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ETHOXYRESORUFIN-*O*-DEETHYLASE (EROD) INDUCING POTENCY OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHs) IN AVIAN HEPATOCYTE CULTURES: INVESTIGATIONS OF THE ABILITY OF AN *IN VITRO* BIOASSAY TO PREDICT THE TOXIC POTENCY OF PAHs

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Abstract

Eighteen polycyclic aromatic hydrocarbons (PAHs) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were examined for their potencies to induce ethoxyresorufin-O-deethylase (EROD) activity in primary cultures of White Leghorn chicken (*Gallus domesticus*), Pekin duck (*Anas platyrhynchos*), and Greater Scaup (*Aythya marila*) embryo hepatocytes. Of the 18 PAHs, only eight induced EROD activity. In chicken hepatocytes the rank order of potency of the eight EROD inducing PAHs was BkF ≥ DahA > Ind > BaA ≥ BghiP ≥ Chr ≥ BaP > BNT. A log-log plot of the ECthr plotted against the LD50 (LD50 data from Brunström *et al.*, 1991) for BkF, DahA, BaA, and BNT resulted in a good correlation ($r^2 = 0.898$) between EROD-inducing potency and *in ovo* lethality. The regression line was used to predict LD50 values for Ind, Chr, and BaP. Although the regression line overestimated the LD50 values, the estimated values appear to be within one order of magnitude of their approximate LD50. The rank order of induction potency of the eight EROD-inducing PAHs in duck and scaup hepatocytes was BkF ≥ DahA > BaP ≥Ind > BghiP ≥ Chr ≥ BaA > BNT, and BkF ≥ DahA > BaP ≥ Ind > BaA ≥ BghiP > Chr, respectively. Among species, there was no clear overall trend in relative sensitivity to EROD induction by individual PAHs. The EROD inducing potency of zebra mussel (*Dreissena polymorpha*) extracts was investigated in hepatocytes of all three species. The rank order of sensitivity to the extracts was chicken > duck ≥ scaup. While TCDD and several PCBs induce porphyrin accumulation in chicken embryo hepatocytes, none of the PAHs elicited this response. Regardless of the lack of porphyrinogenic effects, the EROD data suggests that primary cultures of avian embryo hepatocytes may be a useful model for predicting the *in ovo* toxic potencies of PAHs in avian species.

Resume

On a examiné l'effet de dix-huit hydrocarbures aromatiques polycycliques (HAP) et du 2,3,7,8-tétrachlorodibenzo-p-dioxine (TCDD) sur leur capacité d'induction de l'activité de la 7-éthoxyrésorufine-O-déethylase (EROD) de cultures primaires d'hépatocytes d'embryons de poulet de race Leghorn blanche (*Gallus domesticus*), de canard colvert (*Anas platyrhynchos*) et de fuligule milouinan (*Aythya marila*). Parmi les dix-huit HAP étudiés, seulement huit ont induit l'activité d'EROD. Le rang de la capacité d'induction de ces huit HAP sur les hépatocytes de poulet fut BkF ≥ DahA > Ind > BaA ≥ BghiP ≥ Chr ≥ BaP > BNT. Une représentation graphique log-log de ECthr versus la DL50 (valeurs de DL50 extraites de Brunström *et al.*, 1991) pour BkF, DahA, BaA et BNT a démontré une bonne corrélation ($r^2 = 0.898$) entre la capacité d'induction d'EROD et la létalité *in ovo*. La ligne de régression fut utilisée pour prédire les DL50 de Ind, Chr et BaP. Bien que la ligne de régression ait surestimé les valeurs des DL50, celles-ci se sont avéré être du même ordre de grandeur que leur valeur approximative. Le rang de la capacité d'induction de ces huit HAP sur les hépatocytes de canard fut : BkF ≥ DahA > BaP ≥ Ind > BghiP ≥ Chr ≥ BaA > BNT et pour le fuligule : BkF ≥ DahA > BaP ≥ Ind > BaA ≥ BghiP > Chr. Aucune tendance claire n'a pu être décelée concernant la vulnérabilité relative de l'induction d'EROD pour un HAP en particulier entre les espèces. La capacité d'induction d'EROD par des extraits de moules zébrés (*Dreissena polymorpha*) a été examinée pour les trois espèces. L'ordre du degré de vulnérabilité aux extraits fut le suivant : poulet > canard ≥ fuligule. Bien que le TCDD et plusieurs BPC induisent l'accumulation de porphyrines dans les hépatocytes de poulet, aucun des HAP n'a eu cet effet. Indépendamment de ce manque d'effets porphyrinogéniques, les valeurs de l'activité d'EROD suggèrent que les cultures primaires d'hépatocytes d'embryons aviaires peuvent être un modèle utile pour prédire les limites toxiques *in ovo* des HAP chez les espèces aviaires.

Dedication

This work is dedicated to my parents who over the years have supported me, and encouraged me and at times have just plain put up with me. This work would not have been possible without your understanding and forbearance.

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I can recall few undertakings in my life that have demanded so much dedication to study and experimentation, so much time, energy, thought processes, travel, patience and determination as the preparation of this thesis. As I journeyed along the critical path to completion I realize now that my debts are many.

I now wish to express my gratitude to those who guided me on my way by encouragement, helpful criticism, moral support and the sincere reassurance that they were with me to completion. Let me list them according to their relative order though not necessarily according to their relative importance.

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List of Abbreviations

1-MeP	1-Methylpyrene
AHH	Aryl Hydrocarbon Hydroxylase
AhR	Aryl Hydrocarbon Receptor
Ant	Anthracene
Arnt	Aryl Hydrocarbon Nuclear Translocator
BaA	Benz[a]anthracene
BaP	Benzo[a]pyrene
BeP	Benzo[e]pyrene
BkF	Benzo[k]fluoranthene
BghiP	Benz[ghi]perylene
BNT	Benzo[b]naphtho[2,3-d]thiophene
CEH	Chicken Embryo Hepatocyte
Chr	Chrysene
Cor	Coronene
CYP	Cytochrome P450
DahA	Dibenz[ah]anthracene
DBT	Dibenzothiophene
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
EC50	Median Effective Concentration
ECthr	Effective Threshold Concentration
EROD	Ethoxyresorufin-*O*-deethylase
Fln	Fluoranthene
Flu	Fluorene
GNMT	Glycine N-methyltransferase
HAHs	Halogenated Aromatic Hydrocarbons
IEF	Induction equivalency factor
Ind	Indeno[1,2,3-cd]pyrene
LD50	Median Lethal Dose
MFO	Multifunctional Oxidase
NADPH	Nicotinamide Adenine Dicnucleotide Phosphate Reduced
PAH	Polycyclic Aromatic Hydrocarbon
PCB	Polychlorinated biphenyl
Phen	Phenanthrene
Per	Perylene
Pyr	Pyrene
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin

CHAPTER 1. GENERAL INTRODUCTION

1.1. Global Perspective

Since the mid-1980's the populations of circumpolar sea ducks, including the Greater Scaup, *Aythya marila*, have declined and do not appear to have responded to improvements in water conditions on the breeding grounds. The ecology of these birds is dominated by their migration from wintering sites as far south as the Carribean, to breeding sites located in the far north along the Beaufort Sea in Alaska, and the Yukon. The underlying causes for the observed reductions in breeding success are myriad, and remain unexplained. Although the consumption of contaminated food and concomitant exposure to persistent toxic chemicals (e.g., dioxins/furans, polychlorinated biphenyls (PCBs), pesticides such mirex) may be a contributing factor (Environment Canada, 1991), the loss or degradation of wintering/migratory/breeding habitat, as well as predation, hunting, and disease may be of significance. Climatic variation in the Pacific northeast, where large populations of several species of seabirds breed and overwinter, have resulted in significant ecosystem disturbances. A substantial lowering of secondary productivity and a major decline in seabirds along the coast have been observed (McGowan *et al.*, 1998). Thus, for species already living at the limits of tolerance, a combination of contaminant exposure, habitat loss and climatic change may act synergistically to reduce survivorship and reproductive success.

The Greater Scaup is boreal-subarctic when nesting and is more or less marine in winter. Since the last glaciation the nesting grounds of the Greater Scaup have spread eastward across Canada, along the migratory routes between Alaska and the Atlantic coast. However, the vast majority of Greater Scaup in North America still nest in Alaska. The fall migratory routes of

a large portion of the Alaskan and northwestern Canadian populations are southeast towards Hudson's Bay, and through the Great Lakes towards the Atlantic coast; spring routes are essentially the reverse of the fall routes. The winter range of the Greater Scaup includes the Pacific coast from the Aleutian Islands to southern California, the Atlantic coast from Newfoundland to central Florida and the Carribean, and small numbers on open water almost anywhere in the continental interior, including the Great Lakes (Palmer, 1976).

The Great Lakes basin is known for environmental contamination problems. Extensive laboratory and field studies of the Great Lakes basin ecosystem have provided strong evidence that persistent toxic chemicals have affected the growth and reproduction of at least eleven wildlife species (two mammals, eight birds, and one reptile) (Environment Canada, 1991). Embryolethality, deformities, and reproductive failures have previously been reported for several piscivorus avian species (Gilbertson *et al.*, 1991; Giesy, *et al.*, 1994). Since the introduction of zebra mussels (*Dreissena polymorpha*) into the Great Lakes, several species of diving duck, including the Greater Scaup, have altered their migratory patterns to take advantage of the mussels as an abundant food source (Wormington and Leach, 1992; Custer and Custer, 1996). Zebra mussels are efficient filter feeders and may bioaccumulate more contaminants than do other traditional food items. Because of the shift in migratory patterns of Greater Scaup and other diving ducks through the Great Lakes since the zebra mussel invasion, migrant and wintering populations of diving ducks may be more exposed to contaminants. The decline in Greater Scaup populations over the past 20 years may be partially attributable to consumption of contaminants, including polycyclic aromatic hydrocarbons (PAHs), while staging in the Great Lakes during spring and fall migrations.

1.2. Polycyclic Aromatic Hydrocarbons (PAHs)

1.2.1. Identity, Sources and Entry into the environment

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants of both terrestrial and aquatic ecosystems. PAHs are a broad class of environmental contaminants characterized by a basic structure consisting of three or more fused aromatic rings (Neff, 1979). Two aromatic rings are said to be fused when a pair of carbon atoms is shared; the resulting structure is a molecule with all atoms lying in a single plane. PAHs as a class of chemicals includes about 100 compounds. The term polycyclic aromatic hydrocarbon refers strictly to compounds consisting only of carbon and hydrogen grouped into rings containing five or six carbons. When alkyl, and other types of substituents are introduced to the ring structure, they are called PAH derivatives. PAHs in which a carbon atom in a ring is replaced with an atom of nitrogen, oxygen, or sulfur are called heterocyclic aromatic hydrocarbons. While the number of PAHs of environmental concern (i.e., those with molecular weight ranging from 128 (naphthalene) to 300 (coronene)) includes only about 100 individual compounds, the total number of PAHs with substituents plus heterocyclic aromatic hydrocarbons leads to several thousand possible compounds (Neff, 1979). Because the toxicology of this large number of PAHs would be impossible to study, the US EPA and the WHO have identified 16 unsubstituted PAHs as priority pollutants, several of which were investigated in this report. Figure 1.1 shows the structures of the 18 PAHs investigated in this study.

Pure PAHs are usually colored, crystalline solids at ambient temperature with physical properties varying with molecular weight and structure. Vapor pressure and aqueous solubility

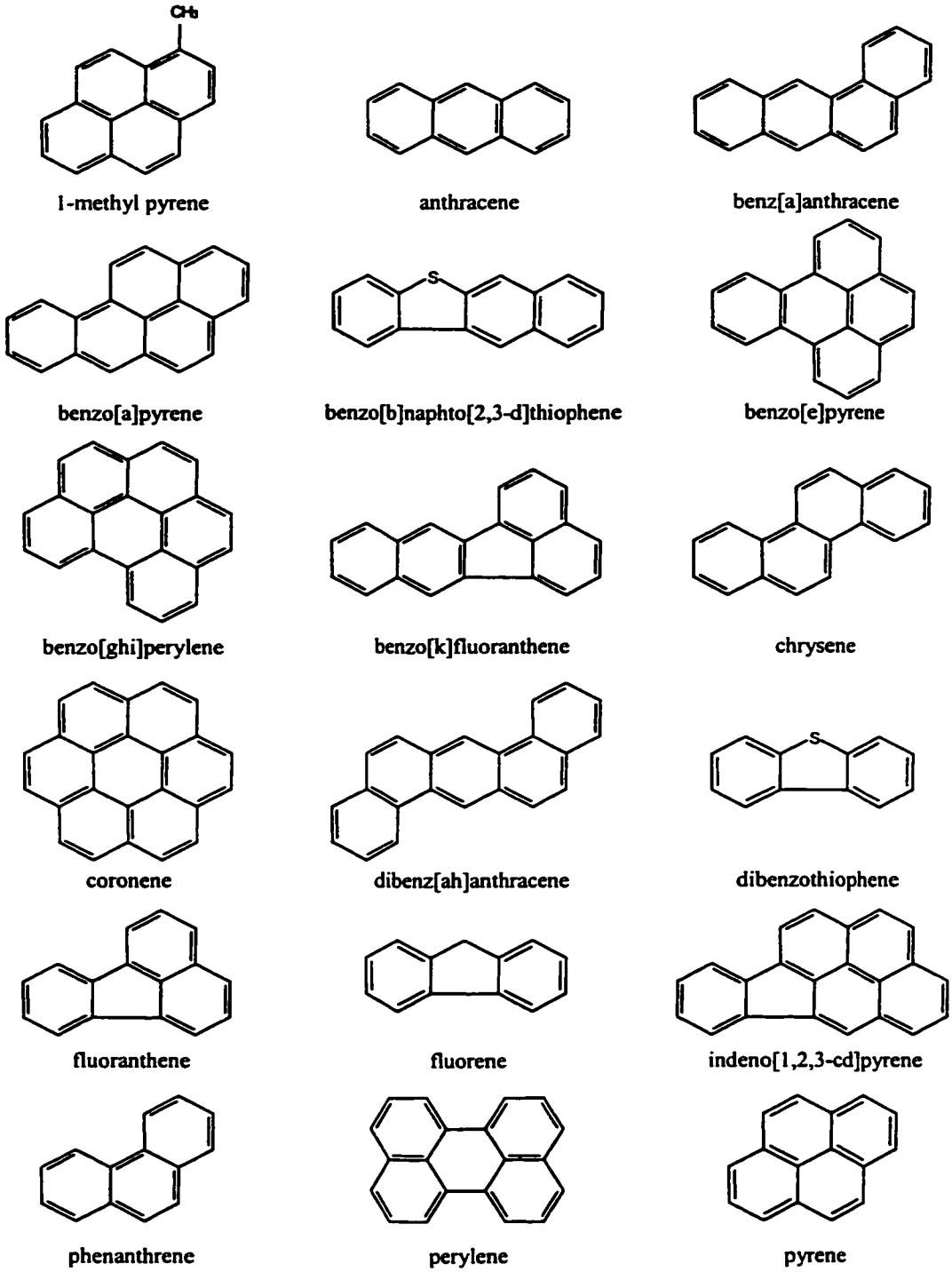

Figure 1.1. Molecular structures of the 18 polycyclic aromatic hydrocarbons (PAHs) investigated in this study. PAHs with alkyl substituents are called PAH derivatives, and PAHs in which a carbon atom is replaced with a sulfur, nitrogen, or oxygen atom are called heterocyclic aromatic hydrocarbons, and are not by definition PAHs. PAHs containing between two and seven aromatic rings are the most environmentally mobile, and several PAHs within this molecular weight group are highly carcinogenic and toxic to numerous species, including avian species.

decrease almost logarithmically with increasing molecular weight. Solubility tends to decrease as the number of aromatic rings or the molecular weight increases. Naphthalene has a solubility of about 30 ppm while five-ring PAHs have solubilities ranging from 0.5 - 5.0 ppm (Neff, 1979). They have relatively high octanol-water partition coefficients (K_{ow}), and have a high potential for adsorption onto suspended particulates in the air and in water, and for bioaccumulation in organisms (Environment Canada, 1994). Two distinct classes of PAHs can be distinguished on the basis of their physical, chemical and biological properties: (a) the lower molecular weight 2-3 ring PAHs (naphthalene, fluorene anthracene); and, (b) the higher molecular weight 4-7 ring PAHs (chrysene to coronene). Of particular concern are PAHs ranging in molecular weight from 128 (naphthalene) to 300 (coronene). PAHs in this group tend to be more water soluble, increasing their potential bioavailability and mobility within the environment (Mackay *et al.*, 1992). Table 1.1 lists some of the physical properties of some of the PAHs in this study.

PAHs are formed whenever organic substances are exposed to high temperatures. Most PAHs are produced during the incomplete combustion, or pyrolysis, of organic materials such as forest fires and the combustion of fossil fuels. However, high temperatures or open flames are not necessary for the production of PAHs; the aromatic hydrocarbons present in crude oil are formed over millions of years at temperatures between 100 and 150°°C (Blumer1976; Neff, 1979). The temperature of formation determines the abundance of PAHs that carry alkyl substituents, collectively called side chains. At high temperatures, the less stable alkyl side chains are rapidly cleaved resulting in a mixture consisting predominantly of unsubstituted PAHs, while at the low temperatures at which crude oils are formed, alkylated PAHs

predominate (Blumer, 1976). Petroleum is composed primarily of alkylated hydrocarbons and is considered a contaminant because of its significant differences from hydrocarbon mixtures made by organisms. The most notable difference between petroleum hydrocarbons and those of plants and animals is the great diversity of molecular structure in petroleum; petroleum contains a vast array of straight chain, branched, cyclic, and aromatic hydrocarbons. In contrast, biological hydrocarbons are rather limited in their variety and do not commonly contain cyclic or aromatic structures.

Table 1.1. Physical properties of selected polycyclic aromatic hydrocarbons.

Compound	Number of rings	Molecular Weight	Log K_{ow}	Water Solubility (mg/ml at 25°C)
Fluorene	3	166	4.18	1.7
Anthracene	3	178.24	4.5	0.045
Pyrene	4	202.26	4.9	0.13
Fluoranthene	4	202.26	5.1	0.21
Benz[a]anthracene	4	228	5.6	0.0094
Benzo[a]pyrene	5	252.32	6.0	0.0038
Benzo[k]fluoranthene	5	252.32	6.06	0.0038
Indeno[1,2,3-cd]pyrene	6	276	6.4	0.00053

Reference: Environment Canada, 1994.

It is estimated that approximately 43,000 tons of PAH are released to the atmosphere with a further 230,000 tons released to the aquatic environment annually, worldwide (Environment Canada, 1991). In Canada, the annual atmospheric emissions of PAHs in 1990 from forest fires was approximately 2010 tons. Anthropogenic sources in the same time period contributed a further 2304 tons (Environment Canada, 1994). Creosote, a pesticide distilled from coal tar, is used extensively as a wood preservative for railway ties, and marine and freshwater pilings. Creosote and other coal tar derivatives contain a variety of PAHs;

approximately 63% of the volume of creosote consists of 17 PAHs, some of which are highly toxic, and known carcinogens. In Canada, production of creosote has decreased from a maximum of 65,000 tonnes in the late 1940s to 20,000 tonnes in 1990. It is estimated that the release of PAHs from creosote treated products to soil and water may be as high as 2,000 tonnes per year (Environment Canada, 1994).

Since natural sources are usually dispersed spatially and temporally over a large area, they do not result in a continuous exposure of any specific area. In contrast, anthropogenic sources of PAHs tend to be highly localized, with sedimentary concentrations rising steeply as one nears a point source; up to 63% of particle bound PAHs in aquatic sediments are located within 100 meters of their point of entry (Neff, 1979). Concentrations of PAHs in sediments from the Great Lakes range from a few parts per billion in remote areas, to up to 1 part per million in contaminated urban environments (Eadie, 1984). Atmospheric deposition is estimated to be the main source of PAHs to soils and sediments. Deposition rates of PAHs to aquatic and terrestrial surfaces in the north-eastern US have been estimated to be 0.8 to 3 ng/cm^2 per year in non-urban areas, and up to 35 ng/cm^2 per year near urban areas (Environment Canada, 1994). In 1990, 484 tonnes/year was estimated to enter the Great Lakes as a result of dry deposition from sources in Canada and the US (Environment Canada, 1994). Road runoff contains a complex mixture of contaminants, including oil, tars and PAHs, and can affect water and sediment quality of the catchement systems, and can alter the benthic structure of the benthic communities (Maltby *et al.*, 1995; Boxall and Maltby, 1997). The major toxicants in the road runoff have been identified as three to five ring PAHs, with anthracene, pyrene, benz[a]anthracene, chrysene, benzo[a]pyrene, and benzo[b & k]fluoranthenes accounting for

over 25% of the volume of the PAHs (Boxall and Maltby, 1997). Estimates of runoff into the Great Lakes range from 10 to 25% of atmospheric input (Eadie, 1984).

Sediments are the ultimate sink for PAHs, where under reducing conditions they may persist indefinitely. Analysis of PAH concentrations in sediment cores from the Tamar Estuary, U.K. revealed an exponential increase in the concentration of PAHs from 30 ng/g prior to 1940 to between 100 and 1000 ng/g in recent sediment samples (Readman *et al.*, 1987). This corresponds to a current PAH input of between 0.23 to 21 mg/m^2 total PAHs to these sediments per year. The PAH composition of contaminated sediment cores from industrial areas shows extraordinary uniformity throughout the core. The PAH composition is dominated by unsubstituted parent PAHs rather than alkyl substituted PAHs, consistent with pyrogenic sources associated with industrial activity and increased use of internal combustion engines. Fluoranthene and pyrene are usually the most abundant, and occur in about equal concentrations (Neff, 1979; Readman *et al.*, 1987).

Most ecotoxicology studies of PAHs have been conducted mostly on aquatic rather than terrestrial organisms. This is a reflection of the tendency of PAHs, as well as other lipophilic substances, to associate with particulate matter and to ultimately become associated with aquatic sediments. PAHs have been shown to adversely affect several aquatic organisms, with a broad range of effects including survival, growth, reproduction and the induction of neoplasmas.

1.2.2. Toxicology of PAHs

In 1775, the English surgeon, Sir Percival Pott, noted an association between the incidence of scrotal cancer in chimney sweeps and their exposure to soot. More than a century later, experiments showing that distillates of coal tar (a major source of PAHs) induced tumors when applied to the skin of mice and rabbits prompted chemists to identify the tumor causing compounds. In 1932, after fractionating 2 tons of coal tar, benzo[a]pyrene, a PAH, was identified as the major tumor-producing compound in coal tar (Kennaway, 1955). Dibenzo[a,l]pyrene, a PAH found in cigarette smoke, coal combustion, and some environmental samples is now considered to be the most potent carcinogen among all PAHs (Shou *et al.*, 1996).

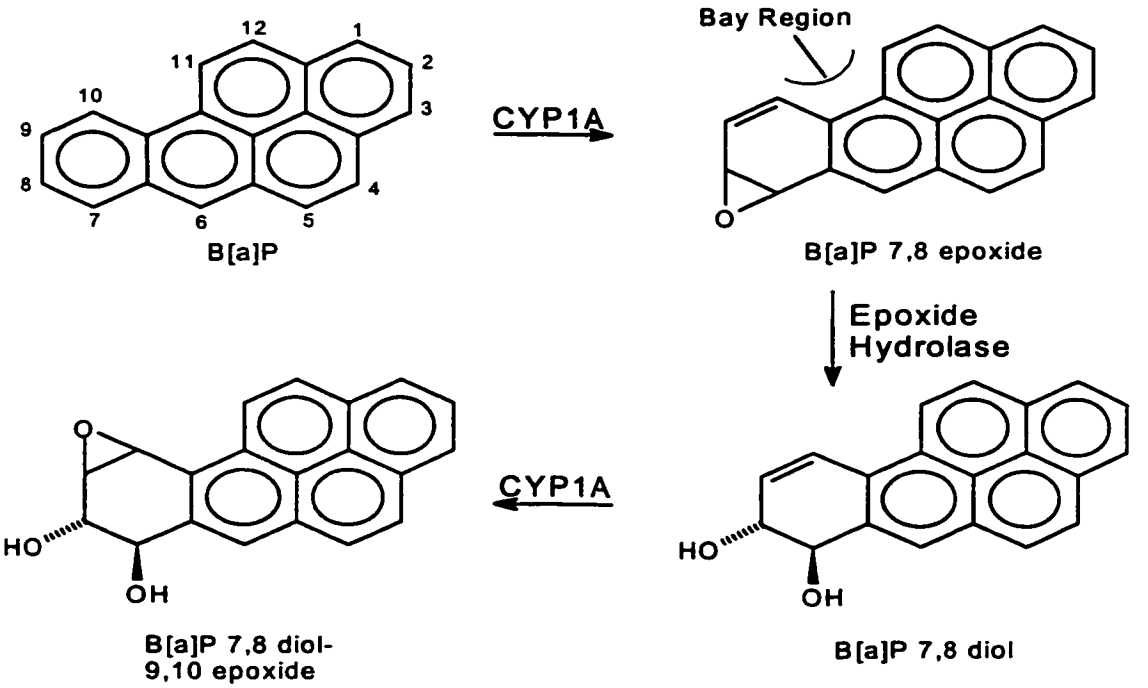

Figure 1.2. Metabolism of B[a]P by CYP1A and epoxide hydrolase. Metabolic activation of BaP by CYP1A is necessary for the bioactivation of BaP to the reactive dihydrodiol and epoxide intermediates.

Because of the differences between petroleum and biological hydrocarbons, most organisms cannot tolerate certain petroleum components, especially the aromatic hydrocarbons which have lethal and sublethal effects on a wide range of aquatic organisms (Albers, 1978; Hoffman, 1979; Rattner, 1981; Meyers, 1984). Large scale mortality and a variety of toxic effects have been reported in sea birds exposed to oil spills. The addition of as little as 2% South Louisiana crude oil to the diet of mallard (*Anas platyrhynchos*) hens resulted in eggs of reduce size and weight, and yolk content (Vanglider and Peterle, 1981). Nesting birds, contaminated externally with petroleum, can transfer petroleum to their eggs in sufficient amounts to cause embryolethality. Laboratory studies have shown that as little as 10 µl of petroleum applied to the eggshell can cause mortality and a variety of developmental defects (e.g., liver necrosis, renal lesions, and edema) in various avian species (Hoffman and Gay, 1981; Ellenton, 1982; Couillard & Leighton, 1990, 1991; Lusimbo and Leighton, 1996).

