 3 days. The day after feeding was completed, 3 control and 3 TCDD-treated fish were i.p. injected with DMSO, whereas the remaining 3 control and 3 TCDD-treated fish were i.p. injected with 400 μg/kg body weight of [^{3}H]AFB_1 (16.6 Ci/mmol) in DMSO. Fish were rinsed with 1 ml of water to remove any residual radioactivity before placement in individual beakers containing 50 ml of water. Rinse water contained less than 5% of the administered dose except for fish 2 of the AFB_1/TCDD treatment group, which contained approximately 8% of the injected dosage. At various time points, fish were removed to fresh beakers of water, the previous water samples were collected, and an aliquot was taken for scintillation counting. At 24 hours, the livers of the fish were sampled and treated as described earlier for enzyme analysis and quantification of DNA adduction.

Water samples were extracted with C_{18} extraction disks and metabolite analysis performed by HPLC as described (Troxel *et al.*, Chapter 2 this thesis). The amount of radioactivity remaining after filtration by the C_{18} filter (i.e., the amount not trapped by

the filter) was higher than expected in some samples, so these water samples were refiltered with another C_{18} filter. Some additional radioactivity was trapped in the 7 and 24 hour samples of the AFB_1/TCDD treated group, suggesting incomplete adsorption in the first filtration. However, there still was a high amount of radioactivity remaining unbound in these doubly filtered water samples. To investigate the nature of this material, the 7 and 24 hour water samples from both AFB_1-treated groups were treated with 0.2 M sodium acetate buffer (pH 5.0), buffer containing β-glucuronidase (4000 units/ml), or sulfatase (20 units/ml, with 40 mM D-saccharic acid-1,4-lactone added to inhibit β-glucuronidase activity) (Fong *et al.*, 1993). The samples were incubated at 37°C for 18 hours before extracting twice with 2 volumes of ethyl acetate. When analysis of the results showed some glucuronidation in the 7 and 24 hour sample from the AFB_1/TCDD treated group and the 24 hour sample from the AFB_1 group, up to 50 ml of the water samples were evaporated down to 1 ml, and the samples were then injected into the HPLC for further analysis. Under these HPLC conditions, AFM_1 and AFL-M_1 were not fully resolved, and therefore are reported together in the analysis.

Immunoblotting

Using bovine serum albumin as the standard, total protein was quantified by the method of Lowry *et al.* (1951). Proteins were separated by SDS-PAGE (Laemmli, 1970) and electrophoretically transferred to nitrocellulose membranes (Towbin *et al.*, 1979). Due to the large number of samples to compare, after verification that only one cross-reacting band was present in blots analyzed for CYP1A protein, subsequent analysis of CYP1A was limited to dot blots. The blots were incubated with rabbit anti-

trout IgG against LM_{4b} (CYP1A) at a concentration of 2 μg/ml. The membranes were next probed with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase. The P450 isozyme was detected using the ECL chemiluminescence detection kit. Densitometry analysis was performed with an HP ScanJet flatbed scanner with NIH Image version 1.54 software (Wayne Rasband, NIH, public domain). Previously quantified βNF-induced trout liver microsomes were used as standards on the blots, and all blots were normalized with the same sample of βNF-induced zebrafish liver homogenate.

Enzyme assays

EROD activity was determined by the method of Prough *et al.* (1978). The assay was conducted at 30°C with a substrate concentration of 2 μM.

The *in vitro* AFB_1 metabolism assay was conducted using the method of Monroe and Eaton (1987), with modifications by Takahashi *et al.* (1996). This assay can indirectly measure bioactivation of AFB_1 by trapping the reactive intermediate with glutathione. Previous studies in which trout microsomes were incubated with AFM_1 instead of AFB_1 have shown the formation of another glutathione adduct with a different HPLC retention time than that of the AFB_1-glutathione adduct, suggesting that trout also bioactivate AFM_1 to a reactive intermediate (data not shown). In this experiment, the metabolism of both AFB_1 and AFM_1 was investigated in zebrafish liver homogenates. The final assay concentration of AFM_1 or AFB_1 was 80 μM, and 0.51 mg/ml of supernatant protein from liver homogenates was used. Assay conditions consisted of a 2 minute pre-incubation at room temperature before the addition of the glutathione and

NADPH. Incubations were carried out at 28°C for 45 minutes before termination with ice-cold 2 M acetic acid and internal standard AFG_1. Preliminary studies showed that this time point was in the linear range of time.

Statistical analysis

Statistical analysis was performed with SAS, version 6.10 (SAS Institute Inc., 1989). Differences in the means between two groups were determined using the t-test procedure (equal or unequal variance t-test). Time or dose response data were analyzed using analysis of variance (GLM procedure), followed by polynomial trend analysis. The dietary βNF study was analyzed by two way analysis of variance (days and βNF dose effects). A p value less than 0.05 was considered significant in all analyses.

Results

Investigation of CYP1A induction in adult zebrafish by Aroclor 1254, βNF, and TCDD

The dietary exposure of adult zebrafish to 500 ppm Aroclor 1254 for up to 9 days failed to increase CYP1A protein quantities above control levels, and did not measurably induce hepatic EROD activity. A subsequent study investigating the induction of CYP1A following i.p. injection of 100 or 200 mg Aroclor 1254/kg body weight also showed no response to this common inducer. A second attempt to induce CYP1A following i.p. injection of 75 or 150 mg Aroclor 1254 was again unsuccessful.

Thus Aroclor 1254 failed to evoke a detectable response in the zebrafish by either dietary or i.p. treatment.

By contrast, a time-course investigation of CYP1A induction following i.p. injection of 150 mg βNF/kg body weight showed strong and maximal induction of both CYP1A protein and EROD activity by 24 hours, the first time-point examined following injection, with a curvilinear decrease over time (linear, $p=0.0001$; quadratic, $p<0.004$) (Figure 3.1, panel A). There was a rapid decrease in both protein and activity during the remaining course of the week, with barely detectable levels of EROD activity and non-detectable levels of protein at day 7. A dietary exposure of 500 ppm βNF for 3 or 7 days demonstrated an approximate 3-fold increase in protein levels and EROD activity above controls ($p<0.04$, βNF main effect), but there was no statistical difference between the days ($p=0.7$) (Figure 3.1, panel B). Noteworthy is that low but detectable levels of CYP1A were observed in the controls from the dietary exposure, whereas control values in the injection experiment were non-detectable. This is suggestive of a dietary component possessing some CYP1A-inducing activity.

Additional studies were conducted to further investigate the response of zebrafish CYP1A following exposure to βNF. Another time-course experiment using a narrower range of time was performed, because CYP1A was already maximally induced at the first time point examined in the previous time-course experiment. This study showed that protein levels displayed an increasing linear response over time ($p=0.003$), and were highest between 24 and 36 hours following injection, while the EROD activity exhibited a significant curved response (quadratic, $p=0.035$), with an observed peak at 16 hours (Figure 3.2, panel A). It is interesting to note that the amount of induction

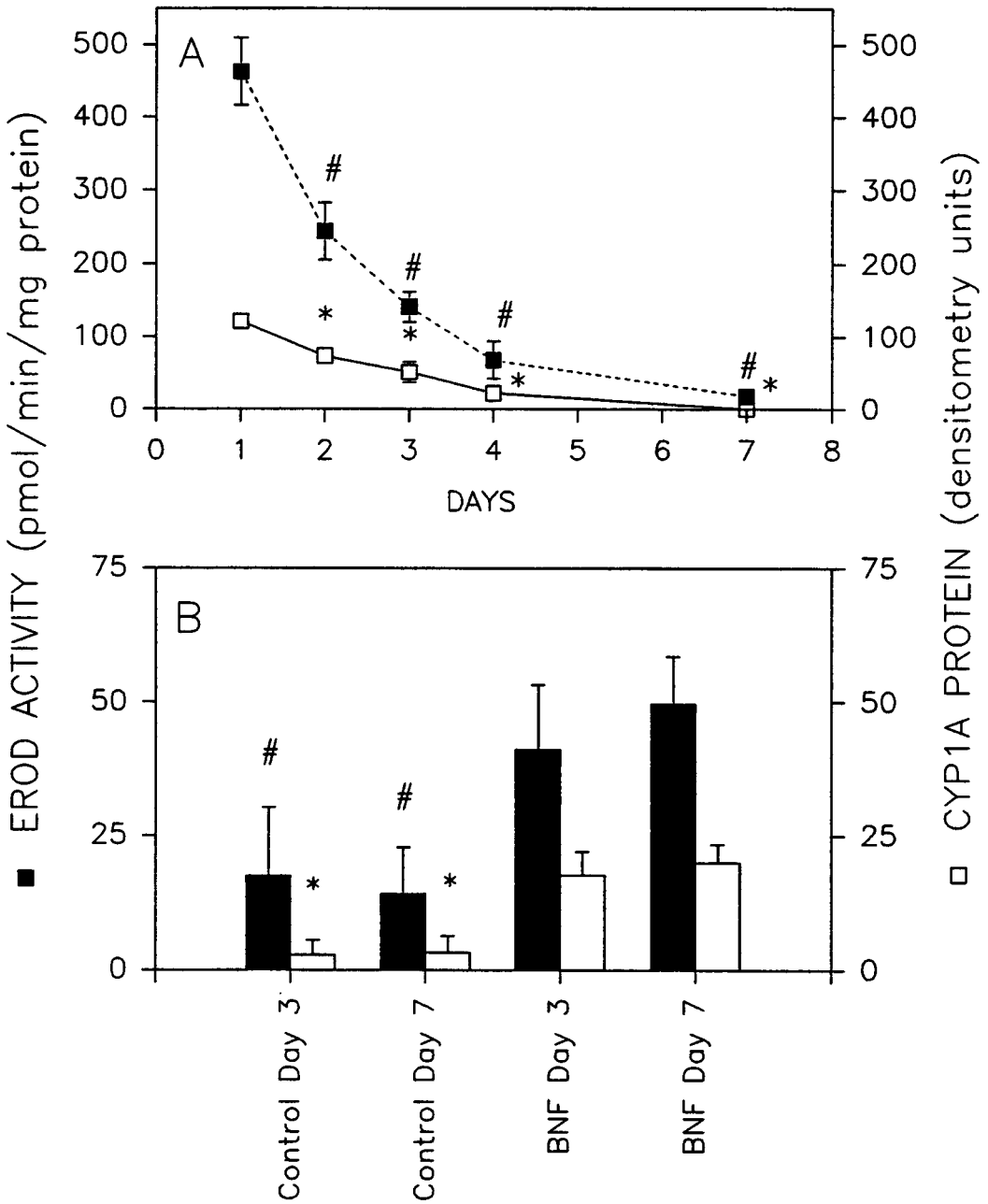

Figure 3.1. Induction of CYP1A protein and EROD activity in adult female zebrafish following i.p. or dietary administration of βNF. A) Single i.p. injection of 150 mg βNF/kg body weight (data are means ± SEM from 3 pools of 2 fish each) B) dietary exposure to 500 ppm βNF (data are means ± SEM from 4 pools of 2 fish each for treated, and 2 pools of 2 fish each for controls). * and # designate statistical difference ($p<0.05$) from the observed peak for protein induction (*) and EROD levels (#).

Figure 3.2. Induction of CYP1A protein and EROD activity in adult female zebrafish by βNF following different treatments. A) single i.p. administration of 150 mg βNF/kg body weight with sampling up to 36 hours after injection B) i.p. administration of 25, 50 100, or 150 mg βNF/kg body weight and sampled 24 hours later C) single i.p. administration of 150 mg/kg body weight of βNF in corn oil instead of the carrier DMSO, sampled up to 4 days following injection. All data are means ± SEM for 4 pools of 2 fish each). * and # designate statistical difference ($p<0.05$) from the observed peak for protein induction (*) and EROD levels (#).