The biological activity of PAHs is dependent on the structure of the individual compounds. A substantial body of evidence has accumulated that suggests that many of the most mutagenic and carcinogenic PAHs are those which are readily metabolized to their ultimate carcinogenic metabolites. Upon biotransformation of PAHs by the cytochrome P450 enzyme system (P450), reactive arene oxides are formed as intermediates (Figure 1.2), which for many are identified as bay-region diol-epoxides (Levin *et al.*, 1982; Jerina and Daly, 1974). The majority of the carcinogenic and mutagenic activity of PAHs is due to the interaction between the electron deficient reactive epoxide metabolites and critical nucleophilic biomacromolecules, such as DNA (Hallett and Brecher 1984; Dipple *et al.*, 1999). Pharmacogenetic studies have shown that some of the toxic effects of PAHs resulting in acute

cell injury are mediated by a cytosolic receptor called the aryl hydrocarbon receptor (AhR) (Poland and Glover, 1977). Certain PAHs are known inducers of several detoxification enzyme systems including certain cytochrome P450 enzymes (e.g., cytochrome P4501A (CYP1A)) (Bigelow and Nebert, 1982; Piskorska-Pliszczynska *et al.*, 1986). However, the action of the P450 system is not always beneficial; some of the most powerful carcinogens are biotransformed *in vivo* by P450 into reactive intermediates. The occurrence of a toxic effect due to a parent molecule is therefore a balance between toxication and detoxication (Vermeulen, 1996).

Although the role of metabolic activation in the generation of reactive metabolic intermediates is well established, the mechanisms by which these toxic species interact with cellular constituents, and how these interactions cause cell injury are poorly understood. Reactions of toxic metabolites may result in covalent bond formation between the metabolite and a target molecule, or they may alter (usually by oxidation or reduction) the target molecule without covalent bond formation. Covalent interactions between reactive metabolites may involve: (1) DNA; (2) peptides and/or proteins; (3) lipids; and (4) carbohydrates. The major target molecule for the initiation of carcinogenesis is probably DNA (Dipple, *et al.* 1999). In contrast, the initiation of acute cell injury resulting from the interaction of reactive metabolites with their target molecules remains poorly characterized. Noncovalent interactions may involve: (1) alterations of GHS/GSSG, NAD(P)/NAD(P)H; (2) generation of reactive oxygen species; (3) lipid peroxidation; and (4) intercalation and complexation (Nelson and Pearson, 1990; Hinson and Roberts, 1992).

The lethal and sublethal effect of PAHs have been investigated for a number of species. In general, the short term lethal and sublethal toxicities are greater for PAHs with higher octanol/water partition coefficients. In *in vitro* studies with rainbow trout gill cells, only two- and three ring PAHs were directly cytotoxic. These PAHs elicited toxic responses rapidly, apparentiy without the need for metabolic activation (Schirmer *et al.*, 1998). Due to their higher octanol/water partition coefficients, the two- and three-ring PAHs are more bioavailable, and are more environmentally relevant than the larger less bioavailable PAHs. In contrast to the smaller molecular weight PAHs, the four- and five-ring PAHs require metabolic activation to elicit toxicity.

Although carcinogenesis has traditionally been the main focus of PAHs, other adverse effects of PAHs have begun to emerge. These include: (1) ability to induce ovarian tumors, cause errors in oocyte meiosis, reduce fertility, and produce reproductive toxicity (MacKenzie and Angevine, 1981; Mattison *et al.*, 1983); (2) the ability of PAHs to induce apoptosis and suppress the immune system (Hardin, 1992; Silkworth, 1995; Davila *et al.*, 1997; Mann *et al.*, 1999); (3) the ability to mimic steroid hormones and disrupt the function of the endocrine system (Santodonato, 1997); (4) generation of reactive oxygen species and oxidative stress following bioactivation (Pearson, 1990; Alsharif *et al.*, 1994; Bondy and Naderi, 1994; Penning *et al.*, 1996; Wells *et al.*, 1997; Nelson and Dalton *et al.*, 1999); and, (5) photoactivation by UV radiation dramatically increases PAH toxicity, especially to organisms in shallow waters (Arfsten, 1997; Schirmer *et al.*, 1998).

1.3. Bioaccumulation

Scientists investigating the behaviour of chemicals in gases, water and other phases developed the principles of our current understanding of bioaccumulation. In 1891 Nernst discovered that a constant partition coefficient, or distribution ratio, controlled the partitioning of a single pure chemical between phases. In 1893, Richet found that the toxic effects of certain ethers, alcohols, aldehydes, and ketones were inversely related to their solubility in water. A major step forward came in 1901 with the discovery by Overton that the narcotic action of a nonelectrolyte is correlated with its lipid-to-water partition coefficient. In the 1950s and 1960s, Hansch *et al.* (1989) suggested the use of the octanol to water partition coefficient (K_{ow}) as the most suitable partitioning phase pair for studying the relationship between partition coefficient and the biological properties of the compound. This relationship has a parabolic form expressed as follows:

$$\text{Log } (1/C) = -k_1 (\log K_{ow})^2 + k_2 (\log K_{ow}) + k_3\sigma + k_4$$

where K_{ow} is the octanol to water partition coefficient, σ is the Hammett constant, and k_1, k_2, k_3 and k_4 are empirically derived constants. This relationship has been used to successfully estimate the aqueous solubility, bioaccumulation, bioconcentration, toxicity and pharmacokinetics of a variety of compounds (Jackson *et al.*, 1993). The lipophilic compounds are defined as those with log K_{ow} values between 2 and 7. Compounds with K_{ow} below 2 do not exhibit a marked affinity for lipid, while compounds with a log $K_{ow} > 7$ are referred to as superhydrophobic. The superhydrophobic chemicals have been shown to require a minimum of 0.5 years to establish equilibrium, increasing to 10 years when log $K_{ow} = 8$, while compounds

with log K_{ow} > 13 are not bioaccumulated to any significant extent (Connell, 1990). The log K_{ow} values for PAHs range from 3.5 for the two-ringed naphthalene to 6.4 for the six-ringed indeno[1,2,3-cd]pyrene (Environment Canada, 1994), with the most toxic PAHs ranging from 5.6 to 6.4. The toxicity of PAHs is therefore directly correlated with their log K_{ow} values indicating that the more lipophilic PAHs will generally be more toxic. Thus, since the bioconcentration factor has been shown to be highly correlated with the octanol to water partition coefficient for a wide variety of chemicals, the octanol to water partition coefficient is a useful physical-chemical parameter for estimating the bioaccumulation of lipophilic compounds (Leo, 1993).

The accumulation of lipophilic contaminants by an organism from its environment involves two opposing forces: uptake and elimination. Uptake occurs either by direct contact between the contaminant and the body of the organism or respiratory surfaces or through ingestion of contaminated food. Elimination may occur with or without metabolism of the contaminant through the body surface, through excretion of metabolic wastes, through defecation of undigested material, through reproduction, or voiding other body tissues. The extent to which an organism bioaccumulates hydrophobic contaminants depends on this dynamic equilibrium between uptake and elimination (Waid, 1986). The amount of a given contaminant which will enter an aquatic organism, and its resultant biological activity, is influenced by its physiochemical properties (i.e., chemical structure, number of carbon atoms, water solubility, and K_{ow}) and is essentially controlled by diffusion and related processes (Connell, 1990).

Experiments with fish and other aquatic organisms have shown that the most important route for both the uptake and elimination of lipophilic compounds is via the gills. The general characteristics of the interaction of xenobiotics with organisms are governed by their physiochemical properties. The water-to-organism partition coefficient is often referred to as the bioconcentration factor and can have a value of up to and above one million; the highest bioconcentration factors are usually seen in organisms with a high lipid content (Connell, 1990). Aquatic organisms may bioaccumulate significant concentrations of xenobiotics from very low concentrations present in the water; it is not uncommon for organisms to accumulate significant amounts of contaminants from waters in which the ambient contaminant concentrations are so low that they cannot be measured by conventional analytical techniques (Connell, 1990).

For filter feeders, exposure to contaminants is through two major routes: (1) filtration of contaminated water, with uptake occurring across the gill membrane; and (2) ingestion of contaminated algae or other suspended material (Bruner *et al.*, 1994; Connell, 1990). This exposure will be particularly high in littoral areas that have high contaminant loads, resulting in large contaminant accumulation. Most freely dissolved, non-polar, hydrophobic substances such as PAHs are readily available for uptake across the gill membrane of aquatic organisms (Spacie and Hamelink, 1985; Fisher *et al.*, 1993). Although direct absorption across the gill membrane is the fastest and most efficient pathway for the accumulation of contaminants, the environmental concentration of dissolved lipophilic contaminants are very low compared to contaminant concentrations in algae and suspended solids (Swackhamer and Skoglund, 1993; Bruner *et al.*, 1994). However, lipophilic contaminants with low aqueous solubilities are usually adsorbed onto organic and inorganic particulate material. For accumulation to occur,

the contaminants must first desorb from the particulates prior to diffusing across gill. Thus, while direct absorption from water is the fastest and most efficient route for accumulation, the concentration of dissolved lipophilic contaminants are usually very low compared to contaminant concentrations adsorbed to particulate material. Consequently, ingested material may represent a more biologically significant route of contaminant exposure than uptake from water, especially in benthic organisms exposed to sediments with high contaminant loads. For benthic organisms such as zebra mussels, ingestion of contaminated sediments is likely to be a major route for exposure to PAHs and other persistent organic compounds (Bruner *et al.*, 1994).

1.4. Zebra Mussels and bioaccumulation of PAHs

The Laurentian Great Lakes St. Lawrence River ecosystem is collectively the largest body of fresh water on the planet, containing an estimated 22,000 cubic kilometers of water representing about 21% of the world's supply of fresh water (Environment Canada, 1991). The ecosystems within the Great Lakes are home to over 100 taxa types and 31 ecological community types. Over the past 200 years, the accelerated rate of human settlement and development within the basin has resulted in extensive changes in the physical and chemical composition of the Great Lakes (Mills *et al.*, 1994). The problems associated with human exploitation have been severely exacerbated by the introduction of nonindigenous species to the Great Lakes ecosystem. Since the 1800s, some of the greatest ecological disasters in the Great Lakes have resulted from biological invasions. It is estimated that the Great Lakes currently host at least 139 exotic species (including aquatic plants, fish, algae, mollusks and crustaceans), which have compromised the biological integrity of the aquatic ecosystem. One

of the most notorious exotic species, the zebra mussel (*Dreissena polymorpha,* introduced ca. 1985), has aggressively colonized the Great Lakes and surrounding watersheds and has caused substantial economic hardship and ecological instability (Mills *et al.*, 1994).

The zebra mussel is a small bivalve common throughout most of Europe and historically native to the Black, Caspian and Azov seas (Stanczykowska, 1977). The zebra mussel was discovered in June of 1988 in the southern part of lake St. Clair (Hebert *et al.*, 1989). Its introduction probably resulted from an ocean-crossing vessel that discharged fresh water ballast containing free swimming larvae (velegers) of the mussel (Griffiths *et al.*, 1991). Since their introduction in 1985, zebra mussels have become firmly established in the Great Lakes ecosystem. By the end of 1990, the mussels had spread through Lake St. Clair and had colonized the littoral and sublittoral zones of the Great Lakes and St. Lawrence River, as well as numerous rivers and inland lakes in the eastern U.S. and Canada.

Zebra mussels preferentially colonize hard substrates such as rock outcrops, and are a well known biofowling organism that can disrupt the operations of water treatment and electrical generating plants (it is estimated that as much as 80% of Lake Erie's bottom sediments have been colonized by zebra mussels) (Dermott and Munawar, 1993). In the absence of hard substrates, zebra mussels can survive as aggregates attached to rocks, pebbles, and shells of unionid clams, or as clumps (druses) on other zebra mussels. Populations of zebra mussels are greatest in littoral and sublittoral zones between 2 and 12 meters, with only a few individuals found below the thermocline (Dermott and Munawar, 1993).

Zebra mussels feed by filtering large volumes of water; mussels 21 mm in length can filter up to 150 cc of water per hour removing algae, phytoplankton, and detritus from the water (Reeders *et al.*, 1989). Fisher *et al.* (1993) experimentally determined the ability of zebra mussels to filter a suspension of Chlorella (avg. conc.: 45,110 particles/ml, size range: 2-20 μm dia.) over a three-hour exposure period. The mean filtering rate for all particle sizes depends linearly on particle concentration. Because of their high filtration rates and high population densities, zebra mussels may have contributed to the decline in the gross primary productivity of the Lake Erie water column (as evidenced by an increase in water clarity). Between 1988 and 1990, Lake Erie experienced a 43% decline in the mean chlorophyll-a concentration, and a concomitant 85% increase in sechi disc transparencies (Leach, 1993). The high filtration capacity of zebra mussels promotes the bioaccumulation of a variety of contaminants. Many of the contaminants bioaccumulated in zebra mussel tissues are persistent halogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polychlorinated biphenyls (PCBs), but non-persistent (i.e., readily metabolized) chemicals such as PAHs may also be significantly bioaccumulated in areas where PAH loads are high.

Although zebra mussels have become a major food resource for a number of Great Lakes species, including several species of diving ducks, they are cause for concern since they possess the ability to rapidly bioaccumulate a variety of toxic contaminants. The organic contaminants of particular concern in the Great Lakes ecosystem, due to their frequency of occurrence and their high concentrations in water, sediments and aquatic biota are PAHs, PCBs, chlorobenzenes and the chlorinated pesticides. Zebra mussels accumulate these contaminants from ambient water, filtered food, and sediments. In littoral and sublittoral zones with high contaminant and

particle loads, ingested material may be a significant route for the bioaccumulation of contaminants.

The bioaccumulation of PAHs by zebra mussels is enhanced by their high lipid content and their relatively poor capability to metabolize xenobiotics (Stegeman and Hahn, 1994; Meador *et al.*, 1995; Gilek *et al.*, 1997). While there is little difference in percent moisture between freshwater bivalves and zebra mussels, there was a significant difference in lipid content: bivalves averaged about 5%, dry weight, while zebra mussels ranged between 9 and 15% dry weight (Comba *et al.*, 1996; Roe and McIsaac, 1998). In a study by Roper *et al.* (1996), zebra mussels were exposed to water and sediment at the Times Beach Confined Waste Disposal Facility in Buffalo, New York. Total PAHs in the water column were below detection limit (0.01 mg/kg), while total PAHs in the sediments averaged 164.41mg/kg. After a 34-day exposure, the total PAH concentration in the tissues of the exposed zebra mussels was 6.58 mg/kg, which is significantly higher than the PAH concentration in the water column.

1.5. Waterfowl and PAH Bioaccumulation

Systematic counts of diving ducks suggest that the ducks have responded to zebra mussels as a novel and abundant food source (Custer and Custer, 1996; Wormington and Leach, 1992). In an enclosure study by Hamilton *et al.* (1994), diving ducks feeding on zebra mussels in Lake Erie at Point Pelee reduced mussel mass by 57% during the period of heaviest feeding. Lack of other foods in duck gizzards suggests that mussels are an important food source for diving ducks in the area. The response of diving ducks to zebra mussels in the Great Lakes, suggests that ducks have altered migratory patterns to take advantage of the zebra

mussels; maximum one-day count of Greater Scaup during fall migrations at Point Pelee in Lake Erie increased from 221 in 1990 to a maximum of 6500 in 1998 (Wormington and Leach, 1992). Prior to the 1997/98 freeze-up of Lakes Erie and Ontario, it is estimated that approximately 45,000 Greater Scaup wintered on the Great Lakes; this represents a large percentage of the eastern Greater Scaup population (Glen Fox, personal communication).

Like the zebra mussel, the bioaccumulation of lipophilic contaminants occurs rapidly in waterfowl. Gebauer and Weseloh (1993) reported that the mallard, *Anas platyrhynchos*, accumulated PCBs to 5300 times initial values within 10 days of exposure. In a European study, de Kock and Bowmer (1993) reported that caged tufted ducks (*Aythya fuliga*) fed a diet of zebra mussels contaminated with a variety of organic contaminants laid fewer eggs, abandoned nests more often, and had higher embryo and chick mortality rates than ducks fed less contaminated mussels. The transfer of dietary PAHs through three trophic levels from sediments to blue mussels to eider ducks was studied by Broman *et al.* (1990). Since PAHs are readily metabolized by higher organisms, their concentrations in higher trophic levels are generally low. Thus, while PAHs were found to be significantly bioaccumulated by the mussels, the PAH concentration in the tissues of the eider ducks was considerably less, indicating that they have been eliminated. The highest PAH concentration in the eider duck was found in the gallbladder, with only small amounts being transferred to the egg. Substantial evidence suggests that in vertebrates, the intestine, and not the liver, is the major site of metabolism of dietary PAHs (Vetter *et al.*, 1985; Kaminsky and Fasco, 1991; Van Veld *et al.*, 1997). Gannon *et al.* (2000) reported that upon exposure to TCDD, several Cytochrome P450 enzymes (reviewed in section 1.6), including Cytochrome P450 1A, were induced in numerous

chick embryo tissues, including the small intestine. Thus, it is possible that in chicken, and other avian species, the intestine may be the primary site of metabolism of dietary PAHs. However, Van Veld *et al.* (1987) reported that dietary fat inhibits the metabolism of PAHs in the intestine of killifish, thereby decreasing the availability of PAHs to the microsomal enzymes. Since zebra mussels have a high lipid content (relative to other bivalves in the Great Lakes), consumption of lipid-rich PAH contaminated zebra mussels by laying hens may promote the absorption of dietary PAHs into the blood where they would be available for incorporation into the egg. However, dietary PAHs are likely to be transferred to the avian egg only when the rate of PAH ingestion exceeds the rate at which they can be metabolized and eliminated.

Hens exposed to dietary PAHs while on the breeding grounds (during the period of rapid development of the ovum, and ovulation) may transfer some of the ingested PAH into the egg. Lebedev *et al.* (1998) analysed the eggs of several aquatic avian species for a variety of contaminants, including PAHs, in the highly contaminated Selenga River estuary of Lake Baikal (Russia). Very high levels of PAHs were detected in mallard (*Anas platyrhynchos*), marsh sandpiper (*Tringa stagnatilis*), and Slavonian Grebe (*Podiceps auritus*) eggs. The hens of these species are carnivorous and feed extensively on aquatic insects, molluscs, and crustaceans. In the mallard, the combined concentrations of benzo[b]fluoranthene and benzo[k]fluoranthene was 3309 μg/kg egg, dry weight (equivalent to ca. 830 μg/kg egg, wet weight). In egg injection studies, 200 μg/kg egg of benzo[k]fluoranthene injected into the yolk of 5 day old domestic duck (*Anas platyrhynchos*) embryos caused 100% mortality (Brunström *et al.*, 1990). Since the total PAH content (13 PAHs were analysed) of the mallard eggs in the

Selenga estuary was approximately 2700 μg/kg egg, it is doubtful that these eggs would have successfully hatched. For PAHs to be transferred to the egg in concentrations sufficient to induce reproductive failures or developmental abnormalities, it would be necessary for the hen to be feeding on highly contaminated foods at the breeding site during the period of rapid yolk formation. In contrast, hens feeding on PAH contaminated food prior to follicle development and yolk formation, will likely metabolize and eliminate dietary PAHs limiting their availability for incorporation into the egg.

While it is unlikely that the concentration of PAHs in the tissues of zebra mussels from the Great Lakes is sufficient to cause overt toxicological symptoms in diving ducks or other organisms consuming them, they may contribute to physiological stress indirectly. Secor *et al.* (1993) examined the element and nutrient composition of zebra mussels from rivers and lakes in New York State, and found that, relative to native clams and oysters, zebra mussels contain low concentrations of vitamin A (38 I.U./100 g dry weight for zebra mussels, versus 559 and 1641 for clams and oysters, respectively). Mallards fed a vitamin A deficient diet developed squamous metaplasia of the mucous surfaces of the respiratory and digestive tracts, and may also have suffered from immunodysfunction (Honour *et al.*, 1995). Vitamin A deprivation leads to congenital abnormalities in many systems including the urogenital, respiratory, and cardiovascular systems. Many of these abnormalities are similar to those caused by TCDD and structurally related HAHs (Walker *et al.*, 1997; Sinning, 1998; Zile, 1998;). Silkworth *et al.* (1995) demonstrated that immunosuppression of 50% or greater was induced in mice exposed to PAHs that were potent EROD inducers. Consequently, a diet consisting exclusively of zebra mussels may adversely affect the health and reproductive

capabilities of ducks through a mechanism involving a combination of PAH toxicity and vitamin A deficiency.

Thus, the ability of zebra mussels to bioaccumulate a wide range of contaminants represents a potential hazard to the organisms that feed on them, as well as serving as effective conduits for the transfer of organic contaminants to higher trophic levels (Roper *et al.*, 1996). The consumption of contaminated zebra mussels may therefore have the potential to adversely affect the reproductive success of waterfowl in North America (Mazak *et al.*, 1997).

1.6. Cytochrome P450: CYP1A induction and metabolism of xenobiotics

The most important mechanism for the detoxification of toxic xenobiotic compounds is carried out by the cytochrome P450 monooxygenase enzyme systems, which are collectively referred to as multifunctional oxidases (MFO). These enzymes metabolize a wide range of substrates by inserting an oxygen atom into the molecule, thereby introducing polar groups to facilitate elimination. All MFOs appear to operate by similar mechanisms that rely on the activation of molecular oxygen by a prosthetic heme iron atom. Some MFOs are involved in the metabolism or biosynthesis of endogenous physiological substrates while others are mainly involved in the metabolism of lipophilic xenobiotics. Although MFOs are found in all tissues, they are most abundant in the liver where they are associated with the smooth endoplasmic reticulum, and contain one or more of the various forms of cytochrome P450 (CYP) enzymes and their associated electron transport systems (Kappas and Alvares, 1975). The superfamily of CYP enzymes are present in all forms of life from bacteria to mammals, and evolved from an ancestral gene which existed approximately 2 billion years ago (Gonzalez and Nebert, 1990).

A characteristic feature of the MFO enzymes is that carbon monoxide binds to the iron of the heme group when the iron is in the reduced state. The reactive site is extraordinarily simple containing only an iron protoporphyrin IX with cysteine as the fifth ligand, leaving the sixth coordination site to bind and activate molecular oxygen. The heme complex possesses an absorption maximum near 450 nm when bound to CO, providing the generic name for these proteins, the cytochromes P450 (CYP). Early attempts at a systematic nomenclature for the various CYP enzymes were based on small differences in absorption maxima (e.g., cytochrome P448, and cytochrome P450). A uniform nomenclature system introduced in 1987 classifies different CYP enzymes involving a two-tiered system using a combination of Roman numerals and letters corresponding to a specific class and subclass. The roman numeral designates a broad class of cytochrome P450s where amino acid sequences of different classes differ by more than 70%, while the letter denotes subclasses that exhibit 70% or greater similarity (Tukey and Johnson, 1990).

New CYP enzymes emerged through gene duplication and divergence events. In mammals, exposure to planar aromatic compounds such as TCDD and PAHs induces the expression of two unique genes, CYP1A1 and CYP1A2. These genes are thought to have originated by a gene duplication event approximately 250 million years ago (Gonzalez and Nebert, 1990). The new CYP genes, being free of the selective pressure imposed on the original gene, diverged by mutation and evolved independently to perform a new function. This led to a high degree of inter-and intraspecies variability, and the emergence of P450s with broad substrate specificities. Genes derived from duplication and divergence events within a species are called paralogs, while genes in different species that share a common ancestor are

called orthologs. Occasionally genes from different species are observed to a have similar nucleotide sequence and code for proteins with similar function, but are not orthologs. For example, in both rodents and chickens, two cytochrome P450s are induced upon exposure to TCDD and PAHs: CYP1A1 and CYP1A2 in rodents, and CYP1A4 and CYP1A5 in chickens (Nakai *et al.* , 1991; Rifkind *et al.* , 1994; Gannon *et al.*, 2000). Although mammalian and chick CYP1A genes are similar in sequence and function, the chick enzymes form a separate branch in the CYP1A family tree distinct from mammalian CYP1A enzymes, and neither can be said to be directly orthologous (Gilday *et al.*, 1996). This provides an interesting example of convergent evolution, whereby two genes performing similar function appear to have developed independently, suggesting that the CYP1A genes performs an essential function in the organism.

The CYP supergene family is now estimated to consist of 74 gene families containing at least 60 and up to more than 500 individual genes (Eastbrook, 1996). The CYP genes are grouped into 11 gene families, six of which encode enzymes involved in steroid and bile acid synthesis. One encodes for P450 enzymes that hydroxylate fatty acids including prostaglandins and arachidonic acid. The mammalian xenobiotic metabolizing P450s have been classified in the first four gene families, and are designated CYP1, CYP2, CYP3, and CYP4. While the P450 enzymes involved in metabolism of endogenous substances are mostly constitutively expressed, the xenobiotic metabolizing P450s are preferentially expressed upon exposure to foreign substances.

Some of the CYP enzymes are substrate inducible; that is, the substance which induces the expression of a particular CYP enzyme, is also the substrate for that enzyme. This property allows organisms to adapt to changes in its chemical environment. Mammalian species exposed to planar aromatic hydrocarbons such as TCDD and PAHs respond with the induction of CYP enzymes in the CYP1A subfamily. The discovery of CYP1A stemmed from the observation that PAHs induce their own metabolism (an excellent P450 historical review is provided by Prough (1996) and references therein). Enzyme induction by TCDD and PAHs reflects the increased expression of the CYP1A gene and associated monooxygenase activity, initially referred to as aryl hydrocarbon hydroxylase (AHH) activity (Schmidt and Bradfield, 1996).

The fundamental breakthrough in the understanding of the regulation of the CYP enzymes was the elucidation of the genetic component in the induction process, culminating in the identification of a cytosolic protein capable of binding PAH and inducing the expression of the CYP1A gene. The observation that AHH inducibility varied significantly among inbred mouse strains exposed to the PAH 3-methylcholanthrene revealed that multiple alleles at a single locus controlled inducibility of AHH (Poland and Knutson,1982; Poland and Glover, 1977). This locus became known as the Ah locus, for aryl hydrocarbon responsiveness, and governs the biological responses to certain aromatic hydrocarbons. Analysis of the induction mechanism using genetic, biochemical and molecular biological techniques have demonstrated that some of the effects of PAHs are mediated by the Ah gene complex. One of the regulatory genes associated with this complex codes for a cytosolic receptor protein, called the Ah Receptor (AhR), which has a high affinity and saturable binding for several aryl and halogenated aryl hydrocarbons, such as PAHs and TCDD (Bigelow and Nebert, 1982;

Whitlock, 1990; Poland and Bradfield, 1992; Hankinson, 1995; Whitlock *et al.*, 1996; Ramana and Kohli, 1998; Whitlock , 1999). It is unclear whether the AhR has a role in normal metabolic processes, since no endogenous ligand to the AhR has yet been identified. Recently, however, Guigal *et al.* (2000) demonstrated that cultured cells treated fetal bovine serum responded with CYP1A gene expression by transcriptional activation independent of the AhR.

The proposed mechanism for the induction of CYP1A is shown in Figure 1.3. This model proposes that a lipophilic inducer (represented by TCDD) binds to the AhR. The cytosolic form of the receptor is maintained in a receptive form by heat shock protein (hsp90) and other cytosolic factors. After ligand binding, hsp90 is released and the complex appears in the nucleus by an unknown mechanism where it heterodimerizes with a related protein, Arnt (Aryl hydrocarbon nuclear translocator) generating a functional transcription factor. The AhR/Arnt heteromer interacts with specific *cis*-acting xenobiotic-responsive enhancers (XRE) located upstream of the CYP1A gene. An initiation complex forms at the promotor activating the transcription of the CYP1A gene, culminating in elevated levels of CYP1A mRNA, and increased levels of CYP1A protein (Whitlock, 1999). Both AhR and Arnt are members of a class of proteins representative of bHLH/PAS (basic helix-loop-helix Per-Arnt-Sim) transcription factors. Arnt is a general heterodimerization factor and has other partners in addition to AhR. Arnt regulates homeostatic responses, such as adaptation to chemicals, light, hypoxia, as well as cell and tissue differentiation (Taylor and Zhulin, 1999). Since Arnt participates in many homeostatic and regulatory processes, cross talk may exist between bHLH/PAS signaling systems. For example, exposure to TCDD may be influenced or

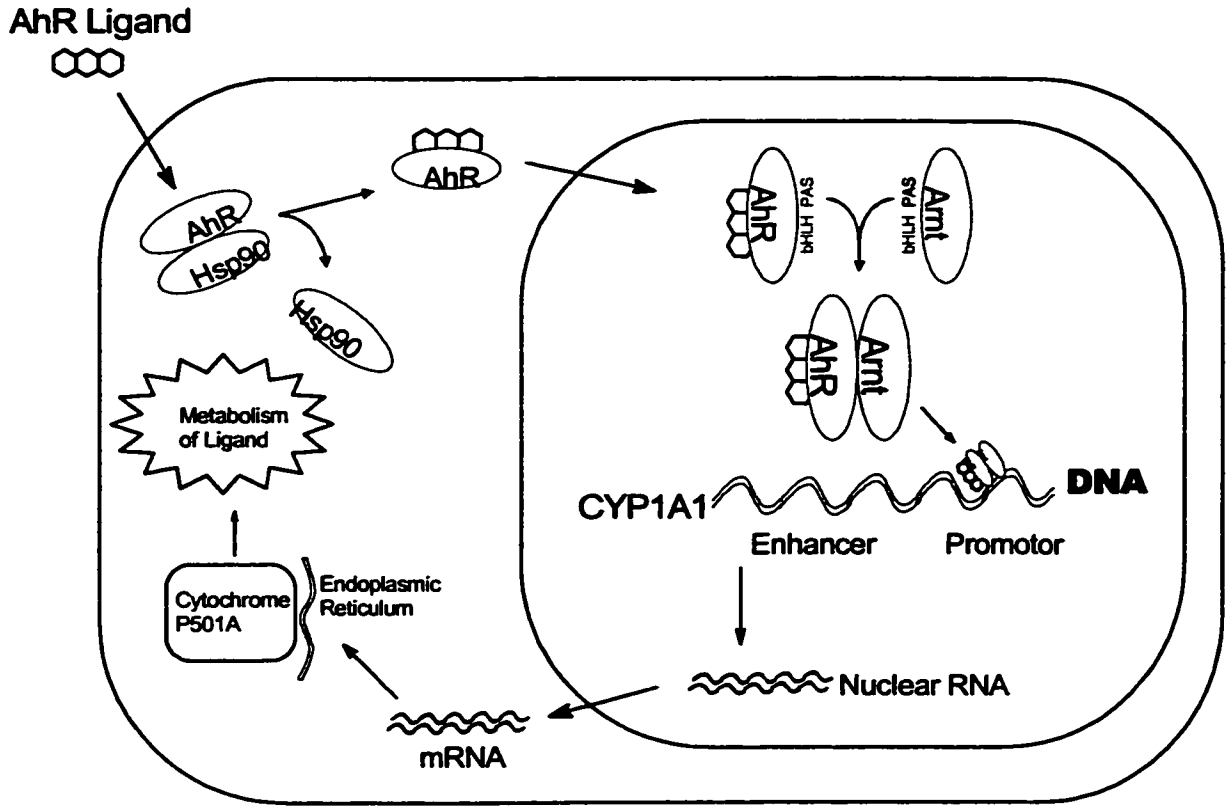

Figure 1.3. Proposed model for the induction of CYP1A by the prototypical AhR agonist TCDD. See text for details. Adapted from Whitlock (1999).

exacerbated by hypoxia (Ponting and Aravind, 1997; Rowlands and Gustafsson, 1997; Wilson and Safe, 1998; Taylor and Zhulin, 1999).

The CYP enzymes catalyze three general types of oxidative reactions: (1) insertion of an oxygen atom into the sigma bond between a hydrogen and a heavier atom (hydroxylation); (2) addition of a oxygen atom across a π-bond (epoxidation); and, (3) addition of an oxygen atom to a non-bonding electron pair (oxidation) (Mansuy, 1998). All CYP enzymes appear to operate by similar mechanisms that rely on the activation of molecular oxygen by a prosthetic heme iron atom. Some CYP enzymes are involved in the metabolism or biosynthesis of

endogenous physiological substrates while others are mainly involved in the metabolism of lipophilic xenobiotics (de Montellano and Correia, 1983). In the overall reaction, equimolar amounts of substrate, O_2, and NADPH are consumed and equimolar amounts of oxidized substrate, H_2O, and $NADP^+$ are formed:

$$P450\text{-}Fe^{III} = O_2 + 2NADPH + 2e^- \rightarrow P450^{\bullet+}(Fe^{IV}=O) + H_2O + 2NADP^+ \quad (1)$$

$$P450^{\bullet+}(Fe^{IV}=O) + X\text{-}H \rightarrow P450\text{-}Fe^{III} + X\text{-}OH \quad (2)$$

The oxygen atom incorporated into the hydroxyl group comes from O_2 rather than H_2O as shown by the use of ^{18}O-labeled O_2 and H_2O (Stryer, 1988). Hydroxylation requires the activation of oxygen and is accomplished by a specialized heme containing protein called cytochrome P450. However, the cytochrome P450 does not act in isolation. Cytochrome P450 is the terminal component of an electron transport chain in liver microsomes. NADPH transfers its high potential electrons to a flavoprotein, called NADPH-cytochrome P450 reductase, which in turn transfers an electron to the oxidized form of cytochrome P450 which then activates O_2 (Stryer, 1988). Other flavoproteins may also be involved in the metabolism of some xenobiotics. Cytochrome b5 and cytochrome b5 reductase may affect some cytochrome P450 mediated xenobiotic metabolism by shunting electrons towards or away from P450 (Morgan and Coon, 1984).

The reduction, oxidation, and hydrolysis reactions, collectively referred to as Phase I metabolic transformation, or biotransformation, are intended to convert lipophilic xenobiotic compounds into more hydrophilic, water soluble metabolites in order to (1) reduce the half life

of xenobiotics, (2) reduce the duration of the exposure to the xenobiotic, (3) avoid accumulation of the parent xenobiotics in the organism, (4) change the biological activity of the xenobiotics, and (5) change the duration of the biological activity of the xenobiotics (Vermeulen, 1996). The Phase I reactions are catalyzed by the cytochrome P450s producing hydroxlyation of aromatic rings or alkyl chains, perform oxidative dealkylation, and N-oxidation. These enzymes exist in multiple forms exhibiting overlapping substrate specificity. Phase II transformations are generally conjugation reactions of the parent xenobiotic or of the phase I metabolite with glucuronic acid, glutathione, glycine, inorganic sulphate, or amino acids. Conjugation reactions facilitate transport and enhances elimination through renal and biliary routes (Vermeulen, 1996).

1.7. CYP1A induction: EROD activity

Upon exposure to certain xenobiotic substances, an organism will respond with the up- or down-regulation of a variety of genes or gene batteries as a mechanism to maintain homeostasis in response to certain types of stress or toxic insult. For chemicals that bioaccumulate, such as the dioxins and PCBs, chemical analysis of body burdens is often used to characterize exposure. Although chemical analysis is capable of measuring a wide range of chemical contaminants with high selectivity and sensitivity, the complex mixtures of environmental contaminants are difficult or impossible to characterize. Even if a compound is found in an organism, it's relevance to the physiology of the organism is difficult to determine in the absence of a thorough understanding of the toxicology of the compound. Furthermore, since the relationship between tissue concentration and toxic effects are poorly

understood, chemical analysis alone may not adequately reflect the effective biological impact of the contaminants to which an organism has been exposed.

Since subcellular biochemical and molecular systems are usually the first to experience alterations upon contaminant exposure, they are more sensitive indicators of exposure than higher levels of organization. Upon exposure to chemical stressors, an organism will typically respond with the induction of a suite of stress proteins intended to minimize or eliminate the stress and re-establish homeostasis. Thus, biochemical disturbances are indicative that exposure has occurred. This has led to the development of a suite of biochemical and physiological assays, or biomarkers, that allow the organism itself to provide information as to whether or not exposure has occurred (Bucheli and Fent, 1995). Since alterations in the biochemical and physiological systems usually precedes the onset of other more serious effects at the organism, population or community levels, biomarkers can be used as a sentinel or early warning indicator for certain classes of toxic environmental contaminants.

Unlike chemical analysis, which represents only a snapshot in time and space, biomarkers integrate the cumulative, synergistic, and antagonistic effect of all the compounds in environmental contaminants on the exposed organism. Biomarkers are especially useful for providing evidence of exposure to compounds that are easily metabolized (i.e., do not bioaccumulate, e.g., PAHs) since the toxic action of the parent compound and its metabolites is taken into consideration. In its broadest sense a biomarker can be defined as: *a measurable response at any level of biological organization that can be related to an impact of contaminants*. For biomarkers at the biochemical level it may be refined as: *a toxicant-induced*

change in gene expression leading to alteration of protein content and enzyme activity that is linked to the amount of the contaminant (Bucheli and Fent, 1995). At present, cytochrome P450 induction, thyroid hormone and vitamin A levels have been shown to be suitable biomarkers for PCDD, PCDF, coplanar PCBs, and PAH exposure.

The biochemical biomarker best studied and characterized so far is the induction of the cytochrome P450 monooxygenases. These enzymes metabolize, or biotransform, endogenous and exogenous hydrophobic substances to more polar metabolites, making them more water soluble, less toxic, and more easy to eliminate. The induction of specific cytochrome P450 isoforms is a sensitive response of exposure of an organism to certain xenobiotics. Exposure to aromatic hydrocarbons such as PAHs and TCDD and other compounds that are ligands to the AhR results in the induction of cytochrome P4501A (CYP1A). Several methods for measuring the extent of induction of CYP1A have been developed and include Western blots, and enzyme linked immunoabsorbant assay (ELISA). However, the most commonly used method for measuring CYP1A induction, due to its ease and cost, is the ethoxyresorufin-*O*-deethylase (EROD) assay.

The induction of CYP1A can be quantitated by fluorimetric measurement of ethoxyresorufin-*O*-deethylase (EROD) activity. Ethoxyresorufin is a surrogate substrate for CYP1A, which when deethylated fluoresces in a discrete narrow band, free of fluorescent contamination from NADPH and proteins. The EROD assay measures the catalytic activity of the CYP1A enzyme by measuring the rate at which the non-fluorescent substrate ethoxyresorufin is deethylated to the fluorescent product, resorufin. Estimates of potency are

measured as efficacy (the maximal activity induced by a compound), and the concentration of inducer required to produce a half maximal response (median effective concentration, EC50). It is important to note that the induction of CYP1A and resultant EROD activity is not in itself a toxic response, but a biochemical response associated with some of the toxic effects of TCDD and other structurally related aromatic hydrocarbons. Since EROD induction is strongly correlated with the toxic (embryolethal) effects of PAHs (i.e., median lethal dose or LD50; Brunström *et al.* , 1991), the EROD assay provides a powerful tool for the estimation of the embryotoxicity of individual PAHs, as well as the toxicity of PAH mixtures extracted from environmental samples (e.g., sediments, eggs, and zebra mussels, etc.) (Brunström *et al.*, 1992).

Previous *in ovo* toxicity studies by Brunström *et al.* (1991) provided the essential *in vivo* data demonstrating the correlation between embryotoxicity (LD50) and EROD-inducing values for several PAHs. However, due to the expense and the large numbers of eggs required for *in ovo* toxicity testing, there has been considerable interest in developing a faster and more economical alternative to egg injection studies. The EROD assay initially developed by Burke *et al.* (1977), and Pohl and Fouts (1980) was modified by Kennedy *et al.* (1993) to allow the assay to be performed *in vitro* using primary cultures of chicken embryo hepatocytes. The *in vitro* EROD assay has several advantages over the egg injection method: (1) since this bioassay is easier and more efficient, large amounts of data were obtainable in a short period of time, with minimal expense; (2) fewer eggs are required to provide comprehensive concentration-response curves since one embryo will provide enough hepatocytes to prepare two 48-well plates in which 24 concentrations (in triplicate) can be tested - this is important when a limited

number of eggs are available; (3) higher doses are possible in the bioassay since embryonic lethality occurring during exposure to high doses is eliminated.

Although CYP1A induction is the most common biochemical endpoint induced upon exposure to HAHs, deregulation of the heme biosynthetic pathway is commonly associated with HAH exposure. While all cells appear to have the ability to synthesize heme (a complex of iron and protoporphyrin IX) for mitochondrial cytochrome synthesis, the liver is the major site of heme synthesis. The liver requires heme for the synthesis of a variety of hemoproteins, particularly the cytochrome P450s (Marks, 1985). The heme biosynthetic pathway involves the carefully regulated action of a series of enzymes, and under normal conditions only small amounts of porphyrins other than protoporphyrin IX are produced. The enzymes of the heme biosynthetic pathway are shown in Figure 1.4. If the regulatory process is disrupted, a variety of porphyrin precursors may accumulate. The pattern of chemically induced porphyrin accumulation is often characteristic of exposure to a particular chemical or class of chemicals (Marks *et al.*, 1985, 1989). HAHs are known to cause a wide spectrum of species-specific toxic and biochemical effects, including CYP1A gene expression and porphyrin accumulation. In chicks, the most toxic HAHs cause massive increases in hepatic concentrations of uroporphyrin (URO) and heptacarboxylporphyrin, inducing disorder similar to an inheritable form of porphyria called porphyria cutanea tarda (Franklin *et al.*, 1997; Scarlett and Brenner, 1998). The accumulation of URO is caused by the oxidation of uroporphyrinogen, and by the inhibition of uroporphyrinogen decarboxylase (likely an intermediate of uroporphyrinogen oxidation) (Elder and Roberts, 1995). In rodents, the CYP1A2 isoform catalyzes the

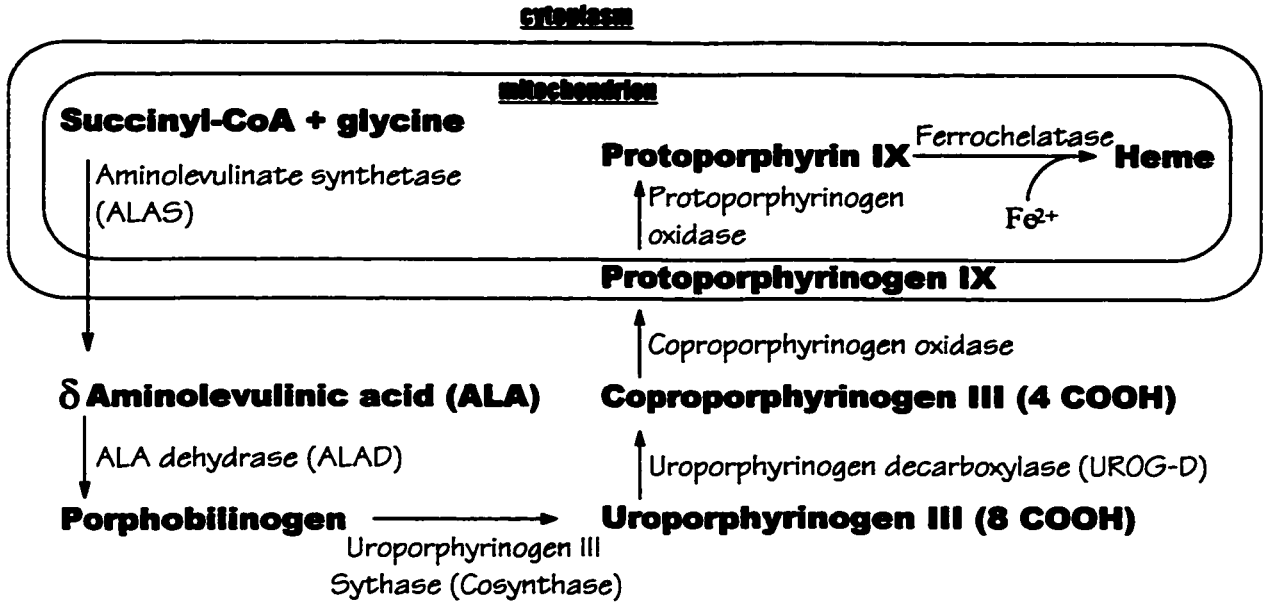

Figure 1.4. Localization of the enzymes of the heme biosynthetic pathway. The enzyme ALA synthase is the rate-limiting enzyme for hepatic heme synthesis. Coproporphyrinogen oxidase catalyzes the oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen IX. This reaction occurs between the inner and outer mitochondrial membranes. In the final step, protoporphyrinogen oxidase catalyzes the conversion of protoporphyrinogen IX to protoporphyrin IX. TCDD and other structurally related halogenated aromatic hydrocarbons cause hepatic uroporphyria by oxidative inhibition of hepatic uroporphyrinogen decarboxylase (UROG-D) activity. Two related human porphyrias, porphyria cutanea tarda and hepatoerythropoietic porphyria, also result from deficiency of this enzyme.

uroporphyrinogen oxidation, while in chick uroporphyrinogen oxidation is catalyzed by CYP1A5 (Sinclair, 1997a). Chicken embryo hepatocytes are thus an ideal model for the analysis of the toxic potency of compounds or environmental samples that have affinity for the AhR, since in addition to responding with the induction of CYP1A protein upon exposure to HAHs, they are sensitive to the disruption of the heme biosynthetic pathway, resulting in the intracellular accumulation of porphyrins.

1.8. Aims of the present study

The goal of the present study was to investigate the potency of 18 PAHs to induce EROD activity and porphyrin accumulation in White Leghorn chicken, Pekin duck, and Greater Scaup embryo hepatocytes. Brunström *et al.* (1991) examined the embryotoxicity and hepatic

EROD-inducing potency of several PAHs in several avian species. In general, the most toxic (embryolethal) PAHs were also the most potent inducers of EROD. The embryolethality and EROD-inducing potency of 24 PAHs was investigated by injecting the PAHs into the air sac of developing chicken embryos. In general, the rank order of toxicity was similar to their EROD-inducing potency. Brunström *et al.* (1992) also investigated the embryotoxic potency BkF, the most embryotoxic PAH tested, in 4 day old chicken, turkey, domestic duck, and eider embryos. The rank order of species sensitivity to BkF was duck > turkey > chicken > eider. Thus, although ducks are much less sensitive to the embryotoxic effects of TCDD and coplanar PCBs than chicken embryos, they are more sensitive to the embryotoxic effects of PAHs than chicken embryos. Thus it was hypothesized that Greater Scaup embryos may likewise be sensitive to the embryotoxic effects of PAHs.

Our goal was to determine if the *in ovo* lethality of PAHs in chick embryos could be predicted from their potency to induce EROD activity in primary cultures of chicken embryo hepatocytes. If it could be established that *in vitro* EROD induction in chicken embryo hepatocytes can be used to predict *in ovo* lethal potency, it might be possible to predict the *in ovo* lethalities of PAHs in Pekin duck or Greater Scaup embryos from the potency of PAH to induce EROD activity in cultures of hepatocytes in the respective species.

In Chapter 3, 18 PAHs were evaluated for their ability to induce EROD activity and to cause porphyrin accumulation in primary cultures of chicken embryo hepatocytes (CEH). Previous studies by Brunström *et al.* (1991) demonstrated that the embryotoxicity of PAH in developing chicken embryos correlate well with in ovo EROD-inducing potencies. I

hypothesize that the EROD-inducing potency of PAHs obtained with the *in vitro* EROD bioassay with CEH cells may be useful for predicting the in ovo toxic potency of PAH that have affinity for the AhR.

In Chapter 4, 18 PAHs were evaluated for their EROD-inducing potency in primary cultures of White Leghorn chicken, Pekin duck, and Greater Scaup embryo hepatocytes. While chick embryos are very sensitive to the toxic effects of TCDD and coplanar PCBs, turkey and domestic duck embryos are much less sensitive to the toxic effects of these compounds. In contrast, the domestic duck is much more sensitive to the embryotoxic effects of PAHs than chicken. My hypothesis is that the *in vitro* EROD-inducing potencies of PAHs in primary cultures of Pekin duck and Greater Scaup hepatocytes is predictive of the embryotoxic potency of PAHs in Pekin Duck and Greater Scaup embryos, respectively.

The EROD-inducing potency of non-polar extracts prepared from zebra mussels collected from three locations in the Great Lakes were also determined. The extracts each contained the equivalent of 10 g of mussel tissue. My hypothesis is that zebra mussel extracts contain sufficient PAHs (or PAH derivatives, and heterocyclic aromatic hydrocarbons) to induce EROD activity in hepatocytes.

Chapter 2. Materials and Methods

2.1. Chemicals

TCDD was a gift from Dr. J. Ryan (Health Canada, Ottawa, Ontario). Fifteen PAHs, two heterocyclic aromatic hydrocarbons, and one substituted PAH were studied (Figure 1.1). Anthracene (Ant), benz[a]anthracene (BaA), benzo[a]pyrene (BaP), benzo[e]pyrene (BeP), benz[ghi]perylene (BghiP), chrysene (Chr), dibenz[ah]anthracene (DahA), fluoranthene (Fln), fluorene (Flu), phenanthrene (Phn), perylene (Per), and pyrene (Pyr) were from Sigma Chemical Co. (St. Louis, MO); 1-methylpyrene (MP) and benzo[k]fluoranthene (BkF) were from Fluka Chemical Co. (Milwaukee, WI, USA); coronene (Cor) and dibenzothiophene (DBT) were from Aldrich Chemical Co. (Milwaukee, WI, USA); indeno[1,2,3-cd]pyrene (Ind) was from Supelco (Mississauga, ON, Canada), and benzo[b]naphtho[2,3-d]thiophene (BNT) was from EQ Laboratories (Atlanta, GA, USA). All compounds were stated to be at least 99% pure by the supplier. Solutions and serial dilutions of PAHs were prepared in dimethyl sulphoxide (DMSO). Sample dilution calculation is shown in Appendix 1. Waymouth's medium was from GIBCO Canada, Inc. (Burlington, ON, Canada). Water (ddH_2O) was treated with a Milli-RO/Milli-Q purification system from Millipore Ltd. (Mississauga, ON, Canada). Collagenase, resorufin, ethoxyresorufin, NADPH, fluorescamine, bovine serum albumin (BSA), penicillin G (sodium salt), streptomycin sulfate, L-thyroxine, and insulin were from Sigma Chemical Co. (St. Louis, MO, USA). DNAse was from Boehringer Manheim (Indianapolis, IN, USA), and Percoll was from Pharmacia Biotech AB (Uppsala, Sweden). Porphyrin standards were from Porphyrin Products (Logan, UT, USA). HPLC-grade acetonitrile was from Caledon Laboratories (Georgetown, ON, Canada). All other chemicals

were reagent grade, and were obtained from Fisher Scientific (Nepean, ON, Canada). A more detailed list is presented in Appendix 2.

2.2. Sources of eggs and incubation conditions

Fertile unincubated White Leghorn chicken eggs (*Gallus domesticus*) were obtained from Agriculture Canada (Ottawa, Ontario, Canada) and were artificially incubated for 19 days in a Petersime Model X1 incubator (Gettysburg, OH) set to 37°C and 60% relative humidity, and were turned automatically every hour. Fertile unincubated Pekin duck eggs were obtained from a local commercial supplier (Les Enterprise Simetin Inc., Mirabel, PQ), and were artificially incubated for 26 days in a Curfew Model RX250 incubator at 37°C and 80% relative humidity. Each afternoon, the incubator was left open for 10 to 15 min, and the eggs sprayed with a mist of ddH_2O (this daily cooling and misting mimics natural conditions where the female leaves the nest for short periods and returns with water in her brood patch causing an increase in nest humidity). Mr. Glen Fox (Canadian Wildlife Service, Hull, Quebec) and Mr. Michael Fournier (Canadian Wildlife Service, Yellowknife, NWT) coordinated the collection of pre-incubated Greater Scaup eggs (the first week of embryological development is critical and eggs should be naturally incubated during this period) from breeding islands in Yellowknife Bay (Yellowknife, NWT, Canada). Greater Scaup eggs were transported in an insulated container to the National Wildlife Research Centre (transportation was a kind gift of Canadian Airlines International), where they were artificially incubated in a Curfew Model RX250 incubator at 37°C and 80% relative humidity. Each afternoon, the incubator door was left open for 10 to 15 min, as described above. Hatching success was about 80%. Since the Scaup eggs were naturally pre-incubated and precise age was unknown (age at collection was

estimated by flotation, and ranged from 10 to 18 days), embryos were sacrificed at pipping. Eggs of each species were candled periodically and sterile eggs and dead embryos were removed.

2.3. Preparation and dosing of hepatocyte cultures

Primary cultures of avian embryo hepatocytes were prepared as previously described (Kennedy *et al.*, 1993, 1995). Briefly, embryos were killed by decapitation and the abdominal cavity opened. The liver was removed, and the gallbladder and fatty tissue carefully separated and discarded. The livers were rinsed twice in Krebs Ringers Buffer (KRB - preparation of solutions and cell culture medium is detailed in Appendix 4) then thoroughly minced. Minced tissue was transferred to a sterile Erlenmeyer flask containing 75 ml of warm collagenase solution (0.5 mg/ml for chicken tissue or 1.0 mg/ml for Pekin duck and Greater Scaup tissue). Chick tissue was digested for 45 min with shaking at 37°C, while Pekin duck and Greater Scaup tissue were digested for a total of 40 min with two intermediate harvests. The cell suspension was then filtered through a nylon screen filtration apparatus (200, 100, 50, 25 μm mesh size) to separate hepatocytes from undigested tissue, and collected into 100 ml of 20 mg/ml BSA solution to dilute collagenase activity. The cell suspension was centrifuged at 300x g for 5 min, the pellet resuspended in percoll\sucrose solution, and centrifuged at 50 x g for 10 min to separate hepatocytes from other cell types. The upper cell layer (hepatocytes) was resuspended in a 0.02 mg/ml DNAse/KRB solution to dissolve tissue clumps, then washed twice in Waymouth's medium. Cells were weighed and resuspended in medium such that addition of 25 μl of cell suspension to each well resulted in a protein concentration of 60 μg/well. Cells were added to each of 36 wells of a 48 well tissue culture plate (Falcon, #3078),

the remaining 12 wells were used for standards. Hepatocytes were cultured in Waymouth's medium (0.5 ml/well) supplemented with L-thyroxine (1 μg/ml) and insulin (1 μg/ml). Appendix 5 outlines the cell culture protocol in more detail. After incubation for 24 hours at 37°C in a 95% air/5% CO_2 atmosphere, DMSO solutions (2.5 μl/well) of TCDD or PAHs were added to 30 of the 36 wells with hepatocytes, and DMSO alone to the other six wells according to the schedule in Appendix 6. Cells were incubated for a further 24 hours, the medium was discarded, the cells washed with phosphate buffered saline and then flash frozen on dry ice and stored at -80°C until analysed.