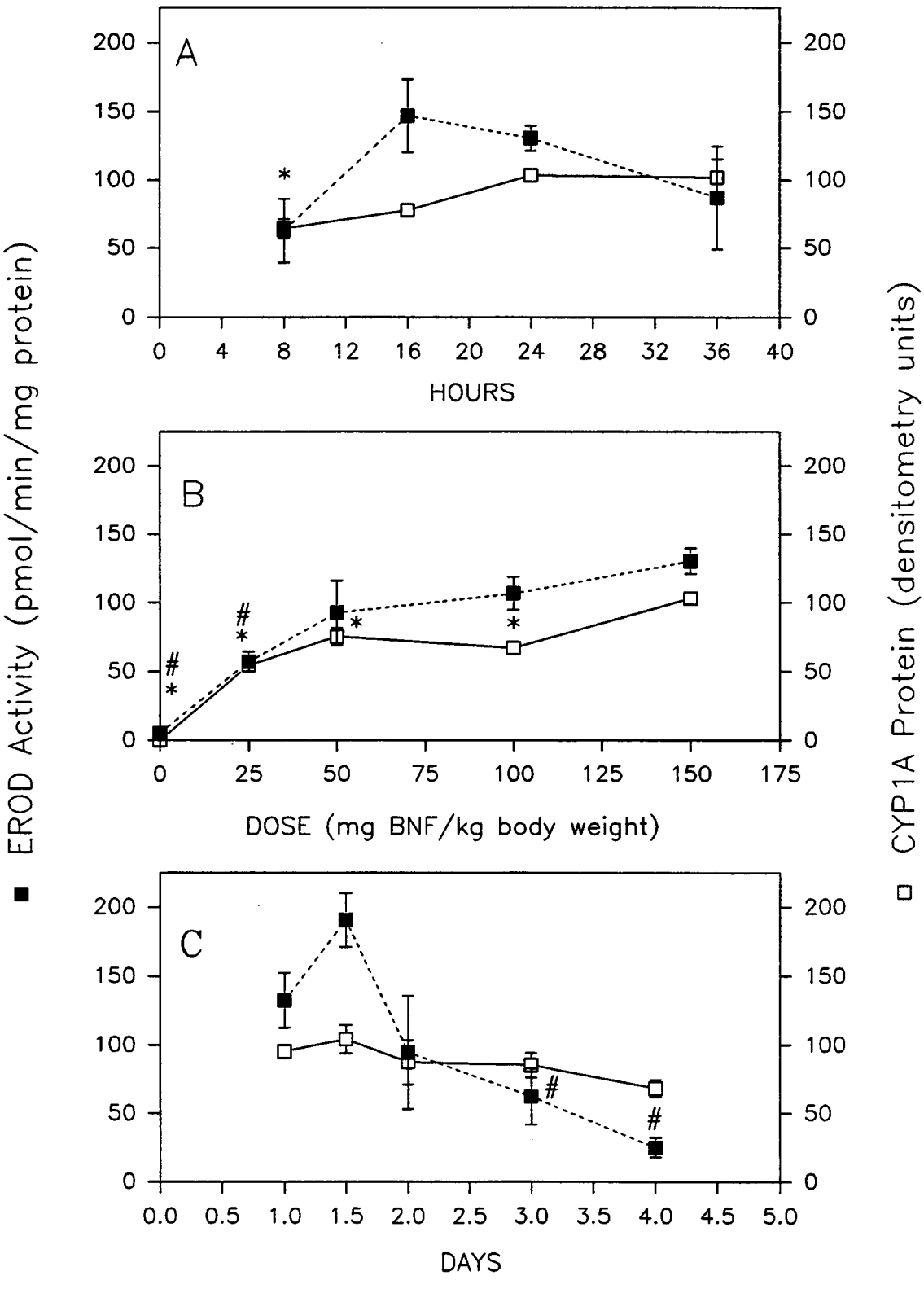
A
B
C
■ EROD Activity (pmol/min/mg protein)
□ CYP1A Protein (densitometry units)
HOURS
DOSE (mg BNF/kg body weight)
DAYS
0
50
100
150
200
0 4 8 12 16 20 24 28 32 36 40
0 25 50 75 100 125 150 175
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0

Figure 3.2.

documented in this experiment is considerably less than that observed in the first study. It should also be noted that these samples showed high variability between groups, especially at the 36 hour time-point, which might explain the discrepancy with maximal induction of EROD activity occurring before peak induction of protein. Intraperitoneal injection of 25, 50, 100, or 150 mg βNF/kg body weight provided dose-responsive induction of protein and EROD activity (Figure 3.2, panel B). The response to βNF was also investigated by conducting another time-course experiment following i.p. injection of 150 mg/kg body weight of βNF in corn oil instead of DMSO (Figure 3.2, panel C). Over the range of four days, there was a decreasing trend for EROD activity ($p=0.0004$), with an observed peak at 36 hours. This time point, however, was not statistically different from the 24 hour time point ($p=0.1$), which is consistent in the experiments with DMSO as a carrier. As in the experiment in Figure 3.2, panel A, levels of CYP1A protein showed less dramatic changes than EROD activity over the period examined.

TCDD, the most potent Ah receptor agonist yet described, was found to be a potent inducer of hepatic CYP1A in this species as well. Zebrafish fed 3 days with 0.75 ppm TCDD in OTD had an EROD activity approximately 17-fold higher than controls (391 ± 29 pmol/min/mg protein compared to 23 ± 5 pmol/min/mg protein). Protein levels were also greatly induced in TCDD-treated fish (96 ± 17 densitometry units versus non-detectable levels in controls).

TCDD modulation of AFB_1 metabolism in vivo

Early excretion kinetics of AFB_1 metabolites following i.p. administration of [^{3}H]AFB_1 was assessed by measuring the amount of radioactivity recovered in the water over 24 hours. The amount of radioactivity did not significantly differ between treatment groups. Approximately 52 $\pm$ 13% of the administered radioactivity was recovered by 24 hours in the TCDD/AFB_1 treatment group, while 45 $\pm$ 7% was accounted for in the AFB_1 treatment group (Figure 3.3). These data show that, overall, early excretion kinetics of [^{3}H]AFB_1 were not strongly affected by preexposure of zebrafish to TCDD.

However, whereas the total amount of radioactivity recovered in the water was similar, the actual metabolic profile of AFB_1 was different between the two groups. The AFB_1 treatment group exhibited a similar metabolic profile to that seen in the earlier metabolism study (Troxel *et al.*, Chapter 2 in this thesis). Aflatoxicol (AFL) accounted for approximately 17% of the original dose administered, followed by unreacted AFB_1 at 6.1% and aflatoxicol-glucuronide (AFL-g) at 5.3% (Figure 3.4, panel A). Less than 1% of the administered dose was recovered as aflatoxin M_1 (AFM_1), aflatoxicol M_1 (AFL-M_1), or aflatoxicol-M_1-glucuronide (AFL-M_1-g) combined. As was expected in the TCDD/AFB_1 treatment group, there was a higher production of AFM_1/AFL-M_1 and of the glucuronides. AFL-M_1-glucuronide was the predominate metabolite, accounting for 8.6% of the original dose, followed by AFB_1 and AFL with approximately 7.5% each, AFM_1 and AFL-M_1 with 5.6%, and lastly AFL-g with 4.0% (Figure 3.4, panel B). Treatment of the water samples with sulfatase demonstrated that

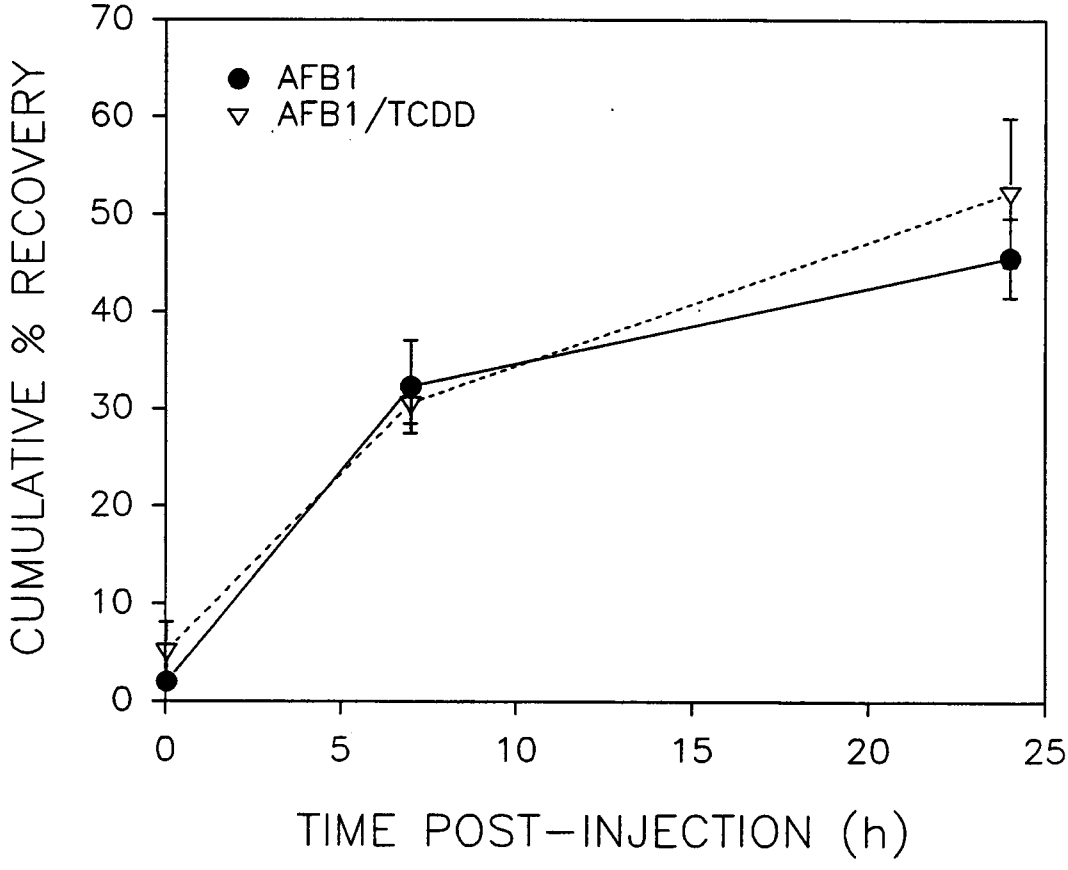

Figure 3.3. The cumulative percentage of radioactivity recovered in the water within 24 hours in control and TCDD-treated zebrafish following i.p. administration of [^{3}H]AFB$_1$.

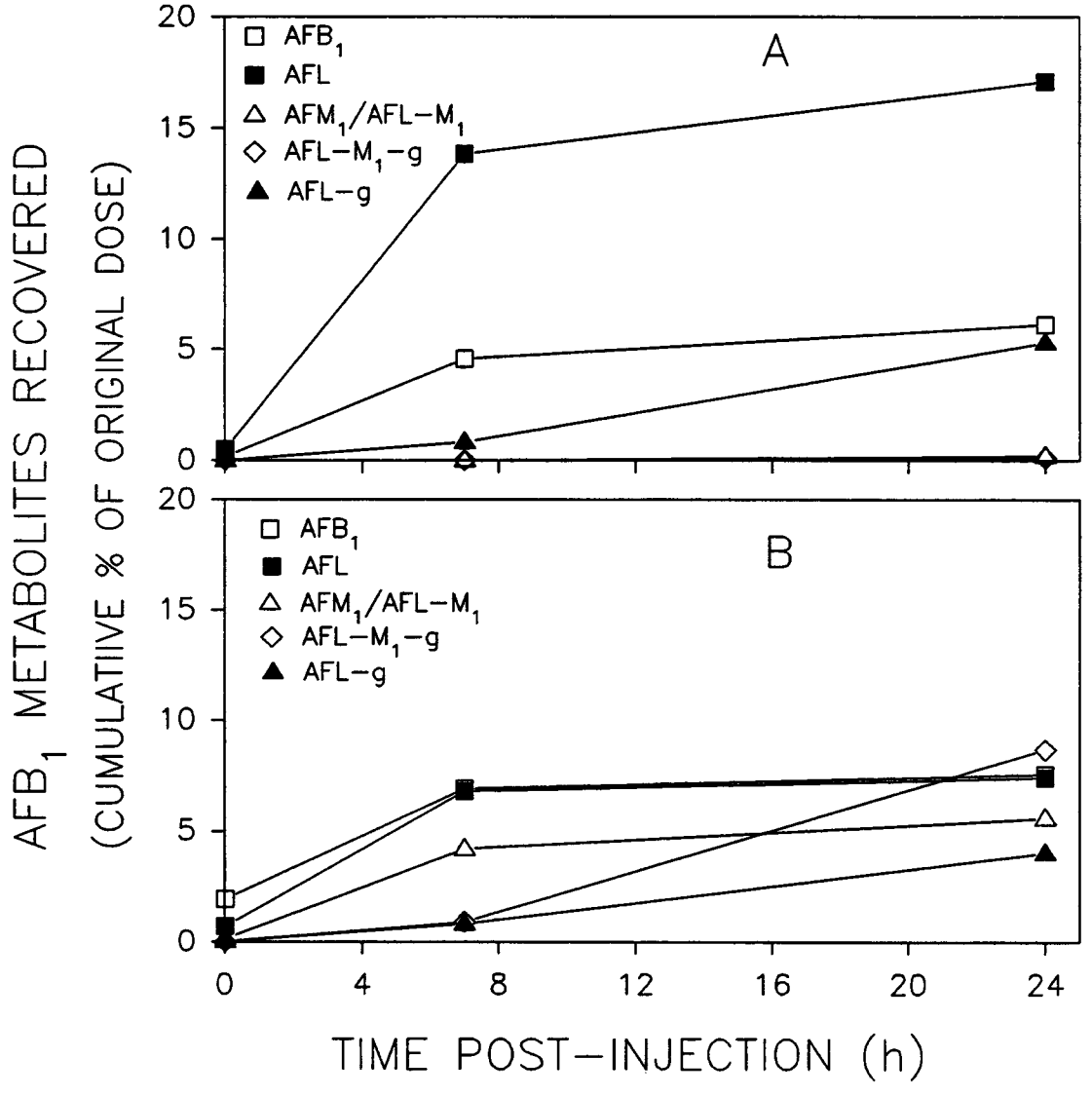

Figure 3.4. The cumulative percentage of individual metabolites recovered in water over 24 hours following i.p. administration of [^{3}H]AFB$_1$, expressed as a percentage of the original dose administered A) in the control-fed group B) in the TCDD-fed group.

sulfate conjugation represented only a minor metabolite (at most representing less that 0.5% of the administered dose) in the zebrafish.