2.4. EROD and total protein assays

EROD, total protein and porphyrin assays were carried out in the 48 well plates as previously described (Kennedy *et al.*, 1995). EROD induction was measured as an increase in the reaction product (resorufin) with a fluorescence plate reader (Cytofluor 2300, Millipore Ltd.). Ethoxyresorufin in sodium phosphate buffer was added to wells containing hepatocytes; the remaining 12 wells were used for standards. Plates were preincubated at 37°C for 15 min, and the reaction started by the addition of NADPH. The reaction was stopped after 7 min by the addition of cold acetonitrile containing 150 μg/ml fluorescamine. Resorufin was measured with 530 nm excitation and 590 emission filters, and total protein was measured with 400 nm excitation and 460 nm emission filters. The EROD assay is described in more detail in Appendix 7. Data were imported into Sigma Plot for analysis and curve fitting.

2.5. Total porphyrin analysis

Total porphyrin induction was measured in the same plate using methods described previously (Kennedy and Jones, 1994; Kennedy *et al.*, 1995). Following analysis of resorufin and total protein, uroporphyrin standard solutions (µl/well) were added to the wells that contained resorufin and BSA standards, and 400 µl of 3 N HCl was added to the wells containing hepatocytes. Since the protein and porphyrin fluorophors have similar excitation spectra, and some overlap of the emission spectra, it is necessary to destroy the protein fluorophors prior to porphyrin analysis. This was accomplished by the addition of 400 µl of 3 N HCl to each of the wells containing hepatocytes, and allowing the plate to sit at room temperature for 60 min. Porphyrins were measured with 400 nm excitation and 645 nm emission filters. Fluorescence data were imported into Sigma Plot for curve fitting. Appendix 7 provides more detail of the assay protocols.

2.6. Porphyrin pattern analysis

Porphyrins patterns were analysed following methods of Kennedy *et al.* (1995). Briefly, after porphyrin analysis with the fluorescence plate reader and analysis with Sigma Plot, the solutions from the set of three wells resulting in the strongest fluorescence signal were pooled, diluted to 50 ml with ddH_2O, and concentrated with Sep-Paks (Water Inc., Mississauga, Ontario, Canada). Sep-Paks were pretreated with 10 ml acetonitrile followed by 15 ml ddH_2O. Porphyrins were eluted with 2.5 ml acetonitrile into glass tubes, and evaporated to dryness under nitrogen. Dried samples were stored at -20°C until analysis on HPLC. Dried samples were prepared for HPLC analysis by addition of 100 µl concentrated HCl to each tube, vortexed for 10 seconds and allowed to stand for 5 min. Water (900 µl) was added to each

tube, shaken by vortex for 10 seconds, and sonicated in an ultrasonicator (Branson #42; Branson Cleaning Equipment Co., Shelton, Conn., U.S.A.) for 5 min. Solutions were filtered through 0.2 μm micro-syringe filters prior to injection onto the HPLC column. Samples were analysed with a Varian 9000 HPLC as described previously (Kennedy and James, 1993).

2.7. Preparation of Zebra Mussel Extracts

Extracts from zebra mussels were prepared using minor modifications of methods used in our laboratory for chemical residue analysis. Zebra mussels were obtained from three locations within the Great Lakes where Greater Scaup are known to feed on zebra mussels: (i) from soft sediments in the Detroit River; (ii) from the Western Basin of Lake Erie, near Monroe Michigan; and, (iii) from Lake Ontario, near Sister Island. In brief, 10 g of mussels from each of three locations in the Great Lakes were shucked, homogenized, dried with anhydrous sodium sulfate, extracted with dichloromethane (DCM)/hexane (1:1), and cleaned up by gel permeation chromatography. Extracts were quantitatively transferred from DCM/hexane to 200 μl DMSO. The DMSO solutions were mixed and dilutions were prepared in DMSO. Details of the extraction and clean up protocol are presented in Appendix 3.

2.8. Data processing

2.8.1. EROD dose-response curves

Data obtained from the fluorescence plate reader were imported into Sigma Plot (Jandel Scientific, V. 3.02) and a modified Gaussian curve was fitted to the data:

$$y(d) = Y_b + (Y_m - Y_b)\exp\{-C[\ln(d) - \ln(d_m)]^2\}, \text{ where}$$

Equation 1

$$C = \frac{\ln(2)}{[\ln(EC_{50}) - \ln(d_m)]^2}$$

$y(d)$ = EROD activity at inducer dose 'd'
Y_b = basal EROD activity
Y_m = maximal EROD activity
d_m = dose where EROD activity is m maximal
EC50 = lower doses where EROD activity is half way between maximal and basal activity

2.8.2. Porphyrins curves

Porphyrin data obtained from the plate reader were imported into Sigma Plot and fitted to a logistic curve:

$$Y(d) = Y_b + (Y_m - Y_b)\{1 + \exp[-g(\ln(d) - \ln(EC_{50}))]\}^{-1}, \quad \text{Equation 2}$$

Where:

$y(d)$ = porphyrin concentration at inducer concentration d
Y_b = basal porphyrin concentration
Y_m = maximal porphyrin concentration
g = a slope parameter
EC50 = the dose at which porphyrin concentration is one-half maximal

2.8.3. ECthreshold (ECthr) calculation

The EC50 is a measure of the potency of a compound to induce CYP1A (e.g., the concentration of compound required to elicit a half maximal response), while the maximal EROD activity is a measure of the efficacy of the compound (e.g., the amount of CYP enzyme the compound induces). However, since several of the PAHs in this study behaved as partial agonists (substances eliciting, at very high concentrations, a lower maximal response than a defined standard (e.g., TCDD), are defined as partial agonists; Kenakin, 1993; Pliska, 1999),

and exhibited non-ideal concentration-response curves (see Results), comparisons of the relative potencies on an EC50 basis may overestimate the potency of a compound (Rovati, 1994; Hahn, 1996; Pliska, 1999). Therefore, we provide an alternate method based on the response at lower concentrations, where inhibition of catalytic activity by residual inducer (or other mechanisms) is minimal, for the estimation of potency. This method, referred to as the threshold dose (ECthr), is calculated as the concentration of a compound required to produce a response equivalent to 10% of the maximal response induced by TCDD in the same species. Appendix 8 provides details of the derivation of the equation.

CHAPTER 3: ETHOXYRESORUFIN-*O*-DEETHYLASE (EROD) INDUCING POTENCY OF 18 POLYCYCLIC AROMATIC HYDROCARBONS (PAHs) IN PRIMARY CULTURES OF CHICKEN (*Gallus domesticus*) EMBRYO HEPATOCYTES

3.1. Abstract

The concentration-dependent effects of 18 polycyclic aromatic hydrocarbons (PAHs) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on cytochrome P4501A (CYP1A) induction in primary cultures of chicken embryo hepatocytes (CEH) were evaluated. CYP1A activity was estimated by measuring ethoxyresorufin-*O*-deethylase (EROD) activity. The PAHs studied were: 1-methylpyrene (MP), anthracene (Ant), benz[a]anthracene (BaA), benzo[a]pyrene (BaP), benzo[b]naphtho-[2,3-d]thiophene (BNT), benzo[e]pyrene (BeP), benz[ghi]perylene (BghiP), benzo[k]fluoranthene (BkF), chrysene (Chr), coronene (Cor), dibenz[ah]anthracene (DahA), dibezothiophene (DBT), fluoranthene (Fln), fluorene (Flu), indeno[1,2,3-cd]pyrene (Ind), phenanthrene (Phn), perylene (Per), and pyrene (Pyr). Of the 18 PAHs examined, only eight induced EROD activity. The most potent PAH, BkF, was almost three orders of magnitude less potent TCDD. Two potency estimates were compared: the concentration of inducer at which EROD activity was 50% of maximal (EC50); and, the concentration of PAH that induced a response equivalent to 10% of the maximal response of TCDD (ECthreshold, ECthr). For both methods, the rank order of potency for the eight EROD inducing PAHs, from most potent to least potent, was: BkF ≥ DahA > Ind > BaA ≥ BghiP ≥ Chr ≥ BaP > BNT. A plot of the ECthr versus LD50 (data from Brunström *et al.*, 1991) had an r^2 of 0.898, suggesting that the ECthr may be useful for predicting the *in ovo* toxic potency of PAHs. Each PAH was tested to determine if it caused porphyrin accumulation in CEH cultures. High performance liquid chromatography (HPLC) analysis indicated that porphyrin accumulation did not occur upon exposure of cells to PAHs. Regardless of the lack of porphyrinogenic effects, the EROD data suggests that primary cultures of chicken embryo hepatocytes may be a useful model for predicting the *in ovo* toxic potencies of PAHs in avian species.

3.2. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental contaminants formed as byproducts of the incomplete combustion of organic materials. Major anthropogenic

sources of PAHs include combustion of fossil fuels, spillage of petroleum, metallurgical and coking plants, and leaching from creosote treated products. As a class of chemical compounds, PAHs comprise about 100 individual compounds, several of which are well known carcinogens/mutagens. Biotransformation of PAHs by the cytochrome P450 enzyme system results in the generation of chemically reactive electrophilic diol-epoxide intermediates (Levin *et al.*, 1982; Jerina and Daly, 1974; Kim *et al.*, 1997; Wells *et al.*, 1997) that covalently bind to critical cellular components (e.g., DNA, proteins). Laboratory studies with various species have demonstrated that PAHs induce a variety of species specific toxic effects, including developmental abnormalities (Brunström *et al.*, 1991), immune system suppression (Silkworth *et al.*, 1995; Santodonato, 1997), reproductive effects (MacKenzie and Angevine, 1981), liver toxicity (Mumtaz *et al.*, 1996), tumor initiation and promotion (Dipple *et al.*, 1999; Ross and Nesnow, 1999), as well as inducing the expression of numerous genes, including the cytochrome P4501A (CYP1A) gene battery (Poland and Knutson, 1982; Poland and Bradield, 1992; Whitlock, 1999).

The induction of CYP1A is a well characterized response of vertebrate animals to PAHs and planar halogenated aromatic hydrocarbons (HAHs) such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). CYP1A is regarded as one of the most sensitive biomarkers of exposure to HAHs (Bucheli and Fent, 1995). Although the role of CYP1A in HAH toxicity is not yet clear, both the toxic and biochemical responses, such as CYP1A induction, appear to be mediated by an initial binding to the aryl hydrocarbon receptor (AhR). While it is not clear whether PAHs and HAHs exert their toxicity via similar mechanisms, many PAHs are known ligands of the AhR (Bigelow and Nebert, 1982; Piskorska-Pliszczynska *et al.*, 1986), and

several of these are highly toxic to developing chick embryos, with the most toxic being potent CYP1A inducers (Brunström, 1991, 1992; Brunström *et al.*, 1991).

CYP1A activity develops early in chick embryos (Powis *et al.*, 1976; Bosveld *et al.*, 1997) and by one week prior to hatching heme synthesis and CYP1A activities are close to adult levels (Hamilton *et al.*, 1983; Heinrich-Hirsch *et al.*, 1990). Administration of TCDD to developing chick embryos results in the production of two CYP1A isoforms. The chick CYP1A isoforms are not strictly orthologous to mammalian CYP1A1 and CYP1A2 and have been classified as CYP1A4 and CYP1A5 (Gannon *et al.*, 2000; Mahajan and Rifkind, 1999; Gorman *et al.*, 1998; Nakai, 1991). In chick, the CYP1A4 isoform is the main catalyst for ethoxyresorufin-*O*-deethylase (EROD) (Rifkind *et al.*, 1994), while the CYP1A5 isoform is responsible for uroporphyrin oxidation associated with porphyrin accumulation (Sinclair *et al.*, 1997a). The chicken and the chick embryo are among the most sensitive species to the toxic effects of TCDD and structurally related halogenated aromatic hydrocarbons (HAHs) (Brunström, 1989, 1991).

Since *in vivo* toxicity testing is difficult due to the number of birds or eggs required, there has been considerable interest in using cell culture bioassays to measure the CYP1A inducing potency of AhR agonists. The most common biochemical endpoint used to determine CYP1A induction is the EROD reaction, which provides a measure of the catalytic activity of the CYP1A enzyme (Kennedy *et al.*, 1993; Kennedy *et al.*, 1995). Strong correlations between potency to induce EROD activity and toxic potency have been shown for HAHs (Safe, 1993) and PAHs. Brunström *et al.* (1991; 1992) investigated the *in ovo* toxicity of 24 PAHs on 7 day

old chick embryos. The rank order of toxicity (LD50) of the four most toxic PAHs was similar to their EROD-inducing potency. Thus, the AhR binding affinities of PAHs correlate well with their *in vitro* EROD induction potencies, and their toxic potencies *in ovo*.

Although CYP1A induction is the most common biochemical endpoint induced upon exposure to HAHs, de-regulation of the heme biosynthetic pathway is commonly associated with HAH exposure. If the regulatory process is disrupted, a variety of porphyrin precursors may accumulate. The pattern of chemically-induced porphyrin accumulation is often characteristic of exposure to a particular chemical or class of chemicals (Marks *et al.*, 1989). In chicks, the most toxic HAHs cause massive increases in hepatic concentrations of uroporphyrin and heptacarboxylporphyrin, inducing a disorder similar to an inheritable form of porphyria called porphyria cutanea tarda (Scarlett and Brenner, 1998; Franklin *et al.*, 1997). Recent studies with mice indicate that CYP1A2 is essential for the accumulation of uroporphyrin; contributions by other CYP forms appear to be minimal (Sinclair *et al.*, 2000). In chick, pheasant, duck, and herring gull hepatocytes, uroporphyrin was the main porphyrin to accumulate in response to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD); heptacarboxyl porphyrin predominated in turkey hepatocytes (De Matteis and Marks, 1996). In rodents, the CYP1A2 isoform catalyzes uroporphyrinogen oxidation, while in chick uroporphyrinogen oxidation is catalyzed by CYP1A5 (Sinclair, 1997a). Chicken embryo hepatocytes are thus an ideal model for the evaluation of the toxic potency of compounds or environmental samples that have affinity for the AhR, since in addition to responding with the induction of CYP1A protein, they are sensitive to the disruption of the heme biosynthetic pathway, resulting in the intracellular accumulation of porphyrins.

The present study was undertaken to extend our previous work with HAHs to PAHs. Specific goals were to: (1) determine the dose-dependent effects of 18 PAHs on EROD activity in CEH cultures; (2) assess the potential of using EROD inducing potencies of PAHs in CEH cultures for predicting the toxic potency of PAHs , *in ovo;* and (3) determine if PAHs cause porphyrin accumulation in CEH cultures.

3.3. Results

3.3.1. Characteristics of the Concentration-Response Curves

Of the 18 PAHs investigated, only eight induced EROD activity in CEH cells. Representative concentration-response curves for TCDD and the eight EROD inducing PAHs are shown in Figure 3.1. TCDD induced a biphasic concentration-response curve characterized by increasing EROD activity at low concentrations, rising to a plateau of maximal activity followed by a decrease in EROD activity at higher concentrations. With concentration plotted on a logarithmic scale, the data were fit to a Gaussian curve. Similar Gaussian curves have been described for TCDD in CEH culture (Kennedy *et al.*, 1996a, 1996b), PLHC-1 (Hestermann *et al.*, 2000; Hahn *et al.*, 1996), H4IIE rat hepatoma cells (Willett *et al.*, 1997) and RTL-W1 trout liver cells (Bols *et al.*, 1999). The concentration-response curves of the eight EROD-inducing PAHs exhibited a biphasic shape similar to TCDD, and were also described by a Gaussian equation. The PAH curves were shifted up to three orders of magnitude to the right with respect to TCDD. Consequently, the EC50s (defined as the concentration of inducer that produces a half maximal response; see section 2.8.1 for calculation) of the PAHs were higher than the EC50 of TCDD. In addition to possessing higher EC50s, the PAHs also

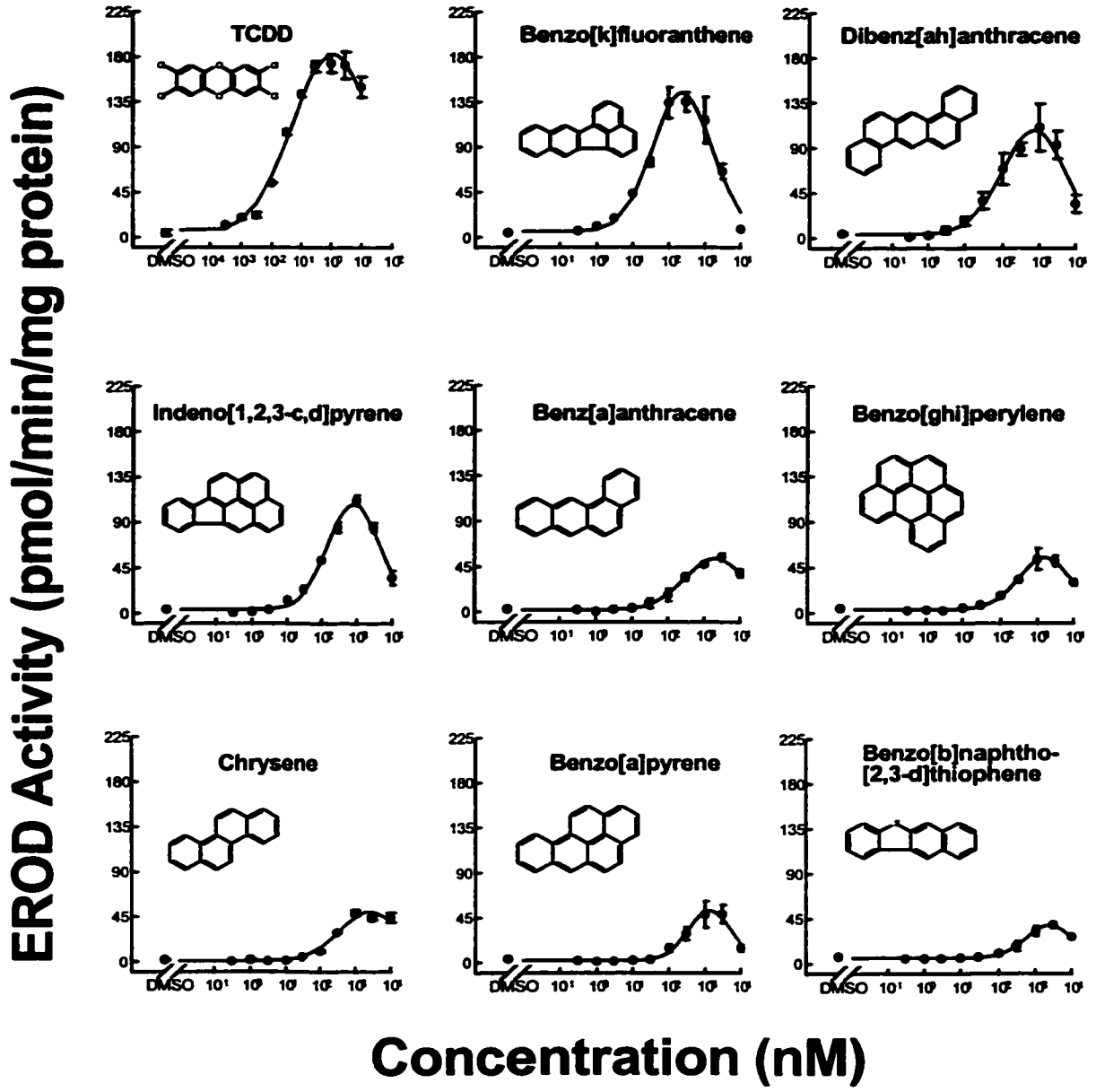

Figure 3.1. Concentration dependent effect of TCDD and PAHs on EROD activity in primary hepatocytes prepared from 19 day old White Leghorn chicken embryos. Cells were exposed for 24 hours, after which they were rinsed, and frozen until analysed for EROD activity as described in the materials and methods section. Each concentration-response curve was obtained from a single 48-well plate. Points represent the mean of triplicates of EROD activity of each concentration and bars represent standard error.

exhibited lower maximal EROD activities than TCDD. The most noticeable trend among the PAHs in Figure 3.1 is the decrease in maximal EROD activity with increasing EC50.

A similar decrease in maximal EROD activity was reported for rainbow trout liver (RTL-W1) cells exposed to high concentrations of PAHs (Bols *et al.*, 1999). The observed decrease in EROD activity at high concentrations of PAH did not appear to be due cytotoxicity, since the appearance of the cells at high concentrations of PAH were similar to the appearance at low concentrations of PAH, but cytotoxicity assays were not carried out to confirm this. Although we did not test for PAH induced cytotoxicity in CEH culture, the appearance of the cells at high concentrations of PAH did not appear to be different from those treated with DMSO only. Previous research in this laboratory has shown that high concentrations of TCDD and PCBs did not cause cytotoxicity (Kennedy *et al.*, 1996a).

3.3.2. EROD-inducing PAHs: Intercompound Comparisons

The rank order of EROD induction potency of the eight PAHs, from most to least potent, was DahA ≥ BkF > Ind > BaA ≥ BghiP ≥ Chr ≥ BaP > BNT. Among the EROD-inducing PAHs, there was considerable variation in potency. The most potent PAHs were DahA and BkF, which had EC50 values substantially lower than the other PAHs (Table 3.1). BkF and DahA were also the most efficient EROD inducers, with maximal EROD activities of 144 and 109 pmol/min/mg protein, respectively, which represents 79% and 60%, of the maximal activity of TCDD. Ind was intermediate in potency, with an EC50 4.3 times higher than BkF. Next in potency were BaA, BghiP, Chr, and BaP all of which had similar potencies and maximal EROD activities. The least potent of the EROD-inducing PAHs, BNT, had the highest EC50 and the lowest maximal EROD activity, suggesting that it has a low affinity for the AhR. Three PAHs, Cor, BeP and 1-MeP, were poor inducers of EROD activity, and we

Table 3.1. Maximal EROD activity (pmol/min/mg protein), EC50 and ECthr values, and Induction Equivalency Factors (IEF; EC50 (or ECthr) TCDD / EC50 (or ECthr) PAH) of the 18 PAHs in White Leghorn chicken hepatocytes.

Polycyclic Aromatic Hydrocarbon (PAH)	N	Max. EROD Activity[a]	EC50 nM ± SE	ECthr nM ± SE	TCDD IEF EC50 based	TCDD IEF ECthr based
EROD Inducers						
TCDD	24	183	0.03	0.0013	1.0	1.0
Benzo[k]fluoranthene	6	144	26.3 ± 1.6	5.4 ± 0.5	1.1e-3	2.4e-4
Dibenz[ah]anthracene	6	109	69.6 ± 6	17.8 ± 1.9	4.3e-4	7.3e-5
Indeno[1,2,3-cd]pyrene	6	109	112 ± 4.3	34 ± 1.1	2.7e-4	3.8e-5
Benz[a]anthracene	6	53	213 ± 24	131 ± 11	1.4e-4	9.9e-6
Benzo[ghi]perylene	6	55	224 ± 23.0	144 ± 8.1	1.3e-4	9.0e-6
Chrysene	6	50	261 ± 29.0	167 ± 41.6	1.1e-4	7.8e-6
Benzo[a]pyrene	8	52	268 ± 21.6	191 ± 23.3	1.1e-4	6.8e-6
Benzo[b]naphtho[2,3-d]thiophene	6	39	422 ± 23.8	483 ± 72.7	7.1e-5	2.7e-6
Non-EROD Inducers						
1-methylpyrene	2	<30	> 1 μM	nr	-	-
Anthracene	2	<30	nr	nr	-	-
Benzo[e]pyrene	2	<30	> 1 μM	nr	-	-
Coronene	2	<30	> 1 μM	nr	-	-
Dibenzothiophene	2	<30	nr	nr	-	-
Fluoranthene	2	<30	nr	nr	-	-
Fluorene	2	<30	nr	nr	-	-
Perylene	2	<30	nr	nr	-	-
Phenanthrene	2	<30	nr	nr	-	-
Pyrene	2	<30	nr	nr	-	-

[a] pmol/min/mg protein; N = number of plates; SE = standard error; nr = no response

were unable to determine reliable EC50 estimates. The remaining PAHs did not induce EROD activity.

All concentration-response curves exhibited non-ideal curves (i.e., maximal EROD activity decreases with increasing EC50, and several of the curves were not parallel). In situations where the maximal activity is reduced, the EC50 may overestimate potency. The non-ideality of the concentration-response curves for compounds with reduced maximal activities led us to develop an alternate method to estimate potency. This potency estimate,

called the ECthreshold (ECthr), estimates the concentration of an inducer required to elicit a response equivalent to 10% of the maximal response produced by TCDD (Appendix 8B describes the theory of the ECthr in more detail). Since the ECthr estimates potency at low concentrations of inducer, it has the advantage of being relatively insensitive to inhibitory effects of inducers on the catalytic activity of CYP1A.

An additional advantage of the ECthr is that potency estimates can be made even though dose-response curves are incomplete (i.e., are not biphasic). The ECthr approach generates a wider range of values than the EC50 (Table 3.1) which may be advantageous when comparing the potencies of compounds having similar EC50s. However, the ECthr approach does have limitations. Since the ECthr concentrations are not always in the linear portion of the dose-response curve, where large changes in dose are needed to induce a change in response, resolving power may be poor and confidence intervals large (Villeneuve *et al.*, 2000).

3.3.3. Induction Equivalency Factors (IEFs) for PAHs

After calculation of EC50 and ECthr values for each PAH, the potency of each PAH was calculated relative to the EC50 and ECthr of TCDD. This ratio, referred to as the induction equivalency factor (IEF; Kennedy *et al.*, 1996a), was determined by dividing the EC50 (or ECthr) of TCDD by the EC50 (or ECthr) of each PAH (Table 3.1). Although this ratio is usually referred to as the toxic equivalency factor (TEF), we use the term induction equivalency factor since EROD (CYP1A) induction is itself not a toxic response, but a biochemical response associated with some of the toxic effects of TCDD and other halogenated aromatic hydrocarbons (Kennedy *et al.*, 1996a). The range in potency between the most potent PAH

(BkF) and the least potent (BNT) was approximately 16-fold on an EC50-basis and approximately 90-fold on an ECthr-basis (Table 3.1).