Figure 3.5 shows the percentage of each metabolite recovered at each time point as a percentage of the original dose administered. Overall, the total percentage of recovery of the metabolites varied slightly between the groups, with 29% of the dose recovered in the AFB_1 group, and 33.2% in the TCDD/AFB_1 group. The slight increase in metabolites recovered in the TCDD/AFB_1 group occurred primarily at the 24 hour time point, where there was the increased production and excretion of the glucuronides of AFL and AFL-M_1. The greater production of AFL-M_1-g compared to AFL-g might reflect substrate availability, or perhaps the more polar AFL-M_1 may be preferentially glucuronidated over AFL.

TCDD modulation of in vivo hepatic AFB-DNA adduction and in vitro activation of AFB_1 and AFM_1

The *in vivo* hepatic AFB-DNA adduction at 24 hours after i.p. injection of 400 μg [^{3}H]AFB_1/kg body weight was determined to be almost 4-fold higher in the group pre-exposed to TCDD than in the control group (1660 $\pm$ 175 pmol AFB_1/mg DNA versus 438 $\pm$ 109 pmol AFB_1/mg DNA, $p < 0.05$) (Table 3.1). To further investigate the cause of this notable difference in adduction levels, *in vitro* AFB_1 and AFM_1 metabolism studies were conducted (Table 3.1). The results from these metabolism experiments showed no statistical difference between the control or TCDD-treated groups in their ability to bioactivate AFB_1 or AFM_1 to the respective reactive intermediate. There was also no statistical difference between the two groups in their

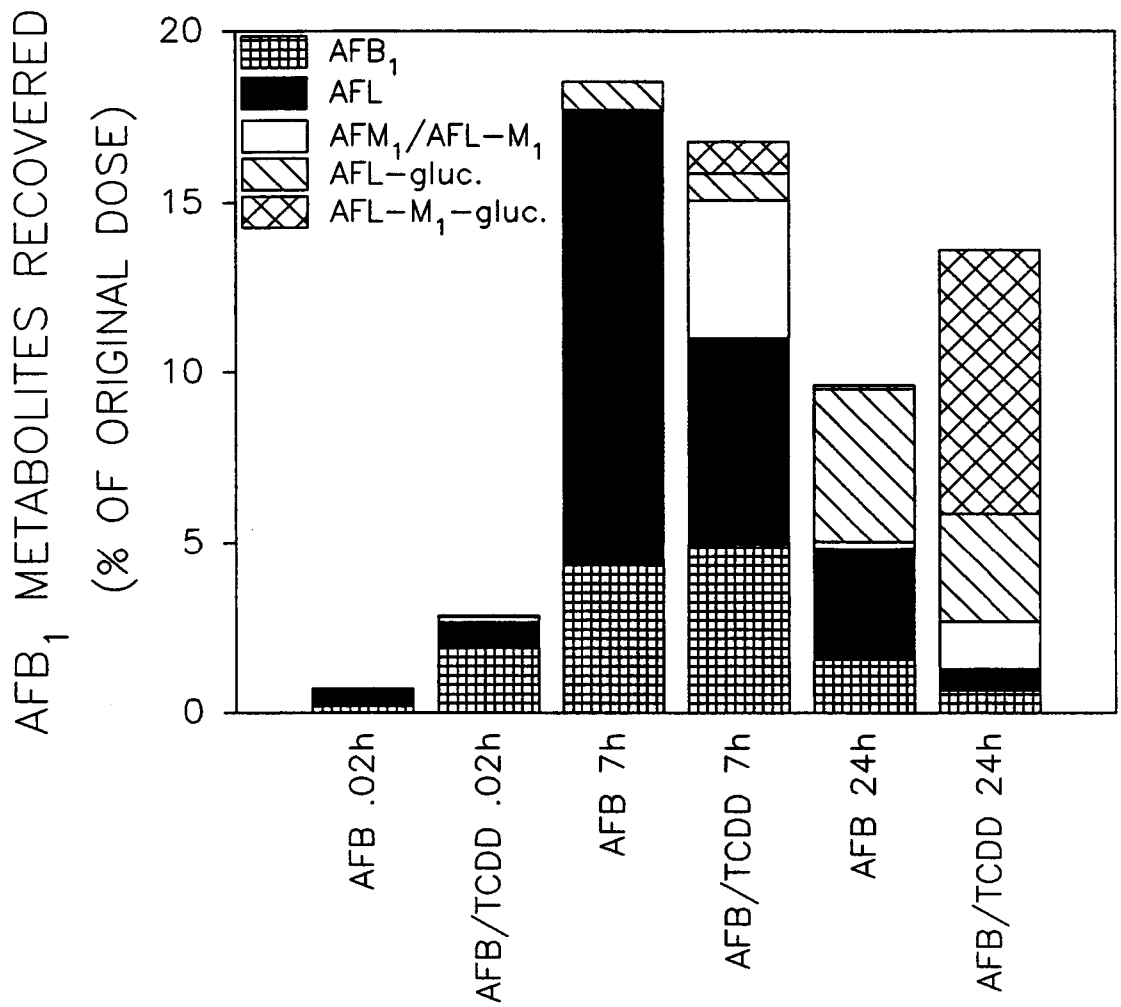

Figure 3.5. The percentage of all metabolites recovered in water at each time point, expressed as a percentage of the original dose in the control-fed group and in the TCDD-fed group.

Table 3.1. Effects of TCDD pretreatment on *in vivo* hepatic [^{3}H]AFB_1-DNA adduction and *in vitro* AFB_1 and AFM_1 bioactivation and metabolism[a].

	AFB_1	TCDD/AFB_1
[a]hepatic DNA adduction		
pmol AFB_1/mg DNA	438.2 ± 109.1	1660.2 ± 175.4
[b]aflatoxin-8,9-epoxide		
pmol/min/mg protein	13.5 ± 5.4	9.5 ± 1.0
aflatoxin M_1 (AFM_1)		
pmol/min/mg protein	1.0 ± 0.3	24.2 ± 3.0
aflatoxicol (AFL)		
pmol/min/mg protein	9.2 ± 2.4	8.3 ± 0.8
[c]aflatoxin M_1-8,9-epoxide		
pmol/min/mg protein	8.2 ± 3.1	6.2 ± 0.6

[a] data are means ± SEM from 3 pools of 4 fish each, and 1 pool of 3 fish each
[b] assays were run in duplicate with a final substrate concentration of 80 μmol AFB_1, with nonenzymatic background subtracted
[c] assays were run singly, with a final substrate concentration of 80 μM AFM_1, with nonenzymatic background subtracted. The epoxide was trapped as the glutathione adduct similarly to the AFB_1-8,9-epoxide.

capability of metabolizing AFB_1 to the primary metabolite AFL. However, the TCDD-treated group did have a 22-fold increase ($p=0.0001$) in their capability to metabolize AFB_1 to AFM_1, a reaction believed to be mediated by CYP1A in zebrafish as has been shown for trout (You *et al.*, unpublished results).

Effects of TCDD on CYP1A protein and EROD activity.

CYP1A protein levels and EROD activity were measured at day 0 (the time that the AFB_1 dosage was administered) in eight control and eight TCDD-treated fish to provide a representative value of CYP1A induction in the zebrafish at the time of AFB_1 dosing. The results were in accordance with the preliminary TCDD-dietary exposure already described earlier in this section. The average EROD activity was 337 $\pm$ 10 pmol/min/mg protein, and protein levels were 128 $\pm$ 4 densitometry units. In this experiment, no CYP1A activity was detectable in the controls. CYP1A induction was also measured in the supernatant from the samples used in the DNA-adduction and metabolism experiments. Protein levels and EROD activity were non-detectable in the samples that received no TCDD treatment (i.e., the groups fed control diet and injected with either DMSO or [^{3}H]AFB_1). CYP1A protein levels and EROD activity were comparable between the TCDD-treated groups (i.e., the groups fed TCDD and injected with DMSO or [^{3}H]AFB_1). The TCDD-treated group that received [^{3}H]AFB_1 had a 1.6-fold higher mean EROD activity than the TCDD-treated group injected with carrier only (550 $\pm$ 64 pmol/min/mg protein versus 344 $\pm$ 68 pmol/min/mg protein), but this difference in mean activity did not achieve significance ($p=0.07$) (Figure 3.6). The

results verify that TCDD treatment in fact did induce hepatic CYP1A in fish used for the AFB_1-DNA binding experiment.

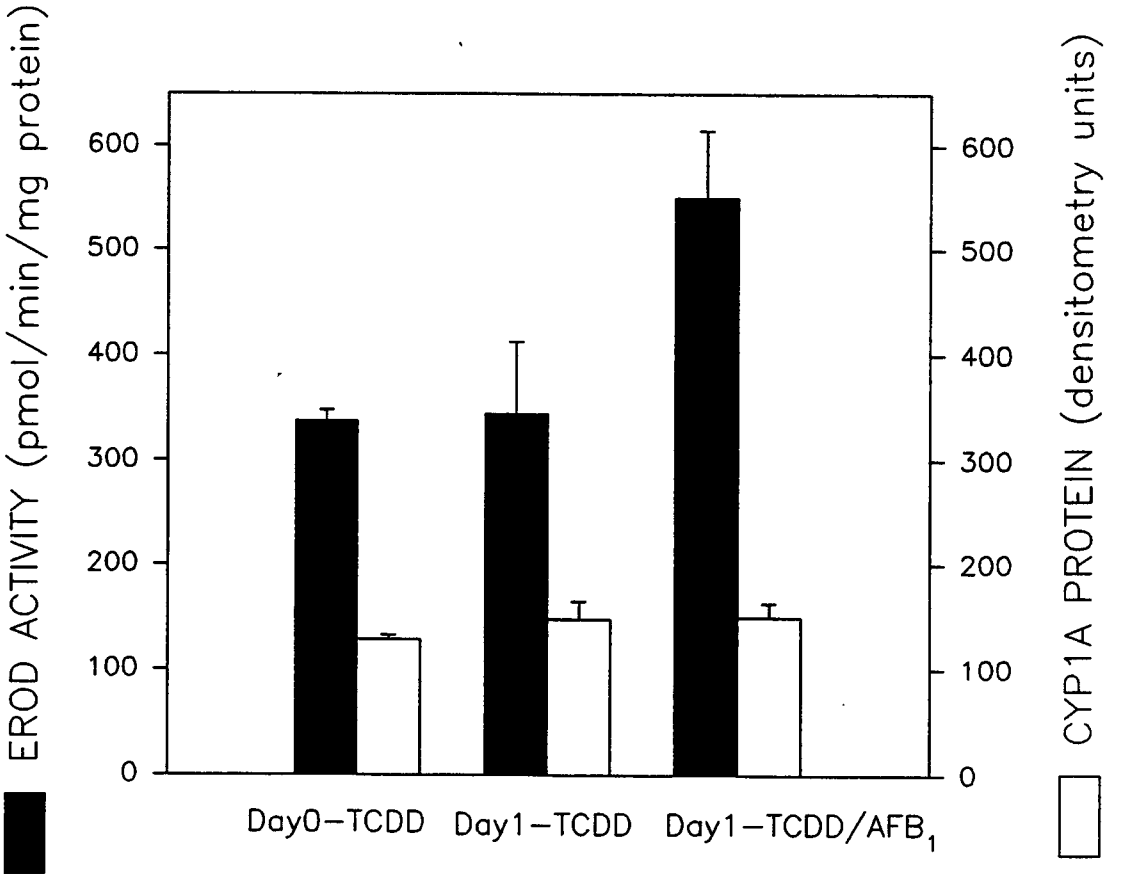

Figure 3.6. Levels of CYP1A protein and associated EROD activity in the supernatant from zebrafish liver homogenates used in the metabolism and DNA adduction experiments. (Data are means ± SEM from 3 pools of 4 fish each, and 1 pool of 3 fish each).