The TCDD- and BaP-based IEFs for CEH, rainbow trout liver cell line (RTL-W1), and the rat hepatoma cell line (H4IIE) are shown in Table 3.2. BkF and DahA were the most potent PAHs in hepatocytes from all three species, with the remaining PAHs exhibiting small interspecies differences in potency ranking. The rank order of IEF of the PAHs in CEH, RTL-W1, and H4IIE cells is shown in parentheses in Table 3.2. With the exception of BaA, H4IIE and CEH cells had similar IEFs (less than 2 fold difference). However, RTL-W1 cells were more sensitive to EROD induction by PAHs (with the exception of BaA) than both H4IIE and CEH cells. The reasons for interspecies differences in potency is unknown, but may be related to concentration or characteristics of the AhR, or other factors downstream of the AhR.

Table 3.2. Induction equivalency factors (IEF) for PAHs derived from White Leghorn chicken embryo hepatocytes (CEH), rainbow trout liver cell line (RTL-W1), and rat hepatoma cell line (H4IIE). All IEFs are based on EC50 values. IEFs expressed as (EC50 TCDD) / (EC50 PAH). The rank order of the PAHs is shown is parentheses.

Polycyclic Aromatic Hydrocarbon (PAH)	CEH cells IEF (rank order)		RTL-W1 cells[a] IEF (rank order)		H4IIE cells[b] IEF (rank order)	
	TCDD-based	BaP-based	TCDD-based	BaP-based	TCDD-based	BaP-based
Benzo[k]fluoranthene	0.0011 (1)	10.2	0.00105 (1)	3.44	0.0048 (1)	15
Dibenz[ah]anthracene	0.00043 (2)	3.8	0.00035 (2)	1.16	0.0020 (2)	6
Indeno[1,2,3-cd]pyrene	0.00027 (3)	2.4	0.00028 (4)	0.92	0.0011 (3)	3
Benz[a]anthracene	0.00014 (4)	1.3	0.000043 (6)	0.14	0.000025 (6)	0.075
Chrysene	0.00011 (5)	1.01	0.000047 (5)	0.16	0.00020 (5)	0.6
Benzo[a]pyrene	0.00011 (6)	1.0	0.000302 (3)	1.0	0.00035 (4)	1.0

[a] Bols *et al.*, 1999
[b] Willett *et al.*, 1997

3.3.4. Effects of Time Course on EROD Induction

There was considerable variation in the shape of the concentration-response curves of the PAHs with varying exposure periods. Figure 3.2 shows the effect of exposure period on EROD activity of CEH cells continuously exposed to BkF for 6, 24, 48, and 72 h. The EC50s shifted to the right with increasing exposure time (6 h: 21 nM; 24 h: 26 nM; 48 h: 49 nM; 72 h: 138 nM). The maximal EROD activities, however, displayed a more complex trend. The highest EROD activity was achieved after 48 hours (165 pmol/min/mg protein), while the 6 hour exposure had the lowest EROD activity, with an activity approximately one fifth that of the 48 hr exposure (31 pmol/min/mg protein). The low activity and potency of the 6 hour exposure suggests 6 hours may be insufficient time to for the CYP1A protein to be fully induced and begin to metabolize the PAH. Since EROD activity was maximal after 48 hours of exposure, an exposure period of greater than 24 hr may be necessary to achieve maximal induction and catalytic activity of the CYP1A enzyme

3.3.5. Porphyrins

Data obtained using the fluorescent plate reader suggested that some PAHs (e.g., Ind) possessed porphyrinogenic activity in CEH cells (Figure 3.3). However, porphyrin pattern analysis by HPLC indicated that the PAHs in the present study did not induce porphyrin accumulation in CEH cells (results not shown).

3.4. Discussion

In the present study, TCDD and 18 PAHs were investigated for their ability to induce EROD activity in chicken embryo hepatocytes. TCDD was the most potent compound tested,

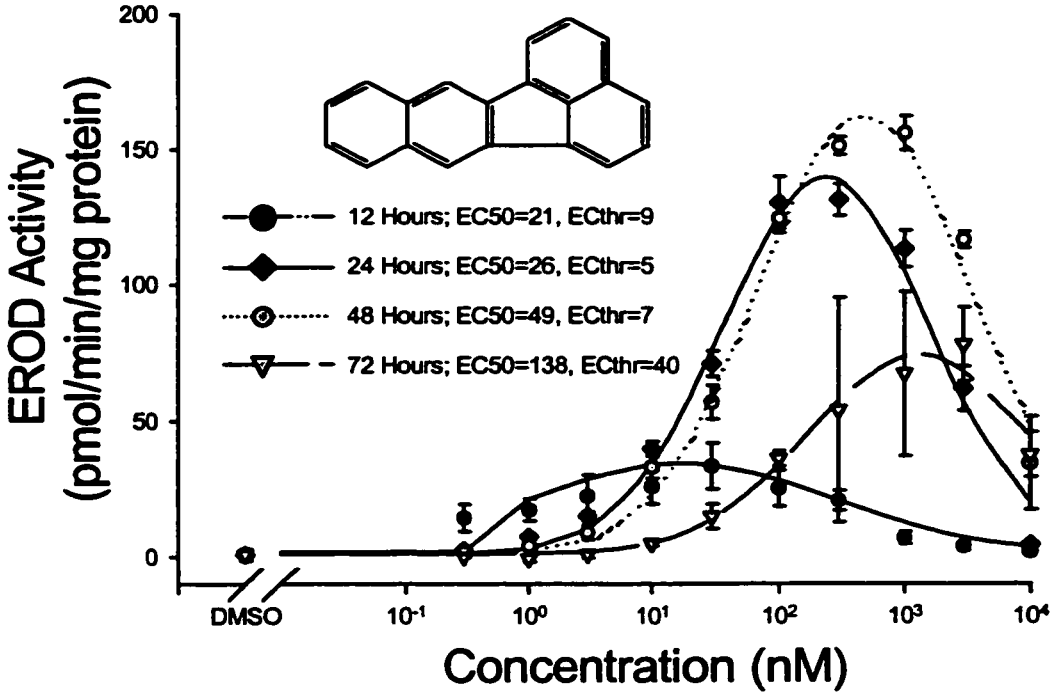

Figure 3.2. Effect of exposure time on EROD induction in chicken embryo hepatocytes by BkF. Cells were exposed to BkF for times ranging from 6 to 72 hours before being assayed for EROD activity. Each curve represents the results from a single plate. Points represent the mean EROD activity of triplicates of each dose, and bars represent standard error.

and was almost three orders of magnitude more potent as an EROD inducer than BkF, the most potent PAH. Of the 18 PAHs examined, only eight induced EROD activity (Figure 3.1). The rank order of induction potency, expressed as EC50, of the these PAHs was BkF ≥ DahA > Ind > BaA ≥ BghiP ≥ Chr ≥ BaP > BNT. The ten remaining PAHs were non-inducers (Table 3.1).

Several of the PAHs that induced EROD activity in this study were also potent inducers of aryl hydrocarbon hydroxylase (AHH) or EROD activity in cultured piscine and mammalian hepatocytes. Villeneuve *et al.* (1995) reported that BkF, BaP, Chr and BaA induced EROD activity in the top minnow hepatoma cell line (PLHC-1). BkF, DahA, Ind, BaP, Chr and BaA

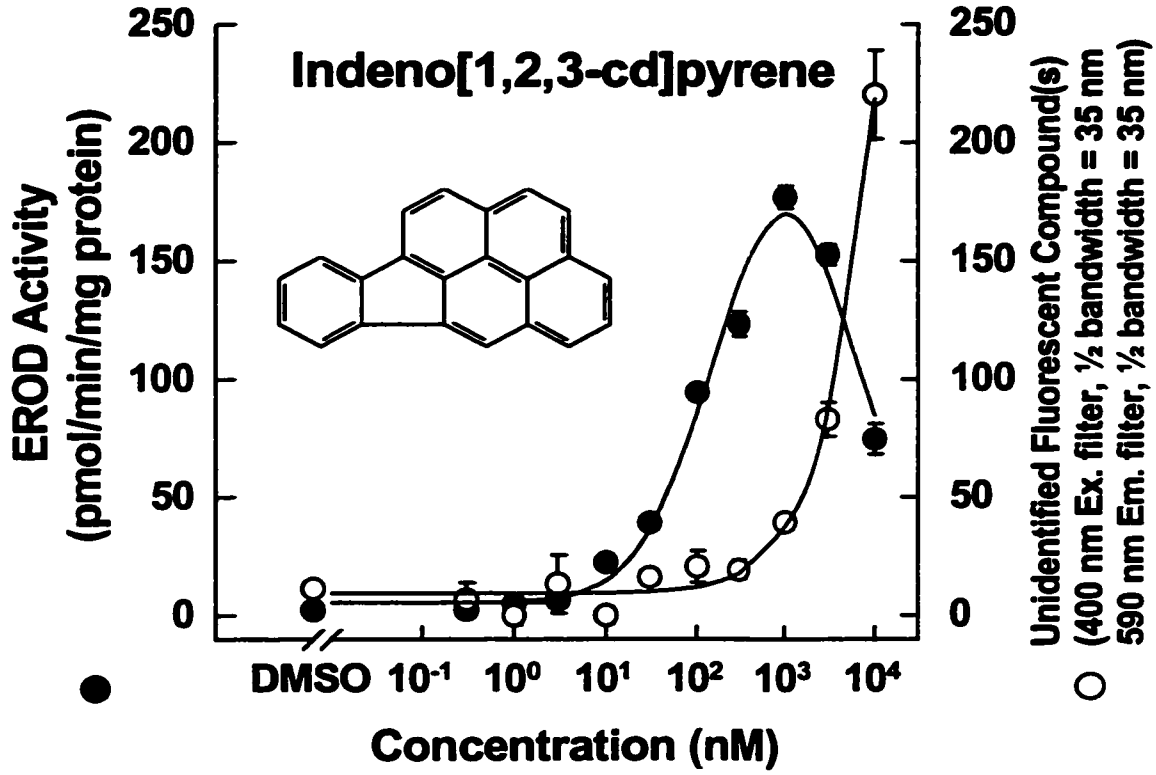

Figure 3.3. EROD (●) and total porphyrin (O) concentration-response curves for chicken embryo hepatocytes exposed to indeno[1,2,3-cd]pyrene. EROD and porphyrin data were obtained on the same multiwell plate using the fluorescence plate reader. While the plate reader indicated the presence of compounds with spectroscopic properties consistent with those of porphyrins, porphyrin pattern analysis by HPLC indicated that indeno[1,2,3-cd]pyrene did not induce porphyrin accumulation. The false positive obtained by the plate reader may have been caused by fluorescence of indeno[1,2,3-cd]pyrene at a frequency similar to that used to detect total porphyrins. Each curve represent s the results of a single 48-well plate. Points represent triplicates of each dose, bars represent standard error.

were potent EROD inducers in the rainbow liver cell line RTL-W1 (Bols *et al.*, 1999), the rat hepatoma cell line H4IIE (Piskorska-Pliszczynska *et al.*, 1986; Willett *et al.*, 1997), and in developing chick embryos (Brunström, 1991). PAHs that were non-inducers in CEH cells were also non-inducers in RTL-W1, and H4IIE cells. With the exception of BaA and BaP, the potency ranking of the EROD-inducing PAHs in this study were similar to the potency ranking reported for RTL-W1 and H4IIE cells.

The concentration-response curves for the EROD-inducing PAHs exhibited the same biphasic response seen for TCDD. There are likely several reasons for the decline in EROD activity at high concentrations of inducer, including loss or inhibition of CYP1A enzyme. A negative regulatory element located upstream of the CYP1A gene suppresses gene expression (Bhat *et al.*, 1996), and may regulate CYP1A expression in response to changes in AhR or Arnt content (Pollenz,1996; Piechocki and Hines, 1998). Hahn *et al.* (1996) showed that while the maximal EROD activity of PCBs 126 and 77 were approximately 42% and 11% of TCDD, respectively, the immuno-detectable CYP1A protein content increased monotonically with increasing dose indicating that they were full agonists relative to TCDD, and not partial agonists as indicated by the EROD assay. Thus, CYP1A content and EROD activity may differ, especially at high concentration of inducer. A partial agonist is defined as a substance that, at high concentrations, induces a lower maximal response relative to a standard that achieves a "full maximal" response, e.g., TCDD (Kenakin, 1993; Patil, 1996; Pliska, 1999).

Although EROD activity is the most economical and sensitive indicator for measuring CYP1A induction, both EROD activity and the immuno-detectable CYP1A protein should be measured simultaneously to obtain a more accurate estimate of potency, especially for compounds that are suspected of being partial agonists. Regardless of the mechanism(s) involved, the decrease in EROD activity occurring with biphasic curves can result in an underestimation of CYP1A content at high doses (Hahn *et al.*, 1993; 1996). Examination of Figure 3.1 shows that the most potent PAHs (e.g., BkF) exhibit biphasic concentration-response curves, suggesting that inhibition of catalytic activity is occurring at the highest doses. Unfortunately, immuno-detectable CYP1A was not determined in the present study, and thus

whether the PAHs were full or partial agonists of CYP1A induction cannot be definitively ascertained at this time. However, Brüschweiler *et al.* (1996) reported that although 3-methylcholanthrene (3-MC; a potent EROD inducing PAH derivative) induced a biphasic EROD concentration-response curve in fish hepatoma cells (PLHC-1), an ELIZA assay indicated that the amount of CYP1A protein increased monotonically with increasing 3-MC concentration , reaching levels similar to that induced by PCBs 77 and 169. Thus, potent EROD inducing PAHs such as BkF and DahA can be classified as full agonists.

Relative to TCDD, the PAH concentration-response curves exhibited lower maximal EROD activities. The mechanism(s) responsible for the decrease in maximal EROD activities of the PAHs are unknown but may involve one or both of the following: (a) the PAHs are partial agonists resulting in lower activation of the AhR signal transduction pathway, and a corresponding decrease in amount of CYP1A protein induced, and/or (b) the catalytic activity of the CYP1A enzymes is inhibited by residual inducer in cells. It should be noted that the decrease in EROD activity associated with a decrease in potency of the compound is distinct from the decrease in activity due to the biphasic shape of the curve.

Piskorska-Pliszczynska *et al.* (1986) examined the relationship between the affinity of PAHs for the AhR and their potency as EROD inducers. The PAHs with the highest affinity for the AhR (e.g., DahA) were the most potent EROD inducers, while PAHs with low AhR affinity (e.g., BghiP) were weak EROD inducers. Therefore, the PAHs with the lowest EROD activity (e.g., BNT and Chr) are likely partial agonists relative to BkF and DahA. However, since the concentration of PAH required to induce EROD activity in CEH cells was

approximately three orders of magnitude greater than for TCDD, it is possible that, at higher concentrations, the PAHs were competitively inhibiting the catalytic activity of the CYP1A enzyme, resulting in lower EROD activity (Hahn *et al.* 1996; Petrulis and Bunce, 1999).

Although bioassay derived potency estimates are easily obtained, interpretation of relative potencies between samples should be approached with caution, especially for compounds that are, or are suspected to be, partial agonists. The accuracy of bioassay derived relative potency estimates is dependent on how well the data meets the assumptions of the mathematical model used to estimate potency. However, a variety of factors, including inhibition of catalytic activity by excess agonist, can influence the shape of the concentration-response curves such that they do not meet the requirements of the mathematical models. In the present study, the concentration-response curves for the PAHs were non-ideal since they did not meet three criteria: (1) the curves were biphasic; (2) maximal EROD activities varied among PAHs (some PAHs were probably acting as partial agonists), and (3) the slope of some curves were not parallel. We therefore evaluated and compared the potency of the EROD-inducing PAHs by an alternate method that relies on estimating potency at a lower dose where inhibition of catalytic activity is minimal. This method, called the ECthreshold method (ECthr), uses the same Gaussian curve used to estimate the EC50 to calculate the concentration of a compound required to induce a response equivalent to 10% of the maximal response produced by TCDD. A value of 10% was chosen since inhibition of CYP1A catalytic activity should be minimal at his concentration. The ECthr, and the corresponding TCDD-based IEFs of the eight EROD-inducing PAHs are shown in Table 3.1. The ECthr appears to have a greater ability to discriminate potency than the EC50; the range in potency between the most

and least potent PAH on an EC50-basis was only 16-fold, while on an ECthr-basis the range was almost 90-fold. Consequently, the ECthr should be especially useful for determining the potency of compounds that are partial AhR agonists or weak EROD inducers.

Although BkF was almost three orders of magnitude less potent an EROD inducer than TCDD, it was an efficient EROD inducer, having a maximal EROD activity 79% of TCDD. DahA the second most potent PAH, had an induction efficacy 60% of TCDD. In Figure 3.1, the PAHs are arranged in order of decreasing potency, with BkF the most potent. A decrease in potency is accompanied by a decrease in maximal EROD activity, indicating that both potency and efficacy decrease as the affinity of PAHs for the AhR decreases. The binding affinities of PAHs for the AhR have been shown to correlate well with their EROD induction potencies (Toftgård, 1985; Piskorska-Pliszczynska *et al.*, 1986). *In vitro* studies with rat and murine cell lines have shown that although TCDD is several orders of magnitude more potent than DahA, they have similar AhR binding affinities (Bigelow and Nebert, 1982; Piskorska-Pliszczynska *et al.*, 1986). Since the affinity with which an agonist binds the AhR determines the extent to which the CYP1A gene is transcribed, TCDD and DahA (and by extension BkF) would be expected to have similar potencies. However, in this and other studies, TCDD is several orders of magnitude more potent than the most potent PAHs (Brunström *et al.*, 1991; Willett *et al.*, 1997; Bols *et al.*, 1999).

Riddick *et al.* (1994) reported that TCDD and 3-methylcholanthrene (MC) had similar affinities for mouse Hepa-1 AhR, and were equipotent in causing the *in vitro* transformation of the AhR to its DNA-binding form. Potency differences between TCDD and MC two hours

after induction were small (0 to 4 fold), while after 14 h TCDD was up to 1,000 time more potent than MC (Riddick *et al.*, 1994). TCDD and DahA are full agonists, and the differences in potency between TCDD and MC are due primarily to their different rates of metabolism; TCDD is poorly metabolized by CYP1A and remains in the cells at high concentrations and persistently activates the AhR pathway (Bock, 1994; Olson *et al.*, 1994). Previous studies in our laboratory have demonstrated that the concentration-response curves of TCDD treated CEH cells were not greatly influenced by exposure time. Exposure periods ranging from 24 to 72 h had little influence on the potency and efficacy of TCDD to induce EROD activity. Persistent activation of the AhR pathway may result in CYP1A mediated oxidation of endogenous substrates, with subsequent generation of reactive oxygen species (ROS). Generation of ROS in combination with the depletion of intracellular antioxidants (e.g., glutathione) leads to oxidative stress and toxicity, and may represent a major pathway by which TCDD elicits its extraordinary toxicity (Bondy and Naderi, 1994; Fantel, 1996).

In contrast to TCDD, PAHs are quickly metabolized to hydrophilic metabolites, and pharmacokinetic factors are likely to play a greater role when CYP1A is induced by PAHs. Time course studies with BkF indicate that the potency of PAHs to induce EROD activity in CEH cells decreases with increasing exposure period (Figure 3.2). This indicates that with increasing exposure time, BkF is metabolically inactivated as a CYP1A inducer by the induced enzyme. The low EROD activity of BkF at 6 h may be due to insufficient time to fully induce CYP1A. Since BkF EROD activity is maximal after 48 h exposure, 48 h may be the optimal exposure period for estimating PAH induced EROD activity in CEH cells. Differences in rates of metabolism may also provide an explanation for the differences in potency and efficacy

between DahA and Ind. Although DahA and Ind have identical maximal EROD activities, DahA is twice as potent as Ind (Table 3.1). The high efficacy of Ind relative to DahA suggests that Ind may be metabolized at a slower rate than DahA, or alternatively, the metabolites of Ind may still have CYP1A induction activity.

The ability of the PAHs to induce EROD activity was expressed relative to TCDD and BaP. This ratio is often referred to as the toxic equivalency factor (TEF). However, since EROD induction is not a toxic response, but a biochemical response that is associated with some of the toxic effects of PAH, we prefer to use the term induction equivalency factor (IEF) as a measure of *in vitro* relative potency. In chicken embryo hepatocytes, BaA was more potent than BaP (Table 3.2), whereas in both RTL-W1 and H4IIE cells, BaP was more potent than BaA. These differences appear to be taxon specific, and may involve species specific differences in the affinity of the AhR for individual PAHs, or to other factors downstream of the AhR. Overall, the species differences in potency ranking of PAHs are subtle, and are probably statistically insignificant.

The *in ovo* toxicity and EROD-inducing potency of PAHs were investigated by Brunström *et al.* (1991) and Machala *et al.* (1996). In the Brunström *et al.* (1991) study, 24 PAHs were injected at a dose of 300 μg/kg-egg into the air cell of 7 day old chicken embryos, and rates of mortality were measured 72 hours later. Of the 24 PAHs injected at this dose, four induced 100% mortality, three induced mortality rates between 55% and 75%, while the remaining PAHs induced 0% mortality. The hepatic EROD activities on day 10 were also determined. Two of the four most toxic PAHs (BkF and DahA) were potent EROD inducers,

while the other two (BaA and BNT) were intermediate inducers. Only six of the non-toxic PAHs (those which caused 0% mortality at 300 μg/kg-egg) induced EROD activity, but at relatively low levels. In general, both Brunström *et al.* (1991) and Machala *et al.* (1996) demonstrated that the EROD-inducing potency of PAHs correlated well with their *in ovo* toxicity. The correlation between the *in ovo* toxicity of PAHs and their potency as CYP1A inducers led us to examine the possibility of using the *in vitro* CEH EROD bioassay to predict the toxic potency of PAHs in developing avian embryos. The rank order of toxicity (LD50) of the four PAHs causing 100% mortality was BkF > DahA > BaA > BNT. This is identical to the rank order of EROD-inducing potency of these PAHs in the present study. In Table 3.3, the PAHs are arranged into three groups according to their *in ovo* toxic potency, with the most toxic (Group 1), inducing 100% mortality.

It should be noted that in the determination of *in ovo* EROD activities by Brunström *et al.* (1991) the large range in embryotoxicity of the PAHs necessitated the administration of different concentrations of PAH to the embryos. Consequently, the concentration between the most toxic (BkF) and the least toxic (BaP) varied twenty times. Moreover, only one concentration of each PAH was used for the determination of EROD potency. Comparisons between the Brunström (1991) EROD data and our EROD potency estimates were further complicated by the varying EROD activities expressed by chicken embryos at different stages of development. Bosveld *et al.* (1997) demonstrated that the EROD activities of a variety of halogenated aromatic compounds (TCDD, TCDF, PCB 126 and PCB 118) from 14 day old embryos were significantly different from hepatocyte cultures from 19 day old embryos and 1 day old hatchlings. This confounding factor may affect the present comparisons since the

embryos used by Brunström *et al.* (1991) were 7 days old at the time of injection, while the hepatocytes in our *in vitro* bioassay were from 19 day old embryos. Consequently, the EROD data obtained by Brunström et al. (1991) is not directly comparable to EROD dose-response data presented in this study.

Table 3.3. *In ovo* toxicity of 18 PAHs compared to EROD-inducing potency in cultured embryo hepatocytes from White Leghorn chicken. In general, those PAHs exhibiting EC50 values less than 500 nM, are toxic to developing embryos. Induction equivalency factor (IEF) is defined as EC50 (or ECthr) of TCDD / EC50 (or ECthr) of PAH for EC50 or ECthr values.

Group	Polycyclic Aromatic Hydrocarbon	*In Ovo* Toxicity % Mortality[a] 300	100	EROD Induction (CEH) EC50 (nM)	ECthr (nM)	IEF EC50-based	ECthr-based
1	Benzo[k]fluoranthene	100	nt[b]	26.3	5.4	0.0013	0.00024
	Dibenz[ah]anthracene	100	nt	69.6	17.8	0.00043	0.000073
	Benz[a]anthracene	100	nt	213	131	0.00014	0.0000099
	Benzo[b]naphtho[2,3-d]thiophene	100	nv[c]	422	483	0.000071	0.0000027
2	Chrysene	75	25	261	167	0.00011	0.0000078
	Indeno[1,2,3-cd]pyrene	65	5	112	34	0.00027	0.000038
	Benzo[a]pyrene	55	0	268	191	0.00011	0.0000068
3	Benzo[ghi]perylene	0	nt	144	nr	0.00013	0.00009
	Dibenzothiophene	5	nt	> 1 μM	nr	-	-
	1-methylpyrene	0	nt	nr	nr	-	-
	Anthracene	0	nt	> 1 μM	nr	-	-
	Benzo[e]pyrene	0	nt	> 1 μM	nr	-	-
	Coronene	0	nt	nr[d]	nr	-	-
	Fluoranthene	0	nt	nr	nr	-	-
	Fluorene	0	nt	nr	nr	-	-
	Perylene	0	nt	nr	nr	-	-
	Phenanthrene	0	nt	nr	nr	-	-
	Pyrene	0	nt	nr	nr	-	-

[a]Percent mortality at 300 μg/g-egg and 100 μg/g-egg; from Brunström *et al.*, 1991.
[b]not tested at this dose
[c]no value given, but tested at this dose
[d]no response

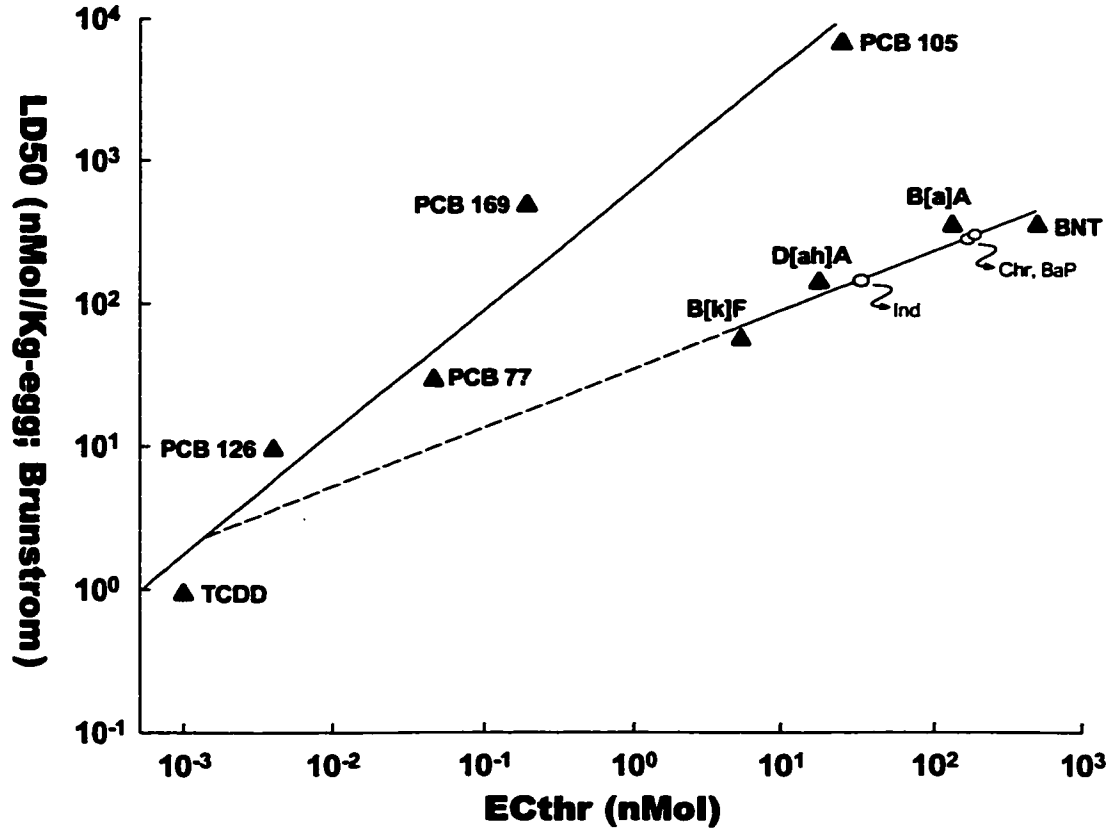

Figure 3.4. Log-Log plot of LD50 vs ECthr for TCDD, PCB and PAH in White Leghorn chicken embryos. LD50s were determined by Brunström *et al.* (1991) by injection of PAH into the air cell of 7 day old embryos. Embryolethality was measured 72 hr after injection. The interpolated LD50 estimated from the PAH regression line for Ind, Chr, and BaP are 145 nM/Kg (40 µg/Kg), 275 nM/Kg (62 µg/Kg), and 285 nM/Kg (71 µg/Kg), respectively.