Discussion

CYP1A in zebrafish

A cytochrome P4501A1-like protein has been found in all fish species so far investigated, including zebrafish. Using anti-trout P4501A1 IgG, Buchmann *et al.* (1993) noted a single cross-reactive band from zebrafish liver microsomes following dietary exposure to TCDD, and Collodi *et al.* (1994) found 2 cross-reactive bands (50,000 and 54,000 kDa) from zebrafish liver homogenates following exposure to a static water bath containing TCDD. Our results showed that a single cross-reacting band was induced in zebrafish liver homogenates following exposure *in vivo* to βNF or TCDD using this same antibody. Interestingly, both an *in vivo* static bath exposure of zebrafish to 50 μg/l of βNF for 48 hours and zebrafish liver cells exposed to βNF *in vitro* showed no detectable induction of a CYP1A-like protein (Collodi *et al.*, 1994; Miranda *et al.*, 1993). Our studies, however, demonstrated that zebrafish CYP1A was indeed responsive to this flavone in the whole animal following i.p. administration or dietary exposure. The explanation for this difference is unknown.

βNF induction of CYP1A

Induction of the CYP1A-like protein and associated EROD activity following exposure to βNF is similar to that seen in other fish species, including the rainbow trout, Japanese medaka, and goldfish (Zhang *et al.*, 1990; Schell *et al.*, 1987; Gooch and Matsumura, 1983). The decreased levels of activity seen between the first and

second time-course experiments is not too surprising. While both experiments were conducted using fish from the same spawning, the experiments were not performed concurrently. The levels and inducibility of CYP1A in fish can vary greatly depending on the reproductive status of the fish (Förlin and Haux, 1990; Larsen *et al.*, 1992). Courtenay *et al.* (1994) investigated CYP1A mRNA expression in Atlantic tomcod and found varying degrees of CYP1A mRNA expression in prespawning, spawning, and spent male and female tomcod. The consequences of continuous spawning in such fish as the zebrafish are not known at this time.

Lack of Aroclor 1254 induction of CYP1A

The lack of induction of CYP1A in zebrafish liver following both dietary exposure and i.p. administration of Aroclor 1254 is puzzling. This commercial mixture of PCBs containing 54% chlorine is usually quite effective as a 3-methylcholanthrene-type inducer in most species. Only seldom is non-responsiveness to this mixture documented. One example is the study by Yawetz *et al.* (1992), in which exposure of a Mediterranean mollusc species to Aroclor 1254 increased overall P450 levels in the digestive gland, but actually decreased EROD activity. In another example, redfish receiving a single i.p. injection of Aroclor 1254 did not show any increase in microsomal mixed function oxidases (Stahl *et al.*, 1984). Studies in fish using high doses of βNF, benzo[*a*]pyrene, or planar PCBs have demonstrated that high doses of these inducers can actually lead to inhibition of activity or mRNA of the very protein being induced (Gooch *et al.*, 1989; Goddard *et al.*, 1987; Haasch *et al.*, 1993; Melancon and Lech, 1983). In the present study, not only was EROD activity non-

detectable, but protein levels were also not induced above control values, even at the lowest dose (75 mg/kg body weight) investigated. Another possible explanation might be that induction by this PCB mixture could be delayed, and simply was not detected by 24 hours following i.p. administration. The dietary exposure, however, was continued for 9 days with continuous feeding, and was also unsuccessful at inducing CYP1A. The Ah receptor has not yet been isolated in zebrafish. While the assumption is that the mechanism of induction of CYP1A in zebrafish is mediated by the Ah receptor, this has not been verified. If the Ah receptor is indeed present, it might be that the agonist binding site is more selective in this species.

TCDD modulation of AFB_1 metabolism

The *in vivo* metabolism of AFB_1 in zebrafish observed in this experiment was similar to the metabolic profile documented in a previous study (Troxel *et al.*, Chapter 2 of this thesis). The predominant metabolites excreted into water over 24 hours were again AFL, AFB_1, and AFL-g. The AFB_1 metabolites excreted by the TCDD pretreated group consisted of AFL, AFB_1, and AFL-g, but additionally included AFM_1/AFL-M_1 and the glucuronide of AFL-M_1. It is believed that CYP1A1 is the enzyme responsible for production of AFM_1, and is postulated that AFM_1 can become further hydroxylated to form AFL-M_1, which can than be glucuronidated, as is the case in rainbow trout (Loveland *et al.*, 1983; 1984; Goeger, *et al.*, 1988; You, publication in progress). Therefore it is no surprise to see increased levels of AFM_1/AFL-M_1 and the glucuronide of AFL-M_1.

The *in vitro* metabolism experiments showed increased formation of AFM_1 in the TCDD-treated liver homogenates, confirming the results of the *in vivo* study. Although there was a significant difference in the *in vivo* production of AFL between the control and TCDD-treated groups (17.1% versus 7.5%), there was no difference in the formation of AFL between the two groups in the *in vitro* metabolism assay. TCDD-treatment also did not affect the ability of the liver homogenates to bioactivate AFB_1 and AFM_1 *in vitro*. Unfortunately, enzyme activities could not be calculated in terms of P450 content, but rather only on a per mg protein basis, since zebrafish livers were too small to permit total P450 quantification. Analysis of CYP1A in the supernatant from zebrafish liver homogenates used in the metabolism and DNA adduction experiments verified that this protein and its associated EROD activity were induced by the TCDD treatment.

TCDD modulation of AFB_1-DNA adduction

The results from the investigation of TCDD modulation of hepatic AFB_1-DNA adduction showed an approximate 4-fold increase in the amount of hepatic DNA-adduction in the TCDD-treated group. A study by Walsh *et al.* (1992) also demonstrated that TCDD pretreatment increased DNA adduction and toxicity following exposure to AFB_1 in a human epidermal cell line. The increased binding in the TCDD-treated group of zebrafish cannot be explained by an increased ability to bioactivate AFB_1 to the reactive *exo*-8-9-epoxide, since the *in vitro* AFB_1 metabolism assay failed to show any difference in this activity between groups. This assay uses mouse cytosolic glutathione transferase which almost exclusively traps the *exo*-, but not the *endo*-AFB_1-

epoxide. The increased binding could be related to the increased production of AFM_1 and/or AFL-M_1, and further activation to a DNA binding species. The *in vitro* AFM_1 metabolism assay did demonstrate that zebrafish are quite capable of bioactivating AFM_1 to a reactive intermediate capable of binding glutathione and forming an adduct. *In vivo* genotoxicity tests of AFM_1 in *Drosophilia melanogaster* showed a only a 3-fold lower potency for damaging DNA compared to AFB_1 in one test, and equal genotoxicity in another test (Shibahara *et al.*, 1995). Work with rainbow trout hepatocytes also demonstrated that AFM_1 and AFL-M_1 had DNA binding values approximately 80% that of AFB_1 (Loveland, *et al.*, 1988).

In conclusion, zebrafish possess a CYP1A1-like protein that is readily inducible following i.p. administration or dietary exposure to βNF, but is refractory to Aroclor 1254 at the doses investigated in this study. Dietary exposure to TCDD, the most potent Ah receptor agonist, was also found to effective. Following exposure to TCDD, the metabolism of AFB_1 was altered in a pattern consistent with increases in CYP1A1, which is usually considered a detoxifying pathway. However, this pathway appears to correlate with an increase in the toxicity of AFB_1 in this species, as measured by an approximate 4-fold increase in hepatic AFB_1-DNA adduction.

Acknowledgements

The authors thank Tom Miller for care and maintenance of the zebrafish colony, Dr. Ulrich Harttig for technical assistance in the *in vitro* AFB_1 metabolism assay, Dr. Cristobal Miranda for assistance in the EROD assay, Dr. Ashok Reddy for aid in injecting the zebrafish, and Dr. Cliff Pereira for assistance in the statistical analysis of

the data. This work was supported by Army grant DAMD17-91-Z-1043 and NIEHS grants 2 T32 ES 07060, ES 00210, and ES 03850. This is technical paper number 11071 from the Oregon State Agricultural Experiment Station.

References

Bailey, G.S., Williams, D.E., and Hendricks, J.D. (1996). Fish models for environmental carcinogenesis: the rainbow trout model. *Environ. Health Perspec.* **104**, 5-21.

Buchmann, A., Wannenmacher, R., Kulzer, E., Buhler, D.R., and Bock, K.W. (1993). Immunohistochemical localization of the cytochrome P450 isozymes LMC2 and LM4B (P4501A1) in 2,3,7,8-tetrachlorodibenzo-p-dioxin-treated zebrafish (*Brachydanio rerio*). *Toxicol. Appl. Pharmacol.* **123**, 160-169.

Cesarone, C., Bolognesi, C., and Santi, L. (1979). Improved microfluorometric DNA determination in biological material using 33258 Hoechst. *Anal. Biochem.* **92**, 188-197.

Collodi, P., Miranda, C.L., Zhao, X., Buhler, D.R., and Barnes, D.W. (1994). Induction of zebrafish (*Brachydanio rerio*) P450 *in vivo* and in cell culture. *Xenobiotica* **24**, 487-493.

Courtenay, S., Williams, P.J., Grunwald, C., Konkle, B., Ong, T.-L., and Wirgin, I.I. (1994). Assessment of within-group variation in CYP1A mRNA inducibility in environmentally exposed and chemically treated atlantic tomcod. *Environ. Health Perspec.* **102**, 85-90.

Daniels, J.M., and Massey, T.E. (1992). Modulation of aflatoxin B_1 biotransformation in rabbit pulmonary and hepatic microsomes. *Toxicology* **74**, 19-32.

DeKoven, D.L., Nunez, J.M., Lester, S.M., Conklin, D.E., Marty, G.D., Parker, L.M., and Hinton, D.E. (1992). A purified diet for medaka (*Oryzias latipes*): refining a fish model for toxicological research. *Lab. Animal Sci.* **42**, 180-189.

Eaton D.L., and Gallagher, E.P. (1994). Mechanisms of aflatoxin carcinogenesis. *Annu. Rev. Pharmacol. Toxicol.* **34**, 135-172.

Fong, A.T., Dashwood, R.H., Cheng, R., Mathews, C., Ford, B., Hendricks, J.D., and Bailey, G.S. (1993). Carcinogenicity, metabolism and Ki-ras proto-oncogene activation by 7,12-dimethylbenz[*a*]anthracene in rainbow trout embryos. *Carcinogenesis* **14**, 629-635.

Förlin, L., and Haux, C. (1990). Sex differences in hepatic cytochrome P-450 monooxygenase activities in rainbow trout during an annual reproductive cycle. *J. Endocrin.* **124**, 207-213.

Fournie, J.W., Hawkins, W.E., Overstreet, R.M., and Walker, W.W. (1987). Exocrine pancreatic neoplasms induced by methylazoxymethanol acetate in the guppy *Poecilia reticulata*. *J. Natl. Cancer Inst.* 78, 715-725.

Gallagher, E.P., and Eaton, D.L. (1995). *In vitro* biotransformation of aflatoxin B_1 (AFB_1) in channel catfish liver. *Toxicol. Appl. Pharmacol.* 132, 82-90.

Goddard, K.A., Schultz, R.J., and Stegeman, J.J. (1987). Uptake, toxicity, and distribution of benzo[*a*]pyrene and monooxygenase induction in the topminnows *Poeciliopsis monacha* and *Poeciliopsis lucida*. *Drug Metab. Dispos.* 15, 449-433.

Goeger, D.E., Shelton, D.W., Hendricks, J.D., and Bailey, G.S. (1986). Mechanisms of anti-carcinogenesis by indole-3-carbinol: effect on the distribution and metabolism of aflatoxin B_1 in rainbow trout. *Carcinogenesis* 7, 2025-2031.

Goeger, D.E., Shelton, D.W., Hendricks, J.D., Pereira, C., and Bailey, G.S. (1988). Comparative effect of dietary butylated hydroxyanisole and β-naphthoflavone on aflatoxin B_1 metabolism, DNA adduct formation, and carcinogenesis in rainbow trout. *Carcinogenesis* 9, 1793-1800.

Goksoyr, A. (1995). Use of cytochrome P450 1A (CYP1A) in fish as a biomarker of aquatic pollution. *Arch. Toxicol.*, Sup. 17, 80-95.

Goksoyr, A., Andersson, T., Buhler, D.R., Stegeman, J.J., Williams, D.E., and Förlin, L. (1991). Immunologic chemical cross-reactivity of β-naphthoflavone-inducible cytochrome P-450 (P450-1A) liver microsomes from different fish species. *Fish Physiol. Biochem.* 9, 1-13.

Gooch, J.W., Elskus, A.A., Kloepper-Sams, P.J., Hahn, M.E., and Stegeman, J.J. (1989) Effects of *ortho* and non-*ortho* substituted polychlorinated biphenyl congeners on the hepatic monooxygenase system in scup. *Toxicol. Appl. Pharmacol.* 98, 422-433.

Gooch, J.W., and Matsumura, F. (1983). Characteristics of the hepatic monooxygenase system of the goldfish (*Carassius auratus*) and its induction with β-naphthoflavone. *Toxicol. Appl. Pharmacol.* 68, 380-391.

Haasch, M.L., Quardokus, E.M., Sutherland, L.A., Goodrich, M.S., and Lech, J.J. (1993). Hepatic CYP1A1 induction in rainbow trout by continuous flowthrough exposure to β-naphthoflavone. *Fundam. Appl. Toxicol.* 20, 72-82.