To determine whether the *in vitro* EROD assay could be used to predict the *in ovo* lethality of PAHs, we compared the LD50 values of the four most toxic PAHs (Group 1, Table 3.3) as determined by egg injection studies (Brunström *et al.*, 1991) against the ECthr values determined in the present study. We chose to use the ECthr instead of the EC50 because of its apparent ability to discriminate potency at low doses where inhibition of CYP1A is minimal. Figure 3.4 presents a log-log plot of the LD50 versus the ECthr for the four Group 1 PAHs. Figure 3.4 also shows the relationship between the *in ovo* LD50 and the *in vitro* ECthr of TCDD and four PCBs. The reason for the different slopes between the PAH and HAH

regression lines is unknown, but may reflect a combination of the differences in mechanism of toxicity , and rates of metabolism. Although the r^2 value for the PAH EC50 regression line (0.939) was higher than the ECthr value (0.898), extrapolation of the ECthr regression line towards the y-axis approaches TCDD whereas extrapolation of the EC50 regression line diverged significantly from TCDD (EC50 line not shown). The significance of this result in not yet clear, but it suggests that the ECthr may be a more accurate method for estimating potency than the EC50.

Using the PAH regression line in Figure 3.4, we estimated the LD50 of the three Group 2 PAHs. The estimated LD50s for Ind, Chr, and BaP were 40 μg/Kg (145 nM/Kg), 62 μg/Kg (270 nM/Kg), and 71 μg/Kg (285 nM/Kg), respectively. Although specific LD50 values are not available for Ind, Chr, or BaP, their LD50 values were bracketed by the upper and lower concentrations used in the Brunström mortality study. Thus, the LD50 of Ind and Chr were between 100 and 300 μg/Kg, and the LD50 of BaP was approximately 300 μg/Kg (Brunström *et al.*, 1991). The *in ovo* mortality rates and estimated LD50s of the Group 2 PAHs are shown in Table 3.4. In the present study, the predicted LD50 values for Ind, Chr, and BaP were 40, 62m, and 71 μg/Kg egg, respectively. Consequently, the predicted LD50 values overestimate their actual *in ovo* LD50 by a factor of 2 to 5 times. Keeping in mind the limitations of comparing small data sets, it appears that ECthr has the capacity to estimate LD50 to within at least one order of magnitude of their actual *in ovo* LD50. Considering the limited *in ovo* toxicity data currently available, these preliminary results suggest that the *in vitro* EROD bioassay and the ECthr may be a promising alternative for predicting the toxic potencies of PAHs *in ovo*.

While it is not clear whether PAHs and HAHs exert their toxicity through similar mechanisms, many PAHs, including several in the present study, are known ligands to the AhR. Consequently, it is probable that at least some of the toxicity of PAHs to developing chicken embryos is mediated by an initial binding to the AhR. However, there may be other cytosolic PAH receptors that contribute to the induction of the CYP1A isoforms. Heintz *et al.* (1981) initially reported the existence of cytosolic 4S binding protein with a saturable high affinity binding for PAHs. This protein has been identified as glycine N-methyltransferase (GNMT). Like the AhR, GNMT is translocated to the nucleus upon interaction with PAHs, and complexes with cis-acting elements in different regulatory regions of the CYP1A gene. Thus, GNMT appears to be both a PAH binding protein, and a mediator of the induction CYP1A (Raha *et al.*, 1994, 1995; Bhat and Bresnick, 1997). Although GNMT does not appear to be as efficient as the AhR in inducing CYP1A, the pattern of P450 isoform expression associated with PAHs is distinct from that induced by TCDD (Gibbons and Babbish, 1992). This indicates that there is at least one AhR-independent pathway for the induction of CYP1A, and that CYP1A isoforms induced by PAHs may differ from those induced by TCDD and related HAHs.

Table 3.4. Comparison of *in ovo* mortality rates of the Group 2 PAHs against LD50 values predicted from Figure 3.4. LD50s for Chr and Ind are between 100 and 300 µg/Kg, and the LD50 of BaP is approximately 30 µg/Kg.

PAH	Mortality Rate[a] µg/Kg egg (% Mortality)	LD50 (Fig .3.3) µg/Kg egg
Indeno[1,2,3-cd]pyrene	100 (5) - 300 (65)	40
Chrysene	100 (25) - 300 (75)	62
Benzo[a]pyrene	100 (0) - 300 (55)	71

[a]Brunström *et al.*, 1991

Several AhR agonists are capable of de-regulating the heme biosynthetic pathway and causing an intracellular accumulation of porphyrins (Marks, 1985; Sinclair *et al.*, 1997b). Since the onset of porphyrin accumulation in hepatocytes often coincides with the attenuation of EROD activity at higher concentrations of inducer, disruption of hepatic heme biosynthesis by HAHs may be mechanistically linked with one or several cytochrome P450 isoforms (Marks, 1989; Tysklind *et al.*, 1995; De Matteis and Marks, 1996). Chicken embryo hepatocytes treated with TCDD and PCB 77 accumulated porphyrins, with uroporphyrin and heptacarboxylporphyrin accounting for over 95% of the porphyrins produced (Lorenzen *et al.* 1997; Sanderson, 1998). The HAHs causing the greatest porphyrin accumulation were also the most potent EROD inducers, indicating that porphyrin accumulation is directly or indirectly associated with CYP1A induction.

In contrast to HAHs, we did not find any evidence to suggest that PAHs induced porphyrin accumulation in chicken embryo hepatocytes. This result is supported by Sinclair *et al.* (1984) who reported that PCB 77 induced uroporphyrin accumulation in chicken embryo hepatocytes, whereas 3-methylcholanthrene and other CYP1A inducing PAHs did not induce uroporphyrin accumulation. Although PAHs do not appear to induce porphyrin accumulation *in vitro*, Francis and Smith (1987) demonstrated that EROD-inducing PAHs such as DahA, BaP and BaA administered to iron loaded mice inhibited hepatic uroporphyrinogen decarboxylase activity and induced porphyria indistinguishable from that induced by HAHs. It would be of interest to determine if the EROD-inducing PAHs in this study are capable of inducing porphyrin accumulation in chicken embryos. As far as we know, no studies

measuring PAH induced porphyrin accumulation in developing chick embryos have been conducted to date.

The fluorescent plate reader can be used to determine whether porphyrins, or compounds with spectroscopic signatures similar to porphyrins, have been induced by a CYP1A inducer. However, a positive response obtained with the plate reader does not mean that porphyrins were induced, only that compounds with spectroscopic characteristics similar to that of porphyrins are present. While the plate reader provides a quick method to scan for "total porphyrins ", it cannot be used to determine porphyrin patterns. It is therefore important to verify by HPLC that porphyrins are indeed present when a positive porphyrin-concentration response curve is obtained with the plate reader. Chromatograms (results not shown) of the PAHs that caused positive porphyrin-concentration curves showed that the PAHs did not induce porphyrin accumulation in chicken embryo hepatocytes, and that positive porphyrin concentration-response curves were "false positives".

Since many PAHs and PAH metabolites are fluorescent, the porphyrin concentration-response curves obtained with the plate reader for Ind may have been caused by the fluorescence of Ind, or its metabolites, at an excitation/emission frequency similar to that used to detect porphyrins. To examine the possibility that the inherent fluorescence of Ind was responsible for the positive porphyrin concentration-response curve in Figure 3.3, we examined the fluorescence of a plate (no cells) containing Ind and the reagents normally used in the porphyrin assay (fluorescamine and 3 N HCl), and scanned the plate with the plate reader (results not shown). The scan was positive for the presence of porphyrins. Since no cells were

present, the positive reading supports the hypothesis that there is some overlap between the excitation/emission frequencies of Ind (and possibly other PAHs) and total porphyrins, and that using the plate reader alone to determine porphyrin accumulation can be misleading. Thus, a positive porphyrin concentration-response must be analysed by HPLC to determine if porphyrins are indeed present. More studies are necessary to confirm whether there is overlap of the excitation/emission frequencies of PAHs and porphyrins. Since the onset of accumulation of porphyrins often coincides with the attenuation of EROD activity at higher concentrations of some halogenated aromatic hydrocarbons (HAHs), some studies suggested that the two events were mechanistically linked. Although the disruption of hepatic heme biosynthesis by certain porphyrinogenic compounds (HAHs) may be indirectly associated with one or several cytochrome P450 isoforms (De Matteis and Marks, 1996), we did not find any evidence to suggest that PAHs induced porphyrin accumulation in the CEH cells.

Huuskonen *et al.* (2000) measured EROD induction and porphyrin accumulation in PLHC-1 cells exposed to extracts of sediments and oil shale deposits collected near a shale oil processing plant. The sediments, which contained PAHs ranging from 4,270 to 150,000 μg/kg dry weight, were found to be potent inducers of both EROD activity and porphyrins. However, since Huuskonen and co-workers (2000) appear to have examined the porphyrinogenic potency of the extracts using only the fluorescence plate reader, it is likely that some, or perhaps most, of the apparent porphyrinogenic activity of the extracts is due to fluorescence of the PAHs in the extracts. Furthermore, since the sediment and oil shale samples were analysed for PAH content only, there may be other compounds (e.g., HAHs) in these samples which were responsible for the putative porphyrin accumulation. Thus, it is unclear at this time whether

the porphyrinogenic activity of the extracts in PLHC-1 cells was a false positive caused by the high PAH content, or to other compounds present in the extracts.

In summary, primary cultures of chicken embryo hepatocytes were used to estimate the ability of 18 PAHs to induce EROD activity, and thus, indirectly, their ability to activate the AhR- dependent CYP1A enzyme system. Of the 18 PAH tested, only eight consistently induced EROD activity. TCDD was approximately three orders of magnitude more potent an EROD inducer than BkF, the most potent PAH. The potency of the PAHs to induce EROD activity was estimated by two methods: (1) the concentration required to induce a half maximal response (EC50); and, (2) the concentration of PAH required to induce a response equivalent to 10% of the maximal response of TCDD (ECthr). Previous studies have demonstrated that there is a good correlation between the EROD-inducing potency of HAHs and their lethality *in vivo* (Safe, 1990; Kennedy *et al.*, 1996a). A comparison of the LD50 of the four most embryotoxic PAHs (BkF, DahA, BaA, and BNT) against their *in vitro* EROD-inducing potency (ECthr) also shows a good correlation, suggesting that the *in vitro* bioassay may be a useful tool to estimate the lethality of PAHs in developing chick embryos. While TCDD and structurally related halogenated aromatic hydrocarbons such as coplanar PCBs disrupt the heme biosynthetic pathway and cause the intracellular accumulation of porphyrins, PAHs did not induce porphyrin accumulation in CEH cells.

CHAPTER 4. ETHOXYRESORUFIN-*O*-DEETHYLASE (EROD) INDUCING POTENCY OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHs) AND ZEBRA MUSSEL EXTRACTS IN PRIMARY CULTURES OF CHICKEN (*Gallus domesticus*), PEKIN DUCK (*Anas platyrhynchos*), AND GREATER SCAUP (*Aythya marila*) EMBRYO HEPATOCYTES

4.1. Abstract

The concentration-dependent effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 18 polycyclic aromatic hydrocarbons (PAHs) on cytochrome P4501A (CYP1A) induction in primary cultures of hepatocytes prepared from White Leghorn chicken (*Gallus domesticus*), Pekin duck (*Anas platyrhynchos*), and Greater Scaup (*Aythya marila*) embryos were evaluated. The PAHs evaluated were the same as in Chapter 3. Two methods for estimating EROD-inducing potency were compared: (1) the concentration of inducer at which EROD activity was half maximal (EC50); and, (2) the concentration of inducer that induced a response equivalent to 10% of the maximal response of TCDD (ECthreshold, ECthr). For each method, the interspecies rank order of sensitivity to EROD induction by TCDD was chicken >> Greater Scaup (scaup) > Pekin duck (duck). Chicken hepatocytes were approximately two orders of magnitude more sensitive than Pekin duck and scaup hepatocytes to TCDD. This is consistent with higher sensitivity of chicken embryos to the embryotoxic effects of TCDD than embryos of many other avian species. Of the 18 PAHs tested, only BkF, DahA, Ind, BaA, BghiP Chr, BaP, and BNT induced EROD activity. There were small differences in the rank order of potency of the PAHs within each species. In contrast to TCDD, all three species of hepatocytes were approximately equally sensitive to the EROD induction by PAHs. Two types of potency comparisons were made: (1) relative potency of compounds within each species expressed relative to TCDD or BkF (induction equivalency factors; IEFs); and, (2) relative potency of each species to EROD induction by each compound expressed relative to chicken. For chicken and scaup, but not Pekin duck, the ECthr-based TCDD IEFs for all PAHs were lower than the EC50-based TCDD IEFs. The ECthr-based BkF IEFs were lower than the EC50-based BkF IEFs for all PAHs in all three species. Among species, Pekin duck hepatocytes were more sensitive to EROD induction by BaP, DahA, and Ind than chicken hepatocytes. The higher sensitivity of duck hepatocytes EROD induction by PAHs is consistent with their higher sensitivity to the *in ovo* toxic potency of PAHs. The EROD-inducing potency of extracts prepared from zebra mussels from three locations in the Great Lakes were evaluated in chicken, Pekin duck, and Greater Scup

hepatocytes. The interspecies rank order of sensitivity to EROD induction by the extracts was chicken > Pekin duck > scaup. These results suggest that the scaup is not more sensitive to environmental contaminants than domestic ducks. Overall, our results suggest that primary cultures of avian embryo hepatocytes may be a useful model for estimating the sensitivity of avian species to the embryotoxic effects of PAHs.

4.2. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants of both aquatic and terrestrial ecosystems. PAHs are formed upon incomplete combustion of organic materials, and are transported in the atmosphere in the vapour phase or as particulate matter. Approximately 50% of the PAHs released to the environment are from natural sources, the most important being forest fires. Major anthropogenic sources of PAHs include combustion of fossil fuels, spillage of petroleum, metallurgical and coking plants, and leaching from creosote treated products. In urban areas, combustion of fossil fuels is probably the most important local source. Birds are exposed to PAHs by respiratory uptake from atmospheric PAHs in the vapour phase, and by ingestion of PAH-contaminated foods. A study of the distribution of PAHs through an aquatic food chain from blue mussels (*Mytilus edulis*) to the common eider (*Sommateria mollissima*) suggested that consumption of contaminated food is the most important exposure route (Broman *et al.*, 1990).

Biotransformation of PAHs by the cytochrome P450 enzyme system results in the generation of chemically reactive diol-epoxide intermediates (Jerina and Daly, 1974; Levin *et al.*, 1982; Kim *et al.*, 1997; Wells *et al.*, 1997) that covalently bind to critical cellular components (e.g., DNA, proteins). Laboratory studies with various species have demonstrated

that PAHs induce a variety of toxic effects, including developmental abnormalities (Brunström *et al.*, 1991), immune system suppression (Silkworth *et al.*, 1995; Santodonato, 1997), reproductive effects (MacKenzie and Angevine, 1981), liver toxicity (Mumtaz *et al.*, 1996), tumour initiation and promotion (Dipple *et al.*, 1999; Ross and Nesnow, 1999), as well as inducing the expression of numerous genes, including the cytochrome P4501A (CYP1A) gene battery (Poland and Knutson, 1982; Poland and Bradfield, 1992; Whitlock, 1999).

The majority of the toxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and structurally related halogenated aromatic hydrocarbons (HAHs) appear to be mediated by binding of HAHs to the aryl hydrocarbon receptor (AhR). CYP1A induction is one of the most sensitive biomarkers for estimating exposure to HAHs and other compounds having affinity for the AhR (Bucheli and Fent, 1995). The most common biochemical endpoint used to estimate CYP1A induction is the ethoxyresorufin-*O*-deethylase (EROD) assay, which provides a measure of the catalytic activity of the CYP1A enzyme (Kennedy *et al.*, 1993, 1995). The CYP1A enzymes induced in chickens upon exposure to HAHs are not strictly orthologous to the mammalian CYP1A1 and CYP1A2 isoforms, and have been classified as CYP1A4 and CYP1A5 (Nakai, 1991; Gorman *et al.*, 1998; Mahajan and Rifkind, 1999; Gannon *et al.*, 2000). In chick, the CYP1A4 isoform is the main catalyst of EROD activity (Rifkind *et al.*, 1994).

Although it is not clear whether PAHs and HAHs exert their toxicities through similar mechanisms, several PAHs are known ligands to the AhR (Bigelow and Nebert, 1982; Piskorska-Pliszczynska *et al.*, 1986), and are known inducers of EROD activity in chick and

domestic duck embryos (Brunström and Halldin, 1998). Brunström *et al.* (1991) investigated the embryotoxic potency of several PAHs by injecting PAHs into the air cell of developing chicken embryos. The most embryotoxic PAHs also proved to be the most potent inducers of EROD activity. Thus, it appears that at least some of the toxicity of PAHs is mediated by the AhR.

Due to the difficulties associated with conducting egg injection studies, especially with wild or endangered avian species, there has been considerable interest in developing alternative methods to determine the sensitivity of avian species to HAHs and other AhR agonists. One promising approach is to examine the *in vitro* induction of EROD activity in primary cultures of avian hepatocytes. Strong correlations between toxic potency and potency to induce EROD activity have been shown for HAHs (Safe, 1993). In an egg injection study, Brunström *et al.* (1991) reported that the rank order of embryotoxicity (LD50) of PAHs was identical to the rank order of their EROD-inducing potency. The EROD-inducing potency of PAHs therefore appears to correlate well with their *in ovo* toxic potency.

Previous studies in our laboratory examined the sensitivity of primary cultures of hepatocytes prepared from domestic chicken (four breeds), duck (three breeds), pheasant, and herring gull to EROD induction by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and six polychlorinated biphenyls (PCBs) (Kennedy *et al.*, 1996a). Chicken hepatocytes proved to be the most sensitive species to EROD induction. These results were supported by egg injection studies that demonstrated that chicken embryos were considerably more sensitive to the embryotoxic effects of HAHs than many other avian species (Brunström, 1991; Brunström and

Reutergårdh, 1986). In contrast, Brunström *et al.* (1990) reported that several avian species exhibited similar or greater sensitivity to the embryolethal effects of BkF, the most embryolethal PAHs in chicken embryos. Although duck embryos were more sensitive to the embryolethal effects of PAHs than chicken embryos, Brunström and Halldin (1998) reported that both chicken and domestic duck embryo livers were approximately equally sensitive to EROD induction by BkF. These studies suggest that the *in vitro* EROD assay may be a useful alternative to egg injection studies for estimating the sensitivity of avian species to the embryotoxic effects of PAHs.

Exposure to halogenated aromatic hydrocarbons and other chemicals that have affinity for the AhR have been correlated with reproductive failures and biochemical abnormalities in populations of wild birds. This has been most intensively studied in colonial fish eating birds in the Great Lakes basin (Fox *et al.*, 1991; Gilbertson *et al.*, 1991). Since the introduction of zebra mussels (*Dreissena polymorpha*) into the Great Lakes, several species of diving ducks, including the Greater Scaup (*Aythya marila*), have altered their traditional migratory patterns to take advantage of the zebra mussels as an abundant food source (Wormington and Leach, 1992; Hamilton *et al.* 1994). Although zebra mussels represent an abundant food source for diving ducks, the high filtering capacity (Reeders *et al.*, 1989; Fisher *et al.*, 1993), their high lipid content (Comba *et al.*, 1996; Roe and McIsaac, 1998), and their limited ability to metabolize xenobiotics (Stegeman and Hahn, 1994; Meador *et al.*, 1995) result in the bioaccumulation of a variety of organic contaminants, including PAHs, in zebra mussel tissues (Secor *et al.*, 1993; Roper *et al.*, 1996).

de Kock and Bowmer (1993) reported that tufted ducks (*Aythya fuliga)* fed a diet of zebra mussels contaminated with a variety of organic contaminants laid fewer eggs, abandoned nests more often, and had higher embryo and chick mortality rates than ducks fed less contaminated mussels. Mallard (*Anas platyrhynchos*), marsh sandpiper (*Tringa stagnatilis*), and Slavonian grebe (*Podiceps auritus*) eggs collected from the highly contaminated Selenga River estuary of Lake Baikal, Russia, were found to contain high levels of PAHs. The hens of these species feed extensively on aquatic invertebrates from the estuary during nesting season, and transfer some of the dietary PAHs to the developing ovum. Thus, it is possible that Greater Scaup and other diving ducks feeding on zebra mussels while staging in the Great Lakes may be exposed to PAHs, and a variety of other contaminants, some of which may be transferred to the developing ovum. (Move to discussion?)

In the present study we examined the ability of 18 PAHs to induce EROD activity in hepatocytes prepared from White Leghorn chicken, Pekin duck, and Greater Scaup embryos. We are interested in determining if primary cultures of avian hepatocytes can be used as an alternative to egg injection studies to predict the sensitivity of wild birds to the *in ovo* toxic potency of PAHs.The purpose of this study was to examine three related issues: (1) the utility of the *in vitro* EROD bioassay to predict the *in ovo* toxic potency of PAHs in developing avian embryos; (2) to estimate the *in ovo* toxic potency of PAHs to Greater Scaup; and, (3) to estimate the sensitivity of chicken, Pekin duck, and Greater Scup hepatocytes to EROD induction by extracts prepared from zebra mussels from the Great Lakes.

4.3. Results

4.3.1. Characteristics of the concentration-response curves

TCDD and 18 polycyclic aromatic hydrocarbons (PAHs) were evaluated for their ability to induce EROD activity in primary cultures of White Leghorn chicken (chicken), Pekin duck (duck), and Greater Scaup (scaup) embryo hepatocytes. Of the 18 PAHs tested, only 8 induced EROD activity. Representative concentration-response curves for TCDD and the eight EROD-inducing PAHs are shown in Figure 4.1. In chicken and duck hepatocytes , TCDD caused biphasic concentration-response curves, characterized by an increase in EROD activity with increasing concentration, and followed by a decline in activity at higher concentrations. Similar Gaussian curves were reported for TCDD in CEH culture (Chapter 3), PLHC-1 cells (Hahn *et al.*, 1996; Hestermann *et al.*, 2000), H4IIE rat hepatoma cells (Willett *et al.*, 1997) and RTL-W1 trout liver cells (Bols *et al.*, 1999). In scaup hepatocytes, the highest TCDD dose (10 nM) did not result in a biphasic response. Consequently, the dose at which TCDD elicits maximal EROD activity in scaup hepatocytes is uncertain. The eight EROD inducing PAHs also induced biphasic concentration-response curves in all three species of hepatocytes. Relative to TCDD, the PAH concentration-response curves were shifted to the right, and exhibited attenuated maximal EROD activities. The decreased potency and efficacy of PAHs is thought to reflect differences in affinity of TCDD and PAHs for the AhR.

4.3.2. Intercompound and Interspecies comparisons

While basal EROD activities for TCDD and the eight PAHs were similar in hepatocytes from all three species, there was considerable interspecies difference in maximal EROD

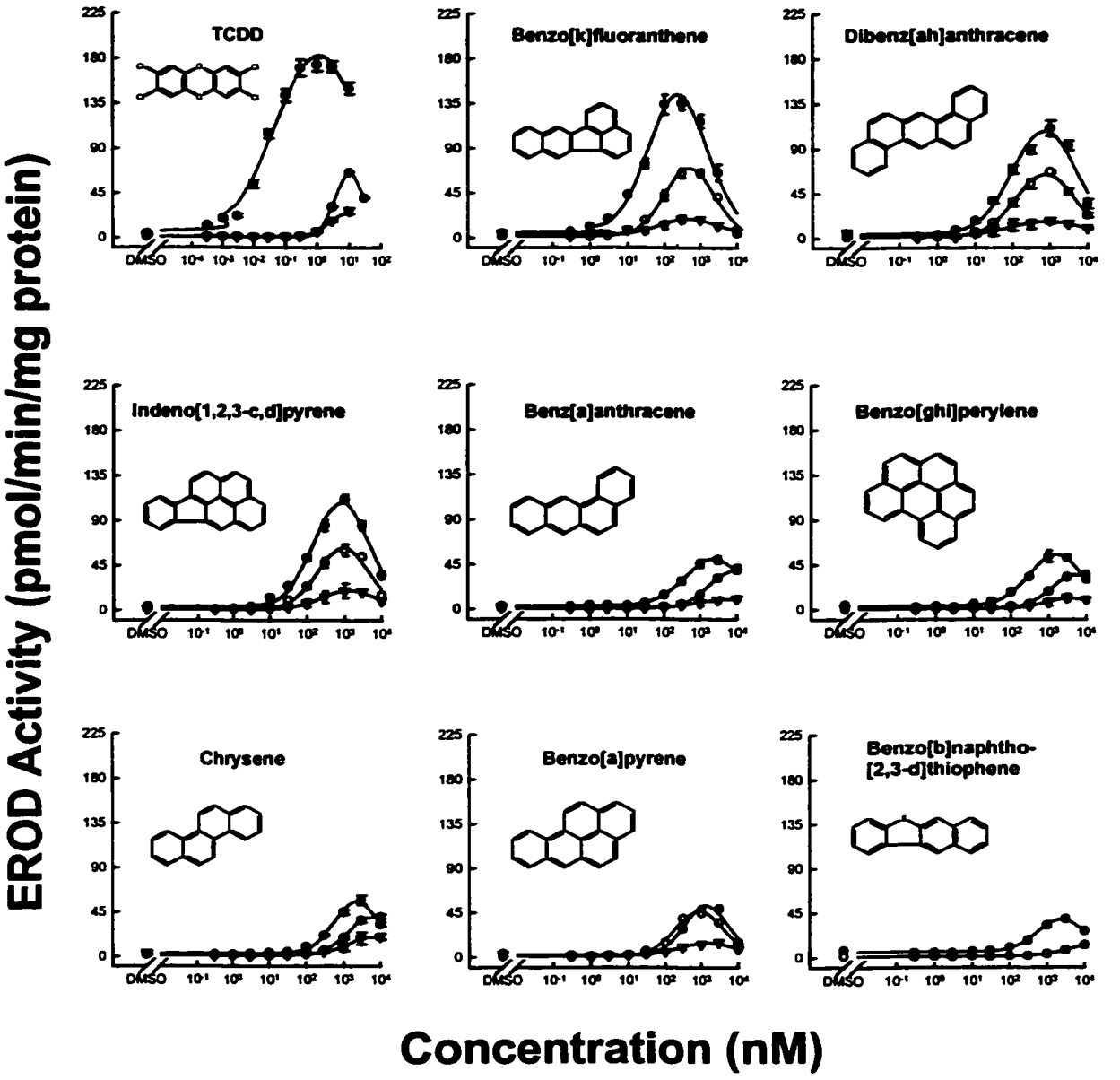

Figure 4.1. Concentration dependent effect of TCDD and PAHs on EROD activity in primary cultures of hepatocytes prepared from chicken (●), Pekin duck (○), and Greater Scaup (▼) embryos. Cells were exposed for 24 hours, after which they were rinsed, and frozen until analysed for EROD activity as described in the materials and methods section. Each concentration-response curve was obtained from a single 48 well plate. Points represent the mean of triplicates of EROD activity of each concentration, and bars represent standard error.

activities. Table 4.1 shows the maximal EROD activity and potency of TCDD and PAHs in

Table 4.1. Maximal EROD activity (pmol/min/mg protein), EC50 and ECthr values of TCDD and the eight EROD-inducing PAHs in White Leghorn chicken, Pekin Duck, and Greater Scaup hepatocytes.