Hankinson, O. (1995). The aryl hydrocarbon receptor complex. *Annu. Rev. Pharmacol. Toxicol.* 35, 307-340.

Hawkins, W.E., Fournie, J.W., Overstreet, R.M., and Walker, W.W. (1986). Intraocular neoplasms induced by methylazoxymethanol acetate in Japanese medaka (*Oryzias latipes*). *J. Natl. Cancer Inst.* 76, 453-465.

Hawkins, W.E., Overstreet, R.M., and Walker, W.W. (1988). Carcinogenicity tests with small fish species. *Aquat. Toxicol.* 11, 113-128.

Husoy, A-M, Myers, M.S., Willis, M.L., Collier, T.K., Celander, M., and Goksoyr, A. (1994). Immunohistochemical localization of CYP1A and CYP3A-like isozymes in hepatic and extrahepatic tissues of atlantic cod (*Gadus morhua L.*), a marine fish. *Toxicol. Appl. Pharmacol.* 1994, 294-308.

Khudoley, V.V. (1984). Use of aquarium fish, *Danio rerio* and *Poecilia reticulata*, as test species for evaluation of nitrosamine carcinogenicity. *Natl. Cancer Inst. Monogr.* No. 65, 65-70.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Larsen, H.E., Celander, M., and Goksoyr, A. (1992). The cytochrome P450 system of Atlantic salmon (*Salmo salar*): II. Variations in hepatic catalytic activities and isozyme patterns during an annual reproductive cycle. *Fish Physiol. Biochem.* 10, 291-301.

Liu, L., Nakatsu, K., and Massey, T.E. (1993). *In vitro* cytochrome P450 monooxygenase and prostaglandin H-synthetase mediated aflatoxin B_1 biotransformation in guinea pig tissues: effects of β-naphthoflavone treatment. *Arch. Toxicol.* 67, 379-385.

Loveland, P.M., Coulombe, R.A., Libbey, L.M., Pawlowski, N.E., Sinnhuber, R.O., Nixon, J.E., and Bailey, G.S. (1983). Identification and mutagenicity of aflatoxicol-M_1 produced by metabolism of aflatoxin B_1 and aflatoxicol by liver fractions from rainbow trout (*Salmo gairdneri*) fed B-naphthoflavone. *Food Chem. Toxicol.* 21, 557-562.

Loveland, P.M., Nixon, J.E., and Bailey, G.S. (1984). Glucuronides in bile of rainbow trout (*Salmo gairdneri*) injected with [^{3}H]aflatoxin B_1 and the effects of dietary β-naphthoflavone. *Comp. Biochem. Physiol.* 78C, 13-19.

Loveland, P.M., Wilcox, J.S., Hendricks, J.D., and Bailey, G.S. (1988). Comparative metabolism and DNA binding of aflatoxin B_1, aflatoxin M_1, aflatoxicol and aflatoxicol-M_1 in hepatocytes from rainbow trout (*Salmo gairdneri*). *Carcinogenesis* 9, 441-446.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265-275.

Melancon, M.J., and Lech, J.J. (1983). Dose-effect relationship for induction of hepatic monooxygenase activity in rainbow trout and carp by Aroclor 1254. *Aquat. Toxicol.* **4**, 51-61.

Miranda, C.L., Collodi, P., Zhao, X., Barnes, D.W., and Buhler, D.R. (1993). Regulation of cytochrome P450 expression in a novel liver cell line from zebrafish (*Brachydanio rerio*). *Arch. Biochem. Biophys.* **305**, 320-327.

Monroe, D.H., and Eaton, D.L. (1987). Comparative effects of butylated hydroxyanisole on hepatic *in vivo* DNA binding and *in vitro* biotransformation of aflatoxin B_1 in the rat and mouse. *Toxicol. Appl. Pharmacol.* **90**, 401-409.

Nakazawa, T., Hamaguchi, S., and Kynono-Hamaguchi, Y. (1985). Histochemistry of liver tumors induced by diethylnitrosamine and differential sex susceptibility to carcinogenesis in *Oryzias latipes*. *J. Natl. Cancer Inst.* **75**, 567-573.

Nixon, J.E., Hendricks, J.D., Pawlowski, N.E., Pereira, C.B., Sinnhuber, R.O., and Bailey, G.S. (1984). Inhibition of aflatoxin B_1 carcinogenesis in rainbow trout by flavone and indole compounds. *Carcinogenesis* **5**, 615-619.

Poland, A., Greenlee, W.F., and Kende, A.S. (1979). Studies on the mechanism of action of the chlorinated dibenzo-*p*-dioxins and related compounds. *Ann. N.Y. Acad. Sci.* **320**, 214-230.

Powers, D.A. (1989). Fish as model systems. *Science* **246**, 352-358.

Prough, R.A., Burke, M.D., and Mayer, R.T. (1978). Direct fluorometric methods for measuring mixed-function oxidase activity. *Methods Enzymol.* **52C**, 372-377.

Raney, K.D., Coles, B., Guengerich, F.P., and Harris, T.M. (1992a). The *endo*-8,9-epoxide of aflatoxin B_1: a new metabolite. *Chem. Res. Toxicol.* **5**, 333-335.

Raney, K.D., Meyer, D.J., Ketterer, B., Harris, T.M., and Guengerich, F.P. (1992b). Glutathione conjugation of aflatoxin B_1 *exo*- and *endo*-epoxides by rat and human glutathione-*S*-transferases. *Chem. Res. Toxicol.* **5**, 470-478.

SAS Institute Inc., *SAS/STAT User's Guide*, Version 6, Fourth Edition, Cary, NC: SAS Institute Inc, **2**, 1-846.

Sato, S., Matsushima, T., Tanaka, N., Sugimura, T., and Takashima, F. (1973). Hepatic tumors in the guppy (*Lebistes reticulatus*) by aflatoxin B_1, dimethylnitrosamine, and 2-acetylaminofluorene. *J. Natl. Cancer Inst.* **50**, 765-778.

Schell, J.D., Cooper, K.O., and Cooper, K.R. (1987). Hepatic microsomal mixed-function oxidase activity in the Japanese medaka (*Oryzias latipes*). *Environ. Toxicol. Chem.* **6**, 717-721.

Shelton, D.W., Goeger, D.E., Hendricks, J.D., and Bailey, G.S. (1986). Mechanisms of anti-carcinogenesis: the distribution and metabolism of aflatoxin B_1 in rainbow trout fed aroclor 1254. *Carcinogenesis* **7**, 1065-1071.

Shibahara, T., Ogawa, H.I., Ryo, H., and Fujikawa, K. (1995). DNA-damaging potency and genotoxicity of aflatoxin M_1 in somatic cells *in vivo* of *Drosophila melanogaster*. *Mutagenesis* **10**, 161-164.

Sinnhuber, R.O., Hendricks, J.D., Wales, J.H., and Putnam, G.B. (1977). Neoplasms in rainbow trout, a sensitive animal model for environmental carcinogenesis. *Ann. NY Acad. Sci.* **298**, 389-408.

Stahl, C.H., Sultatos, L.G., Hacker, C.S., and Murphy, S.D. (1984). Mixed-function oxidase studies in the redfish, *Sciaenops ocellata*, from Galveston Bay, Texas. *Comp. Biochem. Physiol.* **79C**, 177-182.

Stanton, M.F. (1965). Diethylnitrosamine-induced hepatic degeneration and neoplasia in the aquarium fish, *Brachydanio rerio*. *J. Natl. Cancer Inst.* **34**, 117-130.

Stegeman, J.J. (1989). Cytochrome P450 forms in fish: catalytic, immunological, and sequence similarities. *Xenobiotica* **19**, 1093-1110.

Stegeman, J.J., and Hahn, M.E. (1994). Biochemistry and molecular biology of monooxygenases: current perspectives on forms, functions, and regulation of cytochrome P450 in aquatic species. In *Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives* (D.C. Malins and G.K. Ostrander, Ed.), pp 87-206. Lewis Publishers, Boca Raton, Florida.

Stegeman, J.J., Miller, M.R., and Hinton, D.E. (1989). Cytochrome P450IA1 induction and localization in endothelium of vertebrate (teleost) heart. *Mol. Pharmacol.* **36**, 723-729.

Strauss, W.M. (1991). Preparation of genomic DNA from mammalian tissue. In *Current Protocols in Molecular Biology* (Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., Eds.), pp 2.2.1-2.2.2. Greene Publishing Associates and Wiley-Interscience, New York.

Takahashi, N., Harttig, U., Williams, D.E., and Bailey, G.S. (1996). The model Ah-receptor agonist β-naphthoflavone inhibits aflatoxin B_1-DNA binding *in vivo* in rainbow trout at dietary levels that do not induce CYP1A enzymes. *Carcinogenesis* **17**, 79-87.

Takahashi, N., Miranda, C.L., Henderson, M.C., Buhler, D.R., Williams, D.E., and Bailey, G.S. (1995). Inhibition of *in vitro* aflatoxin B_1-DNA binding in rainbow trout by CYP1A inhibitors: α-naphthoflavone, β-naphthoflavone, and trout CYP1A1 peptide antibody. *Comp. Biochem. Physiol.* 110C, 273-280.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* 76, 4350-4354.

Walsh, A.A., Hsieh, D.P.H., and Rice, R.H. (1992). Aflatoxin toxicity in cultured human epidermal cells: stimulation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Carcinogenesis* 13, 2029-2033.

Yawetz, A., Manelis, R., and Fishelson, L. (1992). The effects of Aroclor 1254 and petrochemical pollutants on cytochrome P450 from the digestive gland microsomes of four species of mediterranean molluscs. *Comp. Biochem. Physiol.* 103C, 607-614.

You, L., Harttig, U., and Bailey, G. Cloning, sequencing and functional expression of three rainbow trout CYP1A cDNAs in yeast. [publication in progress].

Zhang, Y.S., Andersson, T., and Förlin, L. (1990). Induction of hepatic xenobiotic biotransformation enzymes in rainbow trout by β-naphthoflavone. Time course studies. *Comp. Biochem. Physiol.* 95B, 247-253.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Zebrafish are efficient at metabolizing AFB_1 to both phase I and phase II metabolites, including bioactivation of AFB_1 to a reactive intermediate capable of binding to DNA, as shown by an *in vitro* AFB_1 metabolism assay and confirmed in the *in vivo* hepatic DNA-binding experiments. These data suggest that zebrafish should be fairly sensitive to the carcinogenic effects of AFB_1. However, Tsai[1] has demonstrated that zebrafish are actually resistant to AFB_1-induced carcinogenesis when the carcinogen is administered in the diet. Hepatic DNA-binding studies conducted following a dietary exposure to [^{3}H]AFB_1 also showed only low levels of adducts (unpublished results). These results suggest a difference in absorbance and distribution of dietary AFB_1 compared to i.p. administration. It seems that if the carcinogen can reach the target organ, zebrafish are quite capable of bioactivating AFB_1. For future studies, this hypothesis could be evaluated by comparing the biodistribution of [^{3}H]AFB_1 following gavage or injection, in order to measure the proportion of doses effectively delivered to the target organ by these two exposure routes. A logical companion study would be to compare the tumor response by dietary and i.p. administration of AFB_1 in zebrafish.

Zebrafish CYP1A responded to the common CYP1A inducers βNF and TCDD, as measured by catalytic activity and antibody detection of protein. CYP1A induction

[1]Tsai, H.-W. (1996). Evaluation of zebrafish (*Brachydanio rerio*) as a model for carcinogenesis. [Ph.D. dissertation]. Oregon State University, Corvallis, OR.

occurred by 48 hours following i.p. administration of βNF. Surprisingly, there was no measurable response following dietary or i.p. administration of Aroclor 1254, a commercial mixture of PCBs that is an effective inducer in trout and other species. It would be interesting to further assess CYP1A response by measuring mRNA levels to see if transcription of this gene was activated or not. One could also explore the affects of other typical CYP1A inducers, such as 3-methylcholanthrene and some of the planar non-ortho substituted chlorinated biphenyls. By conducting investigations using more specific inducers, one could better evaluate if the agonist binding site might be more selective in this species.

The modulation of AFB_1 metabolism by a CYP1A inducer, in this case TCDD, was consistent with experiments conducted by other scientists. Due to increased levels of CYP1A, the metabolic profile shifted to formation of AFM_1 and AFL-M_1-glucuronide, the metabolite and its glucuronide conjugate typically associated with CYP1A. The 4-fold increase in hepatic DNA binding in the TCDD-pretreated group could not be explained by an enhanced capacity for bioactivation of AFB_1. It is postulated that AFM_1 is also bioactivated to a reactive intermediate that can bind to DNA. One approach to investigating this hypothesis is to essentially repeat the previous *in vivo* TCDD-modulation experiment, but this time going a step further. Instead of just measuring the total amount of radioactivity bound to DNA, one could look at the actual adduct profiles produced by the control or TCDD-pretreated groups following AFB_1 exposure. The adduct profile generated from the HPLC analysis should indicate whether AFM_1, or perhaps even AFL-M_1, forms an adduct with DNA, or if some other difference exists between the treatment groups in their DNA-adduction patterns.

The future of the zebrafish as a model for chemical carcinogenesis remains uncertain. Zebrafish do not appear to be sensitive to carcinogenesis following dietary exposures to AFB_1, N-nitrosodiethylamine and N-nitrosodimethylamine, but they did respond to the direct acting carcinogen, methylazoxymethanol acetate[2]. As already mentioned, the lack of response in zebrafish following dietary exposures could be due to lack of absorption and distribution of the carcinogens. It should be noted that zebrafish do not have a stomach, and the effect of the absence of this organ on the process of absorption of compounds is not known. Other factors may play a significant role as well. For example, the dietary exposures could not be initiated until the fish were large enough to eat the diet used in carcinogen exposures. This timing typically coincided with sexual development[2], which in rainbow trout has correlated with decreasing sensitivity to carcinogens. Also, zebrafish are selective eaters, and it is difficult to assure that the proper dose has been given. In contrast to the lack of response of zebrafish following most dietary exposures of carcinogens, static water bath exposures of zebrafish fry and embryos resulted in tumorigenesis. Unfortunately, the metabolism of carcinogens other than AFB_1 has yet to be studied in zebrafish. Additional studies investigating the metabolic capabilities of this species could further elucidate the mechanistic basis for these contrasting results.

Another drawback of this fish model is that it is difficult to conduct biochemical studies in zebrafish. The liver is diffuse and difficult to remove, and large numbers of livers need to be pooled together in order to have enough tissue with which to work.

[2]Tsai, H.-W. (1996). Evaluation of zebrafish (*Brachydanio rerio*) as a model for carcinogenesis. [Ph.D. dissertation]. Oregon State University, Corvallis, OR.

In addition, due to the virtual impossibility of removing the liver intact, it appears that proteases are released during sampling, and if one is not careful, one can quickly lose biological activity of proteins. For a more detailed biochemical analysis, one might have to resort to using *in vitro* test systems, such as zebrafish cells in culture or cloned gene products. One will then have the additional question of the validity of the results as they relate to the *in vivo* system.

Because of the many difficulties and unexpected results encountered in the overall project to develop the zebrafish as a model for chemical carcinogenesis, many questions remain regarding zebrafish and their response to carcinogens.

BIBLIOGRAPHY

Abedi, Z.H., and McKinley, W.P. (1968). Zebra fish eggs and larvae as aflatoxin bioassay test organisms. *J. Assoc. Off. Anal. Chem.* 51, 902-904.

Agustsson, I., and Strom, A.R. (1981). Biosynthesis and turnover of trimethylamine oxide in the teleost cod, *Gadus morhua*. *J. Bio. Chem.* 256, 8045-8049.

Ames, B.N., and Gold, L.W. (1991). Too many rodent carcinogens: mitogenesis increases mutagenesis. *Science* 249, 970-971.

Ames, B.N., Shigenaga, M.K., and Gold, L.S. (1993). DNA lesions, inducible DNA repair, and cell division: three key factors in mutagenesis and carcinogenesis. *Environ. Health Perspec.* 101(Sup), 35-44.

Appleton, B.S., Goetchius, M.P., and Campell, T.C. (1982). Linear dose-response curve for the hepatic macromolecular binding of aflatoxin B_1 in rats at very low exposures. *Cancer Res.*, 42, 3659-3662.

Ashley, L.M., and Halver, J.E. (1968). Dimethylnitrosamine-induced hepatic cell carcinoma in rainbow trout. *J. Natl. Cancer Inst.* 41, 531-552.

Ayres, J.L., Lee,D.J., Wales, J.H., and Sinnhuber, R.O. (1971). Aflatoxin structure and hepatocarcinogenicity in rainbow trout (*Salmo gairdneri*). *J. Natl. Cancer Inst.* 46, 561-564.

Bailey, G.S. (1994). Role of DNA adducts in the cancer process. In *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance* (D.L. Eaton and J.D. Groopman, Ed.), pp 137-148. Academic Press, New York.

Bailey, G.S., Loveland, P.M., Pereira, C., Pierce, D., Hendricks, J.D., and Groopman, J.D. (1994). Quantitative carcinogenesis and dosimetry in rainbow trout for aflatoxin B_1 and aflatoxicol, two aflatoxins that form the same DNA adduct. *Mut. Res.* 313, 25-38.

Bailey, G.S., Williams, D.E., and Hendricks, J.D. (1996). Fish models for environmental carcinogenesis: the rainbow trout. *Environ. Health Perspec.* 104, 5-21.

Bailey, G.S., Williams, D.E., Wilcox, J.S., Loveland, P.M., Coulombe, R.A., and Hendricks, J.D. (1988). Aflatoxin B_1 carcinogenesis and its relation to DNA adduct formation and adduct persistence in sensitive and resistant salmonid fish. *Carcinogenesis* 9, 1919-1926.

Bauer, L., Tulsan, A.H., and Müller, E. (1972). Ultrastructural changes produced by the carcinogen, aflatoxin B1, in different tissues. *Virchows Arch. Zellpath.* 10, 275-285.

Bechtel, D.H. (1989). Molecular dosimetry of hepatic aflatoxin B_1-DNA adducts: linear correlation with hepatic cancer risk. *Reg. Toxicol. Pharmacol.* 10, 74-81.

Bos, J.L. (1989). *Ras* oncogenes in human cancer: a review. *Cancer Res.* 49, 4682-4689.

Braunbeck, T., Görge, G., Storch, V., and Nagel, R. (1990a). Hepatic steatosis in Zebra Fish (*Brachydanio rerio*) induced by long-term exposure to (gamma)-hexachlorocyclohexane. *Ecotoxicol. Environ. Saf.* 19, 355-374.

Braunbeck, T., Storch, V., and Bresch, H. (1990b). Species-specific reaction of liver ultrastructure in zebrafish (*Brachydanio rerio*) and trout (*Salmo gairdneri*) after prolonged exposure to 4-chloroaniline. *Arch. Environ. Contam. Toxicol.* 19, 405-418.

Breinholt, V. (1994). Chlorophyllin anticarcinogenesis in the rainbow trout model [Ph.D. dissertation]. Oregon State University, Corvallis, OR.

Bresch, H., Beck, H., Ehlermann, D., Schlaszus, H., and Urbanek, M. (1990). A long-term toxicity test comprising reproduction and growth of zebrafish with 4-chloroaniline. *Arch. Environ. Contam. Toxicol.* 19, 419-427.

Buchmann, A., Wannenmacher, R., Kulzer, E., Buhler, D.R., and Bock, K.W. (1993). Immunohistochemical localization of the cytochrome P450 isozymes LMC2 and LM4B (P4501A1) in 2,3,7,8-tetrachlorodibenzo-p-dioxin-treated zebrafish (*Brachydanio rerio*). *Toxicol. Appl. Pharmacol.* 123, 160-169.

Cashman, J.R., Olsen, L.D., Nishioka, R.S., Gray, E.S., and Bern, H.A. (1990). S-oxygenation of thiobencarb (Bolero) in hepatic preparations from striped bass (*Morone saxatilis*) and mammalian systems. *Chem. Res. Toxicol.* 3, 433-440.

Cesarone, C., Bolognesi, C., and Santi, L. (1979). Improved microfluorometric DNA determination in biological material using 33258 Hoechst. *Anal. Biochem.* 92, 188-197.

Chang, Y-J., Mathews, C., Mangold, K., Marien, K., Hendricks, J., and Bailey, G.S. (1991). Analysis of *ras* gene mutations in rainbow trout liver tumors initiated by aflatoxin B_1. *Mol. Carcinogen.* 4, 112-119.

Choy, W.N. (1993). A review of the dose-response induction of DNA adducts by aflatoxin B_1 and its implications to quantitative cancer-risk assessment. *Mut. Res.*, 296, 181-198.

Clark, D.J., George, S.G., and Burchell, B. (1991). Glucuronidation in fish. *Aquat. Toxicol.* 20, 35-56.

Collodi, P., Miranda, C.L., Zhao, X., Buhler, D.R., and Barnes, D.W. (1994). Induction of zebrafish (*Brachydanio rerio*) P450 *in vivo* and in cell culture. *Xenobiotica* 24, 487-493.

Coulombe, R.A., Shelton, D.W., Sinnhuber, R.O., and Nixon, J.E. (1982). Comparative mutagenicity of aflatoxins using a *Salmonella*/trout hepatic enzyme activation system. *Carcinogenesis* 3, 1261-1264.

Courtenay, S., Williams, P.J., Grunwald, C., Konkle, B., Ong, T.-L., and Wirgin, I.I. (1994). Assessment of within-group variation in CYP1A mRNA inducibility in environmentally exposed and chemically treated atlantic tomcod. *Environ. Health Perspec.* 102, 85-90.

Daniels, J.M., and Massey, T.E. (1992). Modulation of aflatoxin B_1 biotransformation in rabbit pulmonary and hepatic microsomes. *Toxicology* 74, 19-32.

Dashwood, R.H., Arbogast, D.N., Fong, A.T., Hendricks, J.D., and Bailey, G.S. (1988). Mechanisms of anti-carcinogenesis by indole-3-carbinol: detailed *in vivo* DNA binding dose-response studies after dietary administration with aflatoxin B_1. *Carcinogenesis* 9, 427-432.

Dashwood, R.H., Mariën, K., Loveland, P.M., Williams, D.E., Hendricks, J.D., and Bailey, G.S. (1992). Formation of aflatoxin-DNA adducts in trout and their use as molecular dosimeters for tumor prediction. In *Handbook of Applied Mycology* (Bhatnager, D., Lillehoj, E.B., and Arora, D.K., Eds.), pp. 183-211. Marcel Dekker, New York.

Dauterman, W.C. (1994). Metabolism of toxicants: phase II reactions. In *Introduction to Biochemical Toxicology* (E. Hodgson and P.E. Levi, Ed), pp 113-132. Appleton and Lange, Norwalk, CT.

Dave, G., and Xiu, R. (1991). Toxicity of mercury, copper, nickel, lead, and cobalt to embryos and larvae of zebrafish, *Brachydanio rerio*. *Arch. Environ. Contam. Toxicol.* 21, 126-134.

Degen, G.H., and Neumann H-G. (1981). Differences in AFB_1-susceptibility of rat and mouse are correlated with the capability *in vitro* to inactivate aflatoxin B_1-epoxide. *Carcinogenesis* 2, 299-306.

DeKoven, D.L., Nunez, J.M., Lester, S.M., Conklin, D.E., Marty, G.D., Parker, L.M., and Hinton, D.E. (1992). A purified diet for medaka (*Oryzias latipes*): refining a fish model for toxicological research. *Lab. Animal Sci.* 42, 180-189.

Doll, R., and Peto, R. (1981). The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J. Natl. Cancer Inst.* 66, 1191-1308.

Eaton D.L., and Gallagher, E.P. (1994). Mechanisms of aflatoxin carcinogenesis. *Annu. Rev. Pharmacol. Toxicol.* 34, 135-172.

Eaton, D.L., and Ramsdell, H.S. (1992). Species- and diet- related differences in aflatoxin biotransformation. In *Handbook of Applied Mycology* (Bhatnager, D., Lillehoj, E.B., and Arora, D.K., Eds.), pp. 157-182. Marcel Dekker, New York.

Eaton, D.L., Ramsdell, H.S., and Neal, G.E. (1994). Biotransformation of aflatoxins. In *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance* (D.L. Eaton and J.D. Groopman, Ed.), pp 45-72. Academic Press, New York.

Essigmann, J.M., Croy, R.G., Bennett, R.A., and Wogan, G.N. (1982). Metabolic activation of aflatoxin B_1: patterns of DNA adduct formation, removal, and excretion in relation to carcinogenesis. *Drug Metab. Rev.* 13, 581-602.

Fong, A.T., Dashwood, R.H., Cheng, R., Mathews, C., Ford, B., Hendricks, J.D., and Bailey, G.S. (1993). Carcinogenicity, metabolism and Ki-ras proto-oncogene activation by 7,12-dimethylbenz[*a*]anthracene in rainbow trout embryos. *Carcinogenesis* 14, 629-635.

Förlin, L., and Haux, C. (1990). Sex differences in hepatic cytochrome P-450 monooxygenase activities in rainbow trout during an annual reproductive cycle. *J. Endocrin.* 124, 207-213.

Fournie, J.W., Hawkins, W.E., Overstreet, R.M., and Walker, W.W. (1987). Exocrine pancreatic neoplasms induced by methylazoxymethanol acetate in the guppy *Poecilia reticulata*. *J. Natl. Cancer Inst.* 78, 715-725.

Gallagher, E.P., and Eaton, D.L. (1995). *In Vitro* biotransformation of aflatoxin B_1 in channel catfish liver. *Toxicol. Appl. Pharmacol.* 132, 82-90.