	TCDD				BkF			
Species	N	Max. EROD	EC50 nM	ECthr nM	N	Max. EROD	EC50 nM ± SE	ECthr nM ± SE
W.L. Chicken	24	183	0.03	0.0013	6	144	26.3 ± 1.6	5.4 ± 0.5
Pekin Duck	9	66	3.18	1.18	4	71	79.5 ± 4.9	12.2 ± 1.8
Greater Scaup	9	27	2.28	0.62	2	17	43.9 ± 8.0	15.5 ± 0.1

	DahA				Ind			
	N	Max. EROD	EC50 nM ± SE	ECthr nM ± SE	N	Max. EROD	EC50 nM ± SE	ECthr nM ± SE
W.L. Chicken	6	109	69.6 ± 6	17.8 ± 1.9	6	109	112 ± 4.3	34 ± 1.1
Pekin Duck	2	65	88.8 ± 4.0	10.8 ± 0.4	2	63	151 ± 1.4	25.0 ± 0.1
Greater Scaup	2	17	63 ± 4.9	21.3 ± 3.2	2	21	188 ± 60	64.7 ± 30

	BaA				BghiP			
	N	Max. EROD	EC50 nM ± SE	ECthr nM ± SE	N	Max. EROD	EC50 nM ± SE	ECthr nM ± SE
W.L. Chicken	6	53	213 ± 24	131 ± 11	6	55	224 ± 23	144 ± 8.1
Pekin Duck	2	42	1514 ± 149	325 ± 19	2	37	1036 ± 4.2	252 ± 41
Greater Scaup	2	11	614 ± 132	346 ± 61	2	12	684 ± 78	438 ± 80

	Chr				BaP			
	N	Max. EROD	EC50 nM ± SE	ECthr nM ± SE	N	Max. EROD	EC50 nM ± SE	ECthr nM ± SE
W.L. Chicken	6	50	261 ± 29	167 ± 42	8	52	268 ± 22	191 ± 23
Pekin Duck	2	42	1129 ± 1.0	262 ± 42	2	50	147 ± 47	31.4 ± 15
Greater Scaup	2	20	1136 ± 56	425 ± 47	2	15	179 ± 8.5	86 ± 8.2

	BNT			
	N	Max. EROD	EC50 nM ± SE	ECthr nM ± SE
W.L. Chicken	6	39	422 ± 24	483 ± 73
Pekin Duck	3	19	4943 ± 1649	1704 ± 74
Greater Scaup			not done	not done

N = number of plates; SE = standard error

hepatocytes from all three species. In chicken and scaup hepatocytes, TCDD induced the highest EROD response (183, and 27 pmol/min/mg protein, respectively). In duck hepatocytes, however, BkF induced the highest EROD activity, with an EROD response 108% that of TCDD. Inspection of Table 4.1 shows that TCDD, BkF, DahA, and Ind induced similar maximal EROD responses in duck hepatocytes. Likewise, BkF, DahA, and Ind possessed similar maximal EROD responses in scaup hepatocytes.

There were marked differences in sensitivity of chicken, duck, and Scup hepatocytes to EROD induction by TCDD. For example, the concentration of TCDD required to induce EROD activity above basal activity in chicken hepatocytes was approximately 0.001 nM, while the concentration required to induce EROD activity in duck and scaup hepatocytes was approximately three orders of magnitude higher (Table 4.1). The range in maximal EROD activity within a species was greatest for chicken hepatocytes, and ranged 4.7-fold between the most potent compound (TCDD) to the least potent compound (BNT). The range in maximal EROD activities was less for duck and scaup hepatocytes, and ranged 3.7- and 2.5-fold, respectively. The differences in maximal EROD activity reflects the elevated sensitivity of chicken hepatocytes to TCDD relative to duck and scaup hepatocytes. Interspecies differences in the concentration or affinities of the AhR for PAHs may partially account for some of the differences in potency and efficacy of PAHs observed among the different species.

BkF was the most potent PAH in all hepatocyte cultures. Although BkF was a potent EROD inducer in chicken hepatocytes, inducing a maximal EROD activity that was 78% that of TCDD, it was almost three orders of magnitude less potent an EROD inducer than TCDD.

While there were large differences in sensitivity of chicken hepatocytes to EROD induction by TCDD and BkF, the differences in sensitivity between TCDD and BkF in both duck and scaup hepatocytes were much less. The rank order of EROD induction potency of the PAHs were similar in all three species. The rank order of EROD induction potency of the eight EROD-inducing PAHs is shown in Table 4.2. The reasons for the differences in rank order of induction potency between species is unclear, but may involve species specific differences in the concentration or characteristics of the AhR, or to other factors downstream of the AhR.

Table 4.2. Rank order of EROD-inducing potency of PAHs in hepatocytes from White Leghorn chicken, Pekin duck, and Greater Scaup.

W.L. chicken	BkF ≥ DahA > Ind > BaA > BghiP > Chr > BaP > BNT
Pekin Duck	BkF ≥ DahA > BaP ≥ Ind > BghiP ≥ Chr ≥ BaA > BNT
Greater Scaup	BkF ≥ DahA > BaP ≥ Ind > BaA ≥ BghiP > Chr

Note: BNT was not tested in scaup hepatocytes

To properly compare the potencies of individual PAHs within a species and among species, it is necessary that the concentration-response curves from which the data originated meet certain "ideal" criteria. The concentration-response curves for the PAHs were non-ideal in three critical respects: (1) curves were biphasic; (2) there were differences in maximal EROD activity of the PAHs within a species and between species; and, (3) several of the curves were non-parallel. As a consequence of these non-idealities, determination of the relative potencies of the PAHs within a species and between species was complicated. The reasons for the decrease in maximal EROD activity of the PAHs are unknown, but one of both of the following mechanisms may be partially responsible: (1) the PAHs exhibiting the greatest decrease in EROD activity (e.g., the least potent PAHs) are partial agonists, and only partially activate the

AhR signal transduction pathway; or, (2) the catalytic activity of the CYP1A enzyme is inhibited by residual inducer present in the cells. A partial agonist is defined as a substance that, at high concentrations, induces a lower maximal response relative to a standard that achieves a "full maximal" response, e.g., TCDD (Patil, 1996; Pliska, 1999) (see Appendix 8B for a more complete description of partial agonists). The mechanisms involved in the attenuation of maximal EROD activity are dealt with in fuller detail in Chapter 3.

Regardless of the mechanism(s) involved in the attenuation of EROD activity, the decrease in EROD activity can result in the overestimation of the EC50. Therefore, we developed an alternate method, referred to as the threshold method (ECthr) to estimate potency. The ECthr estimates the concentration of inducer required to elicit a response equivalent to 10% of the maximal response produced by TCDD (Appendix 8B describes the theory of the ECthr in more detail). Since the ECthr estimates potency at low concentrations of inducer, it has the advantage of being relatively insensitive to inhibitory effects of inducers on the catalytic activity of CYP1A. The ECthr may thus be a useful alternative to the EC50 for estimating the potency of compounds that behave as partial agonists. An advantage of the ECthr is that potency estimates can be made even though concentration-response curves are incomplete (i.e., are not biphasic). However, since the ECthr are often estimated in the curvilinear portion of the concentration-response resolving power may be poor and confidence intervals large (Villeneuve *et al.*, 2000).

After the EROD potency of the PAHs was determined, the relative potencies of the PAHs were determined. Two relative potency comparisons were made: (1) comparison of

potency within each species relative to TCDD or BkF (intercompound comparison); and, (2) comparison of the potency of each species to each compound relative to chicken (interspecies comparison). The intercompound comparison was made by dividing the potency (EC50 or ECthr) of TCDD by the potency (EC50 or ECthr) of each PAH for each species (Table 4.3). A second intercompound comparison was made, using BkF instead of TCDD as the reference compound (Table 4.4). Although this ratio has been referred to as the toxic equivalency factor (TEF), we prefer to use the term induction equivalency factor (IEF), since the induction of CYP1A is not a toxic response, but a biochemical response that has been shown to correlate well with some of the toxic effects of compounds with affinity for the AhR. The interspecies comparison was made by dividing the potency (EC50 or ECthr) of each compound in White Leghorn chicken by the potency (EC50 or ECthr) of each PAH in the other species (Table 4.5).

4.3.3 Induction equivalency factors

Chicken hepatocytes were considerably more sensitive to EROD induction by TCDD than duck and scaup hepatocytes. TCDD was almost two orders of magnitude more potent in chicken hepatocytes than in duck and scaup hepatocytes. In contrast, the PAHs were approximately equipotent in all three species of hepatocytes. BkF was about three orders of magnitude less potent than TCDD in chicken hepatocytes, while BkF was only 25 times and 19 times less potent than TCDD in duck and scaup hepatocytes, respectively. Four types of relative potency comparisons were made: (a) EC50-based IEFs relative to TCDD (T50-IEFs); (b) ECthr-based IEFs relative to TCDD (Tthr-IEFs); (c) EC50-based IEFs relative to BkF (B50-IEFs); and, (d) ECthr-based IEFs relative to BkF (Bthr-IEFs).

Table 4.3. Comparison of EROD-inducing potency of PAHs relative to TCDD in hepatocytes from White Leghorn chicken, Pekin duck, and Greater Scaup embryo hepatocytes using EC50-based IEFs and ECthr-based IEFs. IEF defined as EC50 (or ECthr) of TCDD / EC50 (or ECthr) of compound.

	TCDD		BkF		DahA	
Species	EC50 based IEF	ECthr based IEF	EC50 based IEF	ECthr based IEF	EC50 based IEF	ECthr based IEF
Chicken	1.0	1.0	0.0011	2.41e-04	0.0004	7.30e-05
Duck	1.0	1.0	0.0400	9.67e-02	0.0358	1.09e-01
Scaup	1.0	1.0	0.0519	4.00e-02	0.0362	2.91e-02

	Ind		BaA		BghiP	
	EC50 based IEF	ECthr based IEF	EC50 based IEF	ECthr based IEF	EC50 based IEF	ECthr based IEF
Chicken	0.0003	3.82e-05	0.0001	9.92e-06	0.0001	9.03e-06
Duck	0.0211	4.72e-02	0.0021	3.63e-03	0.0031	4.68e-03
Scaup	0.0121	9.58e-03	0.0037	1.79e-03	0.0033	1.42e-03

	Chr		BaP		BNT	
	EC50 based IEF	ECthr based IEF	EC50 based IEF	ECthr based IEF	EC50 based IEF	ECthr based IEF
Chicken	0.0001	7.78e-06	0.0001	6.81e-06	0.0001	2.69e-06
Duck	0.0028	4.50e-03	0.0216	3.76e-02	0.0006	6.92e-04
Scaup	0.0020	1.46e-03	0.0127	7.21e-03	not done	not done

The T50-IEF for BkF in chicken hepatocytes was 36 times lower than the corresponding T50-IEF in duck, and 47 times lower than the scaup T50-IEF. The Tthr-IEF for BkF in chicken hepatocytes was 400, and 165 times lower than the corresponding Tthr-IEF in duck and scaup hepatocytes, respectively (Table 4.3). In general, the TCDD-IEFs for chicken hepatocytes were two orders of magnitude lower than the TCDD-IEFs for duck and scaup hepatocytes. This is a result of the higher sensitivity of chicken hepatocytes to EROD induction by TCDD than duck and scaup hepatocytes. It is interesting to note that for all eight EROD-inducing

PAHs, the Tthr-IEFs were lower than the T50-IEFs for chicken and scaup hepatocytes, while the opposite was observed in duck hepatocytes.

There are two significant differences between EROD induction by PAHs and EROD induction by TCDD: differences in ligand-binding affinities for the AhR, and differences in pharmacokinetic factors governing the rates of metabolism of TCDD and PAHs. TCDD has a higher affinity for the AhR than PAHs, a fact is reflected in the higher EROD-inducing potency of TCDD relative to PAHs. Moreover, TCDD is poorly metabolized by CYP1A, and persistently activates the AhR pathway (Bock, 1994; Olson *et al.*, 1994). In contrast, PAHs have lower affinities for the AhR, and are relatively quickly metabolized and inactivated as CYP1A inducers (Section 3.3.4). Because of the molecular and pharmacokinetic differences between TCDD and PAHs, expressing the IEFs for PAHs relative to TCDD may be inappropriate. Therefore, we calculated a second set of IEFs in which the potency of each compound was determined relative to BkF (Table 4.4). In contrast to TCDD-IEFs which emphasize the interspecies differences in sensitivity to TCDD, the BkF-IEFs demonstrate that all three species of hepatocytes are approximately equally sensitive to EROD induction by PAHs. The range in interspecies BkF-IEFs is relatively small (between 1 and 3, to a high of 14 for BaP) compared to the TCDD-IEFs where the range in values was up to two orders of magnitude. With the exception of the DahA and Ind in duck hepatocytes, the Bthr-IEFs are lower than the B50-IEFs. Since the EC50 approach may overestimate the relative potency of compounds exhibiting attenuated maximal EROD responses relative to TCDD (i.e., partial agonists), the ECthr approach may be a better approach for estimating the potency of compounds that appear to be partial agonists. Although generalizations are difficult to make

Table 4.4. Comparison of EROD-inducing potency of PAHs relative to BkF in hepatocytes from White Leghorn chicken, Pekin duck, and Greater Scaup embryo hepatocytes using EC50-based IEFs and ECthr-based IEFs. IEF defined as EC50 (or ECthr) of BkF / EC50 (or ECthr) of compound.

	BkF		DahA		Ind	
Species	EC50 based IEF	ECthr based IEF	EC50 based IEF	ECthr based IEF	EC50 based IEF	ECthr based IEF
Chicken	1.0	1.0	0.38	0.30	0.23	0.16
Duck	1.0	1.0	0.90	1.1	0.53	0.49
Scaup	1.0	1.0	0.70	0.73	0.23	0.24

	BaA		BghiP		Chr	
	EC50 based IEF	ECthr based IEF	EC50 based IEF	ECthr based IEF	EC50 based IEF	ECthr based IEF
Chicken	0.12	0.041	0.12	0.038	0.10	0.032
Duck	0.05	0.038	0.08	0.048	0.07	0.047
Scaup	0.07	0.045	0.06	0.035	0.04	0.036

	BaP		BNT	
	EC50 based IEF	ECthr based IEF	EC50 based IEF	ECthr based IEF
Chicken	0.10	0.028	0.06	0.011
Duck	0.54	0.40	0.02	0.007
Scaup	0.25	0.18	not done	not done

with limited data sets, it appears that on a BkF-IEF basis, duck and scaup hepatocytes are equally or more sensitive than chicken hepatocytes to EROD induction by PAHs.

4.3.4. Interspecies comparisons

There was no clear trend in interspecies sensitivity to EROD induction by PAHs. Of the eight EROD-inducing PAHs, five were more potent in chicken than in duck or scaup hepatocytes. For BkF, BaA, BghiP, Chr, and BNT, the rank order of sensitivity, from most to least sensitive, to these five PAHs was: chicken > scaup > duck (Table 4.5). For DahA, Ind, and BaP, duck or scaup hepatocytes were more sensitive to EROD induction than chicken

Table 4.5. Interspecies comparison of EROD-inducing potency of PAHs in hepatocytes from White Leghorn chicken, Pekin duck and Greater Scaup expressed relative to the EROD-inducing potency of each compound in chicken. IEFs calculated as EC50 (or ECthr) of chicken / EC50 (or ECthr) species.

	TCDD		BkF		DahA	
Species	chicken EC50/ species EC50	chicken ECthr/ species ECthr	chicken EC50/ species EC50	chicken ECthr/ species ECthr	chicken EC50/ species EC50	chicken ECthr/ species ECthr
Chicken	1.0	1.0	1.0	1.0	1.0	1.0
Duck	0.01	0.001	0.33	0.44	0.78	1.65
Scaup	0.01	0.002	0.60	0.35	1.10	0.84

	Ind		BaA		BghiP	
	chicken EC50/ species EC50	chicken ECthr/ species ECthr	chicken EC50/ species EC50	chicken ECthr/ species ECthr	chicken EC50/ species EC50	chicken ECthr/ species ECthr
Chicken	1.0	1.0	1.0	1.0	1.0	1.0
Duck	0.74	1.36	0.14	0.40	0.22	0.57
Scaup	0.60	0.53	0.35	0.38	0.33	0.33

	Chr		BaP		BNT	
	chicken EC50/ species EC50	chicken ECthr/ species ECthr	chicken EC50/ species EC50	chicken ECthr/ species ECthr	chicken EC50/ species EC50	chicken ECthr/ species ECthr
Chicken	1.0	1.0	1.0	1.0	1.0	1.0
Duck	0.23	0.65	1.82	6.08	0.09	0.28
Scaup	0.23	0.39	1.50	2.22	not done	not done

hepatocytes. Chicken hepatocytes were the most sensitive to EROD induction by TCDD, PCB126, BkF, BaA, BghiP and Chr. For DahA, Ind and BaP, duck was the most sensitive species. Duck and scaup hepatocytes were both more sensitive to EROD by BaP than chicken hepatocytes.

In an egg injection study, Brunström *et al.* (1990) reported that BkF was more toxic in domestic duck embryos than in chicken embryos. While the chicken is the most sensitive species to the embryotoxic effects of TCDD and coplanar PCBs, it was less sensitive to the

embryotoxic effects of BkF than both turkey and the domestic duck. In the present study, Ind and DahA were more potent EROD inducers duck hepatocytes than in chicken hepatocytes, and BaP was more potent in both duck and scaup hepatocytes than in chicken hepatocytes. These results indicate that the *in vitro* EROD bioassay may be useful in predicting the embryotoxicity of PAHs in developing avian embryos.

4.3.5. EROD-inducing potency of zebra mussel extracts

The potency of extracts prepared from zebra mussels to induce EROD activity in hepatocytes from chicken, duck, and scaup embryos were also evaluated. The mussels were collected from three regions within the Great Lakes where scaup and other diving ducks are known to feed on zebra mussels: (1) Detroit River; (2) Western basin of Lake Erie near Monroe, Michigan; and, (3) near Sister Island, Lake Ontario. The concentration-response curves of the mussel extracts in chicken, duck, and scaup hepatocytes is shown in Figure 4.2. Each concentration-response curve corresponds to the amount of compound(s) extracted from 10g of mussel tissue. It should be noted however, that since the extracts were not analysed for PAH and PCB content, some, or perhaps most, of the EROD activity of the extracts may be due to other compounds, including HAHs, which co-extracted with PAHs.

There were marked interspecies differences in the sensitivity of hepatocytes to EROD induction by the extracts. As shown in Table 4.6, chicken hepatocytes were approximately one order of magnitude more sensitive than duck and scaup hepatocytes to EROD induction by the extracts. While all three extracts induced biphasic concentration-response curves in chicken hepatocytes, incomplete concentration-response curves were obtained in both duck and scaup

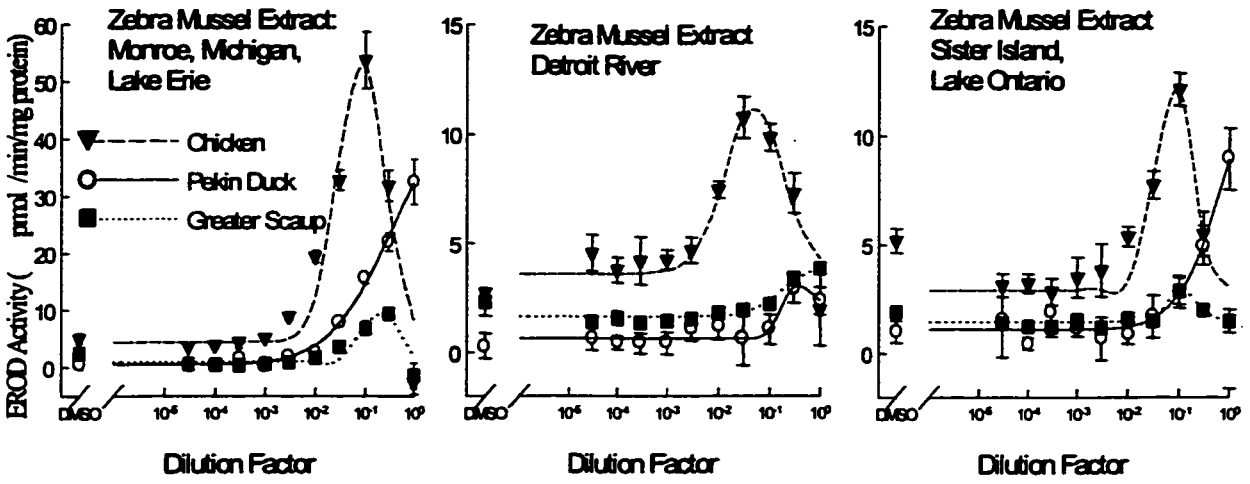

Figure 4.2. Concentration-dependent effect of extracts of zebra mussels collected from various locations in the Great Lakes on EROD activity in primary cultures of hepatocytes prepared from White Leghorn chicken, Pekin duck and Greater Scaup embryos. Each dose-response curve was prepared from the equivalent of 10 g of tissue. Points represent the mean of triplicates of each concentration, and bars represent standard error.

Table 4.6. EROD-inducing potency of zebra mussel extracts in primary cultures of White Leghorn chicken, Pekin duck, and Greater Scaup embryo hepatocytes.

	Monro, L. Erie			Detroit River			Sister Is., L. Ontario		
Species	Max EROD	EC50	ECthr	Max EROD	EC50	ECthr	Max EROD	EC50	ECthr
W.L. Chicken	53.6	0.022	0.016	11.2	0.009	nv	12.6	0.027	nv
Greater Scaup	12.2	0.097	0.08	3.9	0.16	nv	3.1	0.044	nv
Pekin duck	39.7	0.21	0.05	3.4	0.18	nv	14.6	0.73	1.67

nv = no value; max EROD activity was less than 10% of the maximum activity elicited by TCDD.
Two cell culture plates were prepared for each extract; potency estimates are geomean of both plates.

hepatocytes, suggesting that more than 10 g of mussel tissue is necessary to induce biphasic concentration-repose curves in duck and scaup hepatocyte. The rank order of species sensitivity, from most to least sensitive, was chicken >> scaup > duck, and is identical the rank order of sensitivity of in sensitivity to PAHs (Table 4.1). The rank order in EROD response (i.e., efficacy of EROD induction) was chicken > duck > scaup, and is also identical to the rank order of efficacy of PAHs (Table 4.1). The highest EROD response was observed in chicken hepatocytes exposed to the Monroe extract, and was about 28% of the maximal response

induced by TCDD. In contrast, duck and scaup hepatocytes exhibited both reduced maximal EROD activities, and incomplete concentration-response curves. The interspecies differences in sensitivity to EROD induction by the extracts may reflect species specific differences in the concentration or characteristics of the AhR, or other factor downstream of the AhR.

4.4. Discussion

Induction of CYP1A is mediated by an initial binding of a compound to the Ah receptor (AhR). Several studies have shown that a good correlation exists between the structure-binding and structure-activity relationships for various HAHs, supporting the hypothesis that binding of TCDD and structurally related aromatic hydrocarbons to the AhR is responsible for their toxic effects (Poland and Glover, 1973, 1977; Poland and Knutson, 1982; Safe, 1984). Previous studies in our laboratory have shown that chicken embryo hepatocytes possess a functional AhR signal transduction pathway, and respond with the induction of CYP1A enzymes upon exposure to TCDD and other structurally related PCBs (Kennedy *et al.*, 1996a, Lorenzen *et al.*, 1997). CYP1A induction is most commonly measured with the EROD assay which provides a measure of the catalytic activity of the CYP1A enzyme (Kennedy *et al.*, 1993, 1995). Several polycyclic aromatic hydrocarbons (PAHs) are ligands to the AhR, and are inducers of EROD activity (Bigelow and Nebert, 1982; Piskorska-Pliszczynska *et al.*, 1986). Brunström *et al.* (1991) examined the EROD-inducing potency and embryolethality of a suite of PAHs in chicken embryos. The most embryotoxic PAHs were also the most potent EROD inducers. While it is not clear whether HAHs and PAHs exert their toxicity through similar mechanisms, it is probable that at least some of the toxic effects of PAHs are mediated by the AhR.

In the present study, we examined the ability of TCDD and PAHs to induce EROD activity in primary cultures of hepatocytes prepared from chicken, duck and scaup embryos. Detailed concentration-response curves were obtained for the induction of EROD activity by TCDD and 18 PAHs in hepatocytes prepared from embryos in the late stage of embryonic development. In chicken and duck hepatocytes, TCDD induced biphasic concentration-response curves. Biphasic concentration response curves are characterized by an increase in activity with increasing concentration, followed by a decrease in activity at higher concentrations. Similar biphasic concentration-response curves were reported for TCDD in chicken embryo hepatocytes (Kennedy *et al.*, 1996a), PLHC-1 cells (Hahn *et al.*, 1995; Hestermann eta l., 2000), RTL-W1 cells (Bols *et al.*, 1999), and H4IIE rat hepatoma cells (Willett, 1997). The underlying mechanisms responsible for the biphasic response are unclear, but may involve loss or inhibition of CYP1A, or other transcriptionally controlled mechanisms (Bhat *et al.*, 1996; Hahn *et al.*, 1996; Pollenz,1996; Piechocki and Hines, 1998). TCDD did not induce a biphasic concentration-response curve in scaup hepatocytes. This was likely a consequence of administering an insufficient concentration of TCDD to induce a biphasic response. Consequently, the dose at which TCDD elicits maximal EROD activity in scaup hepatocytes is uncertain.