Gallagher, E.P., Stapleton, P.L., Wienkers, L.C., Kunze, K., and Eaton, D.L. (1994). Role of human microsomal and human complementary DNA-expressed cytochromes P4501A2 and P4503A4 in the activation of aflatoxin B_1. *Cancer Res.* 54, 101-108.

George, S.G. (1994). Enzymology and molecular biology of phase II xenobiotic-conjugating enzymes in fish. In *Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives* (D.C. Malins and G.K. Ostrander, Ed), pp 37-85. Lewis Publishers, Boca Raton, FL.

Gibson, G., and Skett, P. (1986). *Introduction to Drug Metabolism*, Chapman and Hall, London.

Goddard, K.A., Schultz, R.J., and Stegeman, J.J. (1987). Uptake, toxicity, and distribution of benzo[*a*]pyrene and monooxygenase induction in the topminnows *Poeciliopsis monacha* and *Poeciliopsis lucida*. *Drug Metab. Dispos.* 15, 449.

Goeger, D.E., Shelton, D.W., Hendricks, J.D., and Bailey, G.S. (1986). Mechanisms of anti-carcinogenesis by indole-3-carbinol: effect on the distribution and metabolism of aflatoxin B_1 in rainbow trout. *Carcinogenesis* 7, 2025-2031.

Goeger, D.E., Shelton, D.W., Hendricks, J.D., Pereira, C., and Bailey, G.S. (1988). Comparative effect of dietary butylated hydroxyanisole and β-naphthoflavone on aflatoxin B_1 metabolism, DNA adduct formation, and carcinogenesis in rainbow trout. *Carcinogenesis* 9, 1793-1800.

Goksoyr, A. (1995). Use of cytochrome P450 1A (CYP1A) in fish as a biomarker of aquatic pollution. *Arch. Toxicol.*, Sup. 17, 80-95.

Goksoyr, A., Andersson, T., Buhler, D.R., Stegeman, J.J., Williams, D.E., and Förlin, L. (1991). Immunologic chemical cross-reactivity of β-naphthoflavone-inducible cytochrome P-450 (P450-1A) liver microsomes from different fish species. *Fish Physiol. Biochem.*, 9, 1-13.

Goldstein, L., and Dewitt-Harley, S. (1973). Trimethylamine oxidase of nurse shark liver and its relation to mammalian mixed function amine oxidase. *Comp. Biochem. Physiol.* 45B, 895-903.

Gooch, J.W., Elskus, A.A., Kloepper-Sams, P.J., Hahn, M.E., and Stegeman, J.J. (1989) Effects of *ortho* and non-*ortho* substituted polychlorinated biphenyl congeners on the hepatic monooxygenase system in scup. *Toxicol. Appl. Pharmacol.* 98, 422.

Gooch, J.W., and Matsumura, F. (1983). Characteristics of the hepatic monooxygenase system of the goldfish (*Carassius auratus*) and its induction with β-naphthoflavone. *Toxicol. Appl. Pharmacol.* 68, 380-391.

Greenblatt, M.S., Bennett, W.P., and Harris, C.C. (1994). Mutations in the *p53* tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res.* 54, 4855-4878.

Grieco, M.P., Hendricks, J.D., Scanlan, R.A., Sinnhuber, R.O., and Pierce, D.A. (1978). Carcinogenicity and acute toxicity of dimethylnitrosamine in rainbow trout (*Salmo gairdneri*). *J. Natl. Cancer Inst.* 60, 1127-1131.

Guengerich, F.P. (1992). Metabolic activation of carcinogens. *Pharmac. Ther.* 54, 17-61.

Haasch, M.L., Quardokus, E.M., Sutherland, L.A., Goodrich, M.S., and Lech, J.J. (1993). Hepatic CYP1A1 induction in rainbow trout by continuous flowthrough exposure to β-naphthoflavone. *Fundam. Appl. Toxicol.* 20, 72-82.

Hankinson, O. (1995). The aryl hydrocarbon receptor complex. *Annu. Rev. Pharmacol. Toxicol.*, 35, 307-340.

Harris, C.C. (1991). Chemical and physical carcinogenesis: advances and perspectives for the 1990s. *Cancer Res.* 51, 5023s-5044s.

Hawkins, W.E., Fournie, J.W., Overstreet, R.M., and Walker, W.W. (1986). Intraocular neoplasms induced by methylazoxymethanol acetate in Japanese medaka (*Oryzias latipes*). *J. Natl. Cancer Inst.* 76, 453-465.

Hawkins, W.E., Overstreet, R.M., and Walker, W.W. (1988). Carcinogenicity tests with small fish species. *Aquat. Toxicol.* 11, 113-128.

Hawkins, W.E., Walker, W.W., and Overstreet, R.M. (1995). Carcinogenicity tests using aquarium fish. In *Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment* (G.R. Rand, Ed), pp 421-446. Taylor and Francis, Washington, D.C.

Hendricks, J.D. (1994). Carcinogenicity of aflatoxins in nonmammalian organisms. In *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance* (D.L. Eaton and J.D. Groopman, Ed.), pp 103-136. Academic Press, New York.

Hendricks, J.D., Cheng, R., Shelton, D.W., Pereira, C.B., and Bailey, G.S. (1994). Dose-dependent carcinogenicity and frequent Ki-*ras* proto-oncogene dependent activation by dietary N-nitrosodiethylamine in rainbow trout. *Fundam. Appl. Toxicol.* 23, 53-62.

Hendricks, J.D., Meyers, T.R., Shelton, D.W., Casteel, J.L., and Bailey, G.S. (1985). Hepatocarcinogenicity of benzo[*a*]pyrene to rainbow trout by dietary exposure and intraperitoneal injection. *J. Natl. Cancer Inst.* 74, 839-851.

Hodgson, E., and Levi, P.E. (1994). Metabolism of toxicants: phase I reactions. In *Introduction to Biochemical Toxicology* (E. Hodgson and P.E. Levi, Ed), pp 113-132. Appleton and Lange, Norwalk, CT.

Husoy, A-M, Myers, M.S., Willis, M.L., Collier, T.K., Celander, M., and Goksoyr, A. (1994). Immunohistochemical localization of CYP1A and CYP3A-like

isozymes in hepatic and extrahepatic tissues of atlantic cod (*Gadus morhua L.*), a marine fish. *Toxicol. Appl. Pharmacol.* **1994**, 294-308.

International Agency for Research on Cancer (IARC). (1993). IARC monographs on the evaluation of carcinogenic risks to humans. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. **56**, 245-396.

James, M.O. (1987). Conjugation of organic pollutants in aquatic species. *Environ. Health Perspec.* **71**, 97-103.

Kalsch, W., Nagel, R., and Urich, K. (1991). Uptake, elimination, and bioconcentration of ten anilines in zebrafish (*Brachydanio rerio*). *Chemosphere* **22**, 351-363.

Kaplan, L.A.E., Schultz, M.E., Schultz, R.J., and Crivello, J.F. (1991). Nitrosodiethylamine metabolism in the viviparous fish *Poeciliopsis*: evidence for the existence of liver P450pj activity and expression. *Carcinogenesis* **12**, 647-652.

Khudoley, V.V. (1984). Use of aquarium fish, *Danio rerio* and *Poecilia reticulata*, as test species for evaluation of nitrosamine carcinogenicity. *Natl. Cancer Inst. Monogr.* **No. 65**, 65-70.

Ladik, J., and Förner, W. (1994) *The Beginnings of Cancer in the Cell.* Springer-Verlag, Berlin, Germany.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Larsen, H.E., Celander, M., and Goksoyr, A. (1992). The cytochrome P450 system of Atlantic salmon (*Salmo salar*): II. Variations in hepatic catalytic activities and isozyme patterns during an annual reproductive cycle. *Fish Physiol. Biochem.* **10**, 291-301.

Liu, L., Nakatsu, K., and Massey, T.E. (1993). *In vitro* cytochrome P450 monooxygenase and prostaglandin H-synthetase mediated aflatoxin B_1 biotransformation in guinea pig tissues: effects of β-naphthoflavone treatment. *Arch. Toxicol.* **67**, 379-385.

Loveland, P.M., Coulombe, R.A., Libbey, L.M., Pawlowski, N.E., Sinnhuber, R.O., Nixon, J.E., and Bailey, G.S. (1983). Identification and mutagenicity of aflatoxicol-M_1 produced by metabolism of aflatoxin B_1 and aflatoxicol by liver fractions from rainbow trout (*Salmo gairdneri*) fed B-naphthoflavone. *Food Chem. Toxicol.* **21**, 557-562.

Loveland, P.M., Nixon, J.E., and Bailey, G.S. (1984). Glucuronides in bile of rainbow trout (*Salmo gairdneri*) injected with [^{3}H]aflatoxin B_1 and the effects of dietary B-naphthoflavone. *Comp. Biochem. Physiol.* 78C, 13-19.

Loveland, P.M., Nixon, J.E., Pawlowski, N.E., Eisele T.A., Libbey, L.M., and Sinnhuber, R.O. (1979). Aflatoxin B_1 and aflatoxicol metabolism in rainbow trout (*Salmo gairdneri*) and the effects of dietary cyclopropene. *J. Environ. Pathol. Toxicol.* 2, 707-718.

Loveland, P.M., Sinnhuber, R.O., Berggren, K.E., Libbey, L.M., Nixon, J.E., and Pawlowski, N.E. (1977). Formation of aflatoxin B_1 from aflatoxicol by rainbow trout (*Salmo gairdneri*) liver *in vitro*. *Res. Commun. Chem. Pathol. Pharmacol.* 16, 167-170.

Loveland, P.M., Wilcox, J.S., Hendricks, J.D., and Bailey, G.S. (1988). Comparative metabolism and DNA binding of aflatoxin B_1, aflatoxin M_1, aflatoxicol and aflatoxicol-M_1 in hepatocytes from rainbow trout (*Salmo gairdneri*). *Carcinogenesis* 9, 441-446.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265-275.

McMahon, G. (1994). The genetics of human cancer: implications for ecotoxicology. *Environ. Health Perspec.* 102, 75-80.

Melancon, M.J., and Lech, J.J. (1983). Dose-effect relationship for induction of hepatic monooxygenase activity in rainbow trout and carp by Aroclor 1254. *Aquat. Toxicol.* 4, 51-61.

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

Miranda, C.L., Collodi, P., Zhao, X., Barnes, D.W., and Buhler, D.R. (1993). Regulation of cytochrome P450 expression in a novel liver cell line from zebrafish (*Brachydanio rerio*). *Arch. Biochem. Biophys.* 305, 320-327.

Monroe, D.H., and Eaton, D.L. (1987). Comparative effects of butylated hydroxyanisole on hepatic *in vivo* DNA binding and *in vitro* biotransformation of aflatoxin B_1 in the rat and mouse. *Toxicol. Appl. Pharmacol.* 90, 401-409.

Moore, M.J., and Myers, M.S. (1994). Pathobiology of chemical-associated neoplasia in fish. In *Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives* (D.C. Malins and G.K. Ostrander, Ed), pp 327-386. Lewis Publishers, Boca Raton, FL.

Nagel, R., Bresch, H., Caspers, N., Hansen, P.D., Markert, M., Munk, R., Scholz, N., and ter Höfte B.B. (1991). Effect of 3,4-dichloroaniline on early life stages of the zebrafish (*Brachydanio rerio*): results of a comparative laboratory study. *Ecotoxicol. Environ. Saf.* 21, 157-164.

Nakazawa, T., Hamaguchi, S., and Kynono-Hamaguchi, Y. (1985). Histochemistry of liver tumors induced by diethylnitrosamine and differential sex susceptibility to carcinogenesis in *Oryzias latipes*. *J. Natl. Cancer Inst.* 75, 567-573.

Nebert, D.W., and Nelson, D.R. (1991). P450 gene nomenclature based on evolution. *Methods Enzymol.* 206, 3-11.

Neilson, A.H., Allard, A-S., Fischer, S., Malmberg, M., and Viktor, T. (1990). Incorporation of a subacute test with zebra fish into a hierarchical system for evaluating the effect of toxicants in the aquatic environment. *Ecotoxicol. Environ. Saf.* 20, 82-97.

Nixon, J.E., Hendricks, J.D., Pawloswki, N.E., Loveland, P.M., and Sinnhuber, R.O. (1981). Carcinogenicity of aflatoxicol in Fischer 344 rats. *J. Natl. Cancer Inst.* 66, 1159-1163.

Nixon, J.E., Hendricks, J.D., Pawlowski, N.E., Pereira, C.B., Sinnhuber, R.O., and Bailey, G.S. (1984). Inhibition of aflatoxin B_1 carcinogenesis in rainbow trout by flavone and indole compounds. *Carcinogenesis* 5, 615-619.

Okey, A.B., Riddick, D.S., and Harper, P.A. (1994). The Ah receptor: Mediator of the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds. *Toxicol. Let.* 70, 1-22.

Phillips, D.H. (1983). Fifty years of benzo(*a*)pyrene. *Nature* 303, 468-472.

Pitot, H.C., and Dragan, Y.P. (1991). Facts and theories concerning the mechanisms of carcinogenesis. *FASEB J.* 5, 2280-2286.

Pliss, G.B., and Khudoley, V.V. (1975). Tumor induction by carcinogenic agents in aquarium fish. *J. Natl. Cancer Inst.* 55, 129-136.

Poland, A., Greenlee, W.F., and Kende, A.S. (1979). Studies on the mechanism of action of the chlorinated dibenzo-*p*-dioxins and related compounds. *Ann. N.Y. Acad. Sci.* 320, 214-230.

Powers, D.A. (1989). Fish as model systems. *Science* 246, 352-358.

Prough, R.A., Burke, M.D., and Mayer, R.T. (1978). Direct fluorometric methods for measuring mixed-function oxidase activity. *Methods Enzymol.* 52C, 372-377.

Purchase, I.F.H. (1994). Current knowledge of mechanisms of carcinogenicity: genotoxins versus non-genotoxins. *Human and Exper. Toxicol.* 13, 17-28.

Raney, K.D., Coles, B., Guengerich, F.P., and Harris, T.M. (1992a). The *endo*-8,9-epoxide of aflatoxin B_1: a new metabolite. *Chem. Res. Toxicol.* 5, 333-335.

Raney, K.D., Meyer, D.J., Ketterer, B., Harris, T.M., and Guengerich, F.P. (1992b). Glutathione conjugation of aflatoxin B_1 *exo*- and *endo*-epoxides by rat and human glutathione-*S*-transferases. *Chem. Res. Toxicol.* 5, 470-478.

Robbins, S.L., and Kumar, V. (1987). *Basic Pathology.* pp 182-213. W.B. Saunders Co., Philadelphia, PA.

Salhab, A.S., and Edwards, G.S. (1977). Comparative *in vitro* metabolism of aflatoxicol by liver preparations from animals and humans. *Cancer Res.* 37, 1016-1021.

SAS Institute Inc., *SAS/STAT User's Guide*, Version 6, Fourth Edition, Cary, NC: SAS Institute Inc, 2, 1-846.

Sato, S., Matsushima, T., Tanaka, N., Sugimura, T., and Takashima, F. (1973). Hepatic tumors in the guppy (*Lebistes reticulatus*) by aflatoxin B_1, dimethylnitrosamine, and 2-acetylaminofluorene. *J. Natl. Cancer Inst.* 50, 765-778.

Schell, J.D., Cooper, K.O., and Cooper, K.R. (1987). Hepatic microsomal mixed-function oxidase activity in the Japanese medaka (*Oryzias latipes*). *Environ. Toxicol. Chem.* 6, 717-721.

Schlenk, D., and Buhler, D.R. (1991a). Flavin-containing monoxygenase activity in liver microsomes from the rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* 20, 13-24.

Schlenk, D. and Buhler, D.R. (1991b). Role of flavin-containing monooxygenase in the *in vitro* biotransformation of aldicarb in rainbow trout (*Oncorhynchus mykiss*). *Xenobiotica* 21, 1583-1589.

Schlenk, D., and Buhler, D.R. (1993). Immunological characterization of flavin-containing monooxygenases from the liver of rainbow trout (*Oncorhynchus mykiss*): sexual- and age- dependent differences and the effect of trimethylamine on enzyme regulation. *Biochim. Biophys. Acta* 1156, 103-106.

Schlenk, D., Ronis, M.J.J., Miranda, C.L., and Buhler, D.R. (1993). Channel catfish liver monooxygenases: immunological characterization of constitutive cytochromes P450 and the absence of active flavin-containing monooxygenases. *Biochem. Pharmacol.* 45, 217-221.

Schoenhard, G.L., Hendricks, J.D., Nixon, J.E., Lee, D.J., Wales, J.H., Sinnhuber, R.O., and Pawlowski, N.E. (1981). Aflatoxicol-induced hepatocellular carcinoma in rainbow trout (*Salmo gairdneri*) and the synergistic effects of cyclopropenoid fatty acids. *Cancer Res.* 41, 1011-1014.

Shelton, D.W., Goeger, D.E., Hendricks, J.D., and Bailey, G.S. (1986). Mechanisms of anti-carcinogenesis: the distribution and metabolism of aflatoxin B_1 in rainbow trout fed aroclor 1254. *Carcinogenesis* 7, 1065-1071.

Shelton, D.W., Hendricks, J.D., and Bailey, G.S. (1984). The heaptocarcinogenicity of diethylnitrosamine to rainbow trout and its enhancement by Aroclors 1254 and 1254. *Toxicol. Letters* 22, 27-31.

Shibahara, T., Ogawa, H.I., Ryo, H., and Fujikawa, K. (1995). DNA-damaging potency and genotoxicity of aflatoxin M_1 in somatic cells *in vivo* of *Drosophila melanogaster*. *Mutagenesis* 10, 161-164.

Shimada, T., and Guengerich, F.P. (1989). Evidence for cytochrome P-450_{NF}, the nifedipine oxidase, being the principle enzyme involved in the bioactivation of aflatoxins in human liver. *Proc. Natl. Acad. Science USA* 86, 462-465.

Sinnhuber, R.O., Hendricks, J.D., Wales, J.H., Putnam, G.B. (1977). Neoplasms in rainbow trout, a sensitive animal model for environmental carcinogenesis. *Ann. NY Acad. Sci.* 298, 389-408.

Sinnhuber, R.O., Wales, J.H., Ayres, J.L., Engebrecht, R.H., Amend, D.L. (1968). Dietary factors and hepatoma in rainbow trout (*Salmo gairdneri*). 1. Aflatoxins in vegetable protein feedstuffs. *J. Natl. Cancer Inst.* 41, 711-718.

Sipes, I.G., and Gandolfi, A.J. (1991). Biotransformation of toxicants. In *Casarett and Doull's Toxicology: The Basic Science of Poisons* (M.O. Amdur, J. Doull, and C.D. Klaassen, Ed.), pp 127-200. Pergamon Press, Maxwell House, New York.

Smith, M.R., Mathews, N.T., Jones, K.A., and Kung, H-F. (1993). Biological actions of oncogenes. *Pharmac. Ther.* 58, 211-236.

Stahl, C.H., Sultatos, L.G., Hacker, C.S., and Murphy, S.D. (1984). Mixed-function oxidase studies in the redfish, *Sciaenops ocellata*, from Galveston Bay, Texas. *Comp. Biochem. Physiol.* 79C, 177-182.

Stanton, M.F. (1965). Diethylnitrosamine-induced hepatic degeneration and neoplasia in the aquarium fish, *Brachydanio rerio*. *J. Natl. Cancer Inst.* 34, 117-130.

Stegeman, J.J. (1993). Cytochrome P450 forms in fish. *Handb. Exp. Pharm.* 105, 279-288.

Stegeman, J.J. (1989). Cytochrome P450 forms in fish: catalytic, immunological and sequence similarities. *Xenobiotica*, 19 1093-1110.

Stegeman, J.J., and Hahn, M.E. (1994). Biochemistry and molecular biology of monooxygenases: current perspectives on forms, functions, and regulation of cytochrome P450s in aquatic species. In *Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives* (D.C. Malins and G.K. Ostrander, Ed), pp 87-206. Lewis Publishers, Boca Raton, FL.

Stegeman, J.J., and Lech, J.J. (1991). Cytochrome P-450 monooxygenase systems in aquatic species: carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. *Environ. Health Perspec.* **90**, 101-109.

Stegeman, J.J., Miller, M.R., and Hinton, D.E. (1989). Cytochrome P450IA1 induction and localization in endothelium of vertebrate (teleost) heart. *Mol. Pharmacol.* **36**, 723-

Strauss, W.M. (1991). Preparation of genomic DNA from mammalian tissue. In *Current Protocols in Molecular Biology* (Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., Eds.), pp 2.2.1-2.2.2. Greene Publishing Associates and Wiley-Interscience, New York.

Swenberg, J.A. (1993). Cell proliferation and chemical carcinogenesis: conference summary and future directions. *Environ. Health Perspec.* 101, 153-158.

Swenson, D.H., Lin, J.-K., Miller, E.C., and Miller, J.A. (1977). Aflatoxin B_1-2,3-oxide as a probable intermediate in the covalent binding of aflatoxins B_1 and B_2 to rat liver DNA and ribosomal RNA *in vivo*. *Cancer Res.* 37, 172-181.

Takahashi, N., Harttig, U., Williams, D.E., and Bailey, G.S. (1996). The model Ah-receptor agonist β-naphthoflavone inhibits aflatoxin B_1-DNA binding *in vivo* in rainbow trout at dietary levels that do not induce CYP1A enzymes. *Carcinogenesis* 17, 79-87.

Takahashi, N., Miranda, C.L., Henderson, M.C., Buhler, D.R., Williams, D.E., and Bailey, G.S. (1995). Inhibition of *in vitro* aflatoxin B_1-DNA binding in rainbow trout by CYP1A inhibitors: α-naphthoflavone, β-naphthoflavone, and trout CYP1A1 peptide antibody. *Comp. Biochem. Physiol.* 110C, 273-280.

Toledo, C., Hendricks, J., Loveland, P., Wilcox, J., and Bailey, G. (1987). Metabolism and DNA-binding *in vivo* of aflatoxin B_1 in medaka (*Oryzias latipes*). *Comp. Biochem. Physiol.* 87C, 275-281.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* 76, 4350-4354.

Tsai, H.-W. (1996). Evaluation of zebrafish (*Brachydanio rerio*) as a model for carcinogenesis. [Ph.D. dissertation]. Oregon State University, Corvallis, OR.

Valsta, L.M., Hendricks, J.D., and Bailey, G.S. (1988). The significance of glutathione conjugation for aflatoxin B_1 metabolism in rainbow trout and coho salmon. *Food Chem. Toxicol.* 26, 129-135.

Van Beneden, R.J., and Ostrander, G.K. (1994). Expression of oncogenes and tumor suppressor genes in teleost fishes. In *Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives* (D.C. Malins and G.K. Ostrander, Ed.), pp 295-325. Lewis Publishers, Boca Raton, FL.

Walsh, A.A., Hsieh, D.P.H., and Rice, R.H. (1992). Aflatoxin toxicity in cultured human epidermal cells: stimulation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Carcinogenesis* 13, 2029-2033.

Weinberg, R.A. (1989a). Oncogenes and multistep carcinogenesis. In *Oncogenes and the Molecular Origin of Cancer.* (R. A. Weinberg, Ed.), pp 307-326. Cold Spring Harbor Laboratory Press, New York.

Weinberg, R.A. (1989b). Oncogenes, antioncogenes, and the molecular basis of multistep carcinogenesis. *Cancer Res.* 49, 3713-3721.

Whitlock, J.P. (1993). Mechanistic aspects of dioxin action. *Chem. Res. Toxicol.* 6, 754-763.

Williams, D.E., and Buhler, D.R. (1983). Purified form of cytochrome P-450 from rainbow trout with high activity toward conversion of aflatoxin B_1 to aflatoxin B_1-2,3-epoxide. *Cancer Res.* 43, 4752-4756.

Williams, G.M., and Weisburger, J.H. (1991). Chemical carcinogenesis. In *Casarett and Doull's Toxicology: The Basic Science of Poisons* (M.O. Amdur, J. Doull, and C.D. Klaassen, Ed.), pp 127-200. Pergamon Press, Maxwell House, New York.

Witham M., Nixon, J.E., and Sinnhuber, R.O. (1982). Liver DNA bound *in vivo* with Aflatoxin B_1 as a measure of hepatocarcinoma initiation in rainbow trout. *J. Natl. Cancer Inst.* 68, 623-628.

Wirgin, I.I., Grunwald, C., Courtenay, S., Kreamer, G.-T., Reichert, W.L., and Stein, J.E. (1994). A biomarker approach to assessing xenobiotic exposure in Atlantic tomcod from the North American Atlantic coast. *Environ. Health Perspec.* 102, 764-770.

Yawetz, A., Manelis, R., and Fishelson, L. (1992). The effects of Aroclor 1254 and petrochemical pollutants on cytochrome P450 from the digestive gland

microsomes of four species of mediterranean molluscs. *Comp. Biochem. Physiol.* 103C, 607-614.

You, L., Harttig, U., and Bailey, G. Cloning, sequencing and functional expression of three rainbow trout CYP1A cDNAs in yeast. [publication in progress].

Zhang, Y.S., Andersson, T., and Förlin, L. (1990). Induction of hepatic xenobiotic biotransformation enzymes in rainbow trout by β-naphthoflavone. Time course studies. *Comp. Biochem. Physiol.* 95B, 247-253.

Ziegler, D.M. (1993). Recent studies on the structure and function of multisubstrate flavin-containing monooxygenases. *Annu. Rev. Pharmacol. Toxicol.* 33, 179-199.