There were considerable interspecies differences in the sensitivity of the hepatocytes to EROD induction by TCDD. Chicken hepatocytes were the most sensitive to EROD induction by TCDD, and were approximately two orders of magnitude more sensitive than duck and scaup hepatocytes. The rank order of species sensitivity to TCDD was chicken >> scaup > duck. Chicken hepatocytes were also the most responsive to EROD induction, with a

maximal EROD response of 183 pmol/min/mg protein for TCDD, while scaup hepatocytes exhibited the lowest EROD response of 27 pmol/min/mg protein. These observation are consistent with egg injection studies in which chicken embryos proved to be much more sensitive than embryos of other avian species to the *in ovo* toxic effects of TCDD (Brunström and Reutergårdh, 1986; Brunström, 1988; Sanderson and Bellward, 1995).

Of the 18 PAHs examined for their ability to induce EROD activity in chicken, duck, and scaup hepatocytes, only eight induced EROD activity. In all three species of hepatocytes, PAHs were considerably less potent EROD inducers than TCDD. In contrast to TCDD, all three species of hepatocytes were approximately equally sensitive to EROD induction by PAH. With a few exceptions, the rank order in species sensitivity to the PAHs was chicken > scaup > duck. In chicken hepatocytes, the rank order of potency of the PAHs was BkF > DahA > Ind > BaA > BghiP > Chr > BaP > Chr. The potency ranking in duck hepatocytes was identical to the rank order in chicken hepatocytes except that the ranking of BaP and BaA were reversed. The rank order of potency in scaup hepatocytes was also identical to the rank order in chicken hepatocytes except that BaP ranked after Ind in potency (see Table 4.2). Thus, with the exception of BaP, the rank order of EROD-inducing potency of the PAHs was identical in all three species. This suggests that all three species possess a functional AhR signal transduction pathway. The interspecies differences in response to PAHs may be due to species specific differences in the concentration or characteristics of the AhR.

Recently, Karchner *et al.* (2000) reported that the basic-helix-loop-helix and Per-Arnt-Sim (bHLH-PAS) domains of the AhR in chicken and duck possessed a high degree of

conservation of amino acid sequence (97% amino acid identity). The percent identity between bird, mouse or mudpuppy was lower at 79%. The bHLH-PAS domains of the AhR are involved in ligand binding and protein interactions. Full length coding sequences are expected to be useful for investigating ligand binding affinities of the AhR. Determination of coding sequences, AhR mRNA, and protein levels may provide insight into the interspecies differences in response to TCDD and other AhR ligands. Sanderson and Bellward (1995) examined the EROD-inducing potency and AhR binding affinity of TCDD in four avian species. The EC50 of EROD induction was between one and two orders of magnitude lower in chicken than in the other species. Consistent with this, the affinity of TCDD for the chick hepatic cytosolic AhR was about 15 times higher than in the other species. Similar results for chicken and turkey were reported by Brunström and Halldin (1998). Thus, while all three species of hepatocytes appear to possess a functional AhR pathway, the differences in sensitivity to EROD induction by PAHs is likely due to differences in AhR ligand binding affinities.

In chick, two CYP1A isoforms are induced in chick embryos upon exposure to TCDD. These isoforms are not strictly orthologous to mammalian CYP1A1 and CYP1A2, and have been classified as CYP1A4 and CYP1A5 (Gannon *et al.*, 2000; Mahajan and Rifkind, 1999; Gorman *et al.*, 1998; Nakai, 1991). Recently, Gorman *et al.* (1998) examined the immunocross-reactivity of 3-methylcholanthrene (a potent EROD-inducing PAH) treated duck hepatic microsomes against polyclonal anti-chick CYP1A4/1A5 antibodies. The chick CYP1A4/1A5 antibodies reacted strongly against the duck CPY1A protein, revealing the existence of two bands of proteins, probably CYP1A4 and CYP1A5, suggesting that similar

CYP1A proteins are induced in chick and duck microsomes. Since scaup hepatocytes responded with EROD induction when exposed to PAHs, it is likely that CYP1A isoforms similar to those induced in chicken and duck were also expressed in scaup. Unfortunately, immuno-detectable CYP1A studies were not performed in the present study.

Although the EROD-inducing potency of individual PAHs were similar in all three species of hepatocytes, there were large differences in maximal EROD response (i.e., efficacy of induction). As the EROD-inducing potency of the PAHs decreased, the concentration-response curves shifted to the right, and exhibited reduced maximal EROD activities. Induction of EROD activity by TCDD differs from EROD induction principally in terms of their affinity for the AhR, and in the pharmacokinetics of metabolism. TCDD has a high affinity for the AhR than PAHs , and is poorly metabolized by CYP1A. TCDD therefore has a long biological half life and persistently activates the AhR pathway. In contrast to TCDD, PAHs are quickly metabolized and inactivated as CYP1A inducers, further decreasing their potency and efficacy as EROD inducers. Thus, relative to TCDD, PAHs are appear to act as partial agonists.

The decrease in maximal EROD response associated with decreasing potency is thought to be caused by the inhibition of CYP1A1 catalytic activity by high concentrations of residual inducer. Hahn *et al.* (1996) demonstrated that biphasic concentration-response curves are caused when CYP1A catalytic activity is inhibited or inactivated by residual inducer present in the cells. Since the EC50 may overestimate the potency of partial agonists exhibiting an attenuated maximal EROD response, we developed an alternative method of estimating potency

at a lower concentration where inhibition of catalytic activity is minimal. This method, called the ECthreshold (ECthr) method, estimates the concentration required to elicit a response equivalent to 10% of the maximal EROD response induced by TCDD. The ECthr has the advantage of being relatively insensitive to the inhibitory effects of PAHs on CYP1A activity. In a previous study (chapter 3) we demonstrated that the ECthr is a useful alternative for estimating potency, especially for compounds that are, or are suspected of being partial agonists (e.g., PAHs).

The EROD-inducing potency of the eight PAHs was expressed relative to the EROD-inducing potency of TCDD or BkF on both an EC50 and ECthr basis. This ratio is usually referred to as the toxic equivalency factor (TEF). However, since induction of EROD activity is not a toxic response, but a biochemical response we prefer to use the term induction equivalency factor (IEF) (Kennedy *et al.* 1996a). In contrast to the TCDD-IEFs in which the range of interspecies values was approximately two orders of magnitude, the range in interspecies BkF-IEFs values was relatively small. When the potency of the PAHs was expressed relative to BkF, all three species of hepatocytes were approximately equally sensitive to EROD induction. Among species, the duck and scaup BkF-IEFs were higher than chicken IEFs. In particular, the ECthr BkF-IEF values for duck and scaup were consistently equivalent or higher than the chicken ECthr BkF-IEF values, indicating that the PAHs were more potent EROD inducers in duck and scaup hepatocytes than in chicken hepatocytes. This observation is consistent with the results of Brunström *et al.* (1990) who demonstrated that duck embryos were more sensitive than chicken embryos to the *in ovo* toxic effects of PAHs. The ECthr method therefore appears to be a useful alternative to the EC50 for estimating the potency of

compounds that act as partial agonists. Due to the pharmacokinetic and AhR ligand-binding differences between TCDD and PAHs, we therefore recommend using the BkF-IEFs rather than TCDD-IEFs for determining the relative potency of compounds that are partial agonists and exhibit higher EC50s and lower maximal EROD activities relative to TCDD.

Although the induction of EROD activity in not a toxic response, several studies have demonstrated that there is a good correlation between the potency of a compound to induce CYP1A and its potency to induce toxic effects (Poland and Knutson, 1982; Safe, 1994). Brunström *et al.* (1991) injected PAHs on day 7 of development into the air cell of chick embryos, and determined mortality rates 72 h later. Hepatic EROD activities on day 10 of development were also determined. The most embryotoxic PAHs were also the most potent EROD inducers. The PAHs that caused the highest embryolethality are all known to bind to the mouse or rat AhR with high affinity (Bigelow and Nebert, 1982; Toftgård *et al.*, 1985; Piskorska-Pliszczynska, 1986). BkF, which has the highest affinity for the AhR, was the most toxic to developing embryos.

Very few studies have examined the differences in sensitivity of avian species to the *in ovo* toxic effects of PAHs. To our knowledge, the only egg injection studies examining the embryotoxic potency of PAHs in the same species used in the present study were those of Brunström *et al.* (1990, 1991). Brunström *et al.*, (1990) injected BkF into the yolk on day 4 or 5 of development of chicken, Pekin duck, turkey (*Meleagris gallopavo*), and common eider (*Somateria mollisima*) embryos, and determined mortality rates on day 18 or 24. Duck embryos were the most sensitive to the embryolethal effects of BkF. A dose of 200 µg/g egg

induced 100% mortality in duck embryos, while the same dose caused only 25% mortality in chick embryos. Thus, duck embryos are at least 4 times more sensitive than chicken embryos to the *in ovo* toxic effects of BkF. Brunström and Halldin (1998) reported that the EC50 for EROD induction by BkF in chicken and duck livers *in vitro* was 0.6 μM and 1.1 μM, respectively. The good agreement between EROD-inducing potency and embryolethality suggests that EROD induction is associated with an increase in AhR mediated CYP1A induction. Thus, the question arises as to whether it is possible to predict the *in ovo* toxicity of PAHs in duck embryos from the *in vitro* data obtained from hepatocyte cultures.

We previously demonstrated that the induction of EROD activity in primary cultures of chicken embryo hepatocytes may be a useful alternative to egg injection studies for the prediction of the toxic potency of PAHs in chicken embryos (Chapter 3; see Figure 3.4 and related discussion). Unfortunately, there are no published LD50 values for PAHs in duck embryos. However, we do Brunström *et al.* (1990) reported that duck embryos are approximately four times more sensitive than chicken embryos to the *in ovo* toxic effects of BkF. In the present study we obtained EC50 values for BkF in chicken and duck hepatocytes of 26.3 nM and 79.5 nM, respectively. The ratio of the EC50 for EROD induction by BkF in chicken and duck hepatocytes in the present study is similar to the ratio for EROD induction in the Brunström study suggesting that it may be possible to estimate LD50 values for duck embryos from duck EROD potency estimates obtained in this study.

Brunström *et al.* (1991) determined the LD50 values of the four most embryotoxic PAHs in chicken embryos (BkF, DahA, BaA, and BNT) by injecting the PAHs into the air cell.

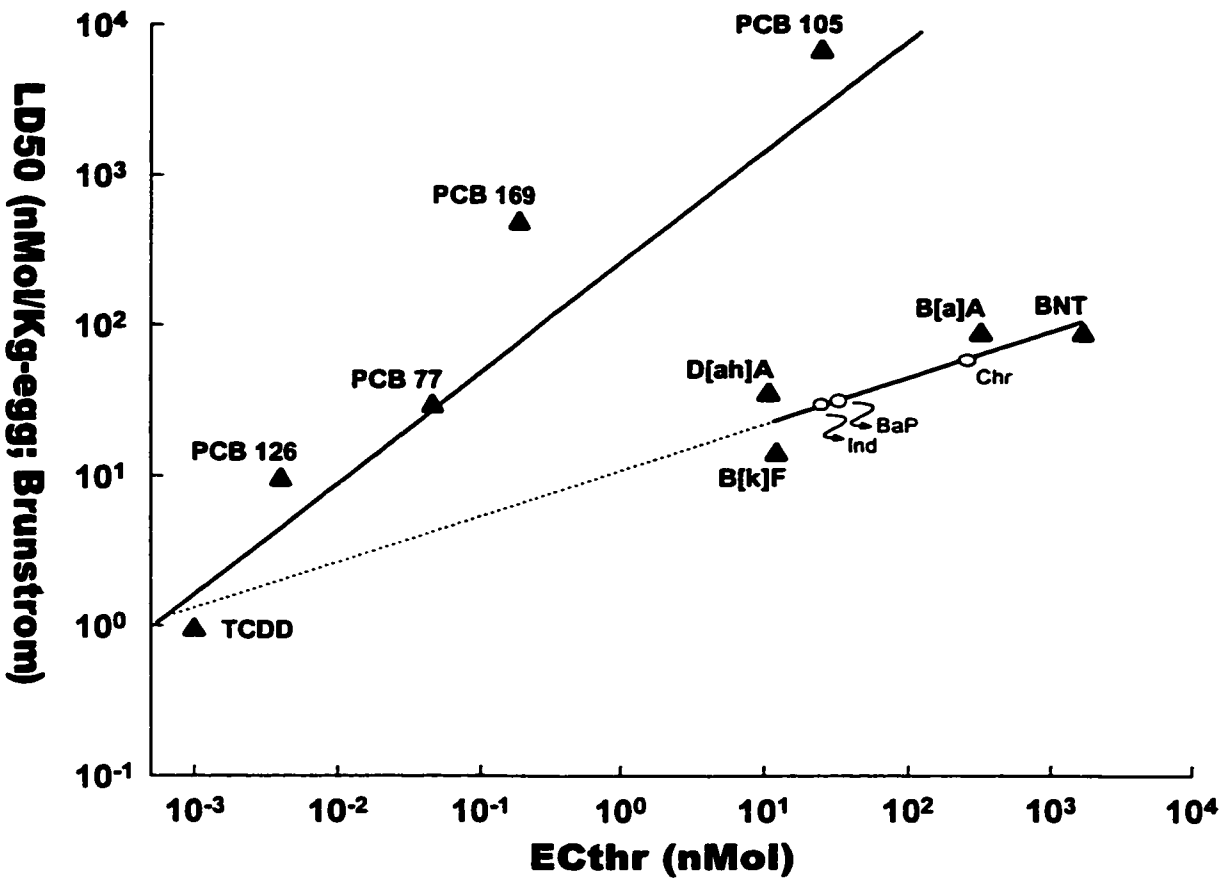

Figure 4.3. Log-log plot of the LD50 of PAHs in White Leghorn chicken embryos versus the ECthr of the same PAHs in Pekin duck hepatocytes. Since there are no published LD50 values for PAHs in duck embryos, chicken LD50 values were used. However, Brunström *et al.* (1990) reported that duck embryos were four times more sensitive than chicken embryos to the embryotoxic effects of BkF. Consequently, the LD50 values were divided by four to reflect the higher embryotoxicity of BkF in duck embryos. The LD50 values for Ind, BaP, and Chr predicted from the regression line were 29, 31, and 59 nMol, respectively. The TCDD/PCB regression line was included as a reference.

These PAHs caused 100% mortality when injected at a dose of 300 ng/g egg. Figure 4.3 shows a log-log plot of the LD50 of these four PAHs in chicken embryos versus the ECthr of the PAHs in duck hepatocytes. Figure 4.3 also shows the relationship between the *in ovo* LD50 and the *in vitro* ECthr of TCDD and four PCBs. Extension of the BkF regression line towards the Y-axis intersects the TCDD/PCB regression line. The similarity in origin of the regression lines may reflect a combination of the differences in mechanism of toxicity and rates of metabolism between TCDD and PAHs. Since BkF was four times more toxic in duck embryos

than in chicken embryos when injected into the yolk, the LD50 was dived by four to reflect the higher toxicity of the PAHs in duck embryos. From Figure 4.3, we predicted the LD50 values or Ind, BaP, and Chr. These PAHs exhibited intermediate mortality rates in the Brunström study. The predicted LD50 values for Ind, BaP, and Chr were 29, 31, and 59 nM, respectively. However, these LD50 values must be approached with caution since the rank order of EROD-inducing potency of the PAHs in chicken hepatocytes differed slightly from the rank order in duck hepatocytes. Although we realize that these results are questionable at best due to the assumptions and generalizations employed, they represent the first predictions of the *in ovo* toxic potency of PAHs in duck embryos determined from the EROD-inducing potency of PAHs in primary cultures of duck hepatocytes. Egg injection studies are necessary to validate these predictions.

Due to the lack of *in ovo* toxicity data for Greater Scaup, we hesitate to make any LD50 predictions based on *in vitro* EROD estimates. Based on ECthr estimates, it appears that scaup hepatocytes are less sensitive to EROD induction by PAHs than duck hepatocytes. This is supported by several studies that reported that wild birds are less sensitive to AhR agonists than domestic avian species (Brunström *et al.*, 1990; Kennedy *et al.*, 1996a; Lorenezen *et al.*, 1997; Brunström and Halldin, 1998). Thus, scaup hepatocytes are not more sensitive to TCDD or PAHs compared to our duck model.

Since binding of TCDD or PAHs to the AhR is a prerequisite for the manifestation of their toxic properties, it is difficult to explain why the chick embryos are more sensitive than most other avian species to the *in ovo* toxic effects of TCDD, but are less sensitive than duck

embryos to *in ovo* toxic effects of BkF. While it is tempting to suggest that species specific differences in the binding affinity of the AhR for TCDD and PAHs is a major factor contributing to species differences in sensitivity to TCDD and PAHs, there are factors other than AhR concentration or binding affinity that contribute to species sensitivity. For example, the rate of translocation of the AhR-ligand complex to the nucleus; the affinity of the AhR/Arnt dimer for the 5'-regulatory regions of the CYP1A gene (also known as XREs or DREs), and pharmacokinetic factors all contribute to the responsiveness of a species to AhR ligands (Okey, 1994).

Recent studies indicate that there are cytosolic receptors other than the AhR that possess affinity for PAHs. Bhat and Bresnick (1997) and Raha *et al.* (1995) reported that a cytosolic 4S PAH binding protein identified as glycine N-methyltransferase (GNMT) has saturable binding affinity for PAHs, translocates to the nucleus upon binding to PAHs, and is capable of inducing the expression of CYP1A. Thus, GNMT appears to function as a PAH receptor that mediates the induction of CYTP1A through an AhR independent pathway. These studies indicate that multiple CYP1A isoforms, possessing different substrate specificities, may be induced by TCDD and PAHs. Differences in the concentration or characteristics of expression of GNMT may explain some of intercompound and interspecies differences in sensitivity observed in response to exposure to TCDD and PAHs. For example, if duck embryos have a higher concentration of GNMT than chick embryos, this may partially account higher sensitivity of duck embryos to the *in ovo* toxic effects of BkF.

However, the PAHs may exert their toxicity through a mechanism other than bioactivation by CYP1A. Recent studies have shown that PAHs can be classified as bifunctional inducers since they can induce gene expression by two distinct mechanisms: (1) unmetabolized PAHs bind the AhR inducing the expression of CYP1A; and, (2) PAHs that have been metabolized to electrophilic trans-dihydrodiols (by CYP1A) activate the expression of dihydrodiol dehydrogenase (DD), isoforms of aldo-keto reductases (AKRs) (Ciaccio *et al.*, 1994). The AKRs are a superfamily of cytosolic NADP(H)-dependent oxidoreductases that catalyze the interconversion of aldehydes and ketones to alcohols of drugs and xenobiotics (Hara *et al.*, 1996). Dihydrodiol dehydrogenase catalyzes the oxidation of PAH *trans*-dihydrodiols to reactive PAH *ortho*-quinones (Smithgall *et al.*, 1988). The products of the DD reaction are transient catechols which auto-oxidize to PAH *ortho*-quinones with the concomitant generation of reactive oxygen species (ROS). PAH *ortho*-quinones enter into futile redox cycles thus amplifying the generation of ROS, and causing cytotoxic and genotoxic effects. Flowers-Geary *et al.* (1996) reported that treatment of rat hepatoma cells with PAH *ortho*-quinones resulted in the generation of *ortho*-semiquinone radicals, and the subsequent production of superoxide anion and the depletion of glutathione. Since CYP1A catalyzes the conversion of *trans*-dihydrodiol to diol-epoxides, the diversion of *trans*-dihydrodiols form diol-epoxides to *ortho*-quinones may represent an alternative pathway for the toxic potency of PAHs. The interspecies differences in sensitivity to PAHs may be related to differences in the expression of CYP1A and dihydrodiol dehydrogenase.

We also examined the potency of extracts prepared from zebra mussels to induce EROD activity in chicken, duck, and scaup hepatocytes. The mussels were colleted from locations in

the Great Lakes where Greater scaup are known to feed extensively on zebra mussels. Since the invasion of zebra mussels into the Great Lakes, several species of diving duck, including the Greater Scaup, have altered their traditional migratory patterns to take advantage of the abundant supply of zebra mussels. Relative to native clams and oysters, zebra mussels have a high lipid content, and a high rate of filtration. Consequently, the high filtering capacity (Reeders *et al.*, 1989; Fisher *et al.*, 1993), their high lipid content (Comba *et al.*, 1996; Roe and McIsaac, 1998), and their limited ability to metabolize xenobiotics (Stegeman and Hahn, 1994; Meador *et al.*, 1995) result in the bioaccumulation of a variety of organic contaminants, including PAHs, in zebra mussel tissues (Secor *et al.*, 1993; Roper *et al.*, 1996).

We were interested in determining the sensitivity of scaup hepatocytes to EROD induction by zebra mussel extracts relative to chicken and duck hepatocytes. Each extract was prepared from 10 g of mussel tissue. Although biphasic concentration-response curves were obtained in chicken hepatocytes, some of the extracts failed to induce biphasic concentration-response curves in duck and scaup hepatocytes. This suggest that either insufficient mussel tissue was used in the preparation extracts, that the mussel tissue was relatively uncontaminated, or that the duck or scaup hepatocytes were relatively insensitive to the extracts. Since the extracts were not analysed for their individual PAH and HAH content, it is possible that some of the activity of the extracts was due to halogenated aromatic compounds that coextracted with the PAH fraction.

In summary, the ability of 18 PAHs and TCDD to induce EROD activity in primary cultures of hepatocytes prepared from chicken, duck and scaup embryos was investigated.

Chicken hepatocytes were approximately two orders of magnitude more sensitive than duck or scaup hepatocytes to EROD induction by TCDD. This result is consistent with the higher sensitivity of chicken embryos relative to other avian species to the *in ovo* toxic effects of TCDD. Of the 18 PAHs tested only eight were EROD inducers. In contrast to TCDD, all three species of hepatocytes were approximately equally sensitive to EROD induction by PAHs. The interspecies rank order of sensitivity to EROD induction by the EROD inducing PAHs was chicken > Pekin duck ≥ Greater Scaup. BkF and DahA were the most potent PAHs in all three species of hepatocytes. Although there were small differences in the intercompound rank order of remaining six PAHs, these differences may insignificant. In general, scaup hepatocytes do not appear to be more sensitive to TCDD or PAHs compared to our duck model. Brunström *et al.* (1990) reported that domestic duck embryos were at least four times more sensitive than chicken embryos to the *in ovo* toxic effects of BkF. Although duck and scaup hepatocytes were less sensitive than chicken hepatocytes to EROD induction by PAHs, the interspecies difference in sensitivity is much less than that observed for TCDD. While there may be species specific differences in the mechanism(s) by which TCDD and PAHs exert their toxicity, these results suggest that the *in vitro* EROD assay has potential for screening the *in ovo* toxic potency of PAHs in developing avian embryos.

CHAPTER 5: GENERAL DISCUSSION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants of both aquatic and terrestrial ecosystems. The toxicology of PAHs is difficult to study for several reasons: (1) PAHs are structurally, and therefore chemically diverse; (2) PAHs occur in the environment as complex mixtures, including substituted PAHs and heterocyclic aromatic hydrocarbons; and, (3) PAHs exert their toxicity via several mechanisms.

Several PAHs are known ligands to the AhR and are potent inducers of the cytochrome P450 (CYP1A) enzyme system in the liver and other tissues. It is through the activation of the AhR signal transduction pathway that many of the manifestations of toxicity of these compounds are produced. Ethoxyresorufin-*O*-deethylase (EROD) is a relatively selective and sensitive biomarker for the induction of CYP1A activity by compounds with affinity for the AhR. Egg injection studies by Brunström *et al.* (1991) have shown that several PAHs are highly toxic to developing chick embryos. The most embryotoxic PAHs were also potent inducers of hepatic EROD activity in chick embryos. Brunström *et al.* (1991) reported that the EROD-inducing potency of PAHs correlated well with their *in ovo* toxicity. The correlation between the *in ovo* toxicity of PAHs and their potency to induce hepatic EROD activity in chick embryos reported by Brunström *et al.* (1991) led us to investigate the possibility of using the *in vitro* EROD bioassay to estimate the *in ovo* toxic potency of PAHs in three species of avian embryos, including Greater Scaup. The goal of the present study was two fold: (1) to evaluate the utility of the *in vitro* chicken embryo hepatocyte (CEH) EROD bioassay for predicting the *in ovo* lethality of PAHs in developing chick embryos, and (2) to use the *in vitro*

EROD bioassay to estimate the sensitivity of toxic potency of Pekin duck and Greater Scaup embryos to the *in ovo* toxic potency of PAHs.

In chapter 3, the ability of 18 PAHs to induce EROD activity in primary cultures of chicken embryo hepatocytes was examined. Of the 18 PAHs tested, only eight were found to induce EROD activity. The rank order of potency of the EROD-inducing PAHs was BkF > DahA > Ind > BaA > BghiP > Chr > BaP > BNT. The rank order *in ovo* lethality (LD50) of the four most toxic PAHs reported by Brunström *et al.* (1991) was identical to the *in vitro* rank order of EROD-inducing potency of the PAHs in chicken hepatocytes. Relative to TCDD, the PAH concentration-response curves were shifted to the right, and exhibited attenuated maximal EROD response. Compounds exhibiting concentration-response curves with these characteristics are referred to as partial agonists. A compound may behave as a partial agonist because it has a lower affinity for the AhR relative to TCDD, is metabolized faster than TCDD, or both. Finally, a compound may exhibit a reduced maximal EROD activity, when it in fact has a high affinity for the AhR, and induces the expression of the same amount of CYP1A protein as TCDD. This can occur when the catalytic activity of the CYP1A enzyme is inhibited by residual inducer.

An alternate method for estimating the potency of compounds exhibiting an attenuated maximal EROD response was evaluated. This method, called the EC threshold method (ECthr), estimates the concentration of inducer required to elicit a response equivalent to 10% of the maximal response produced by TCDD. The ECthr is less sensitive to errors of overestimation since potency is estimated at a lower concentration where inhibition of the

enzyme is less likely to occur. A log-log plot of *in ovo* LD50 plotted against the ECthr resulted in a good correlation (r2 = 0.898). Using the regression curve, we were able to predict the LD50 of the PAHs to within one order of magnitude of their actual value. This indicates that the induction of EROD activity in primary cultures of avian hepatocytes may be used to predict the toxic potency of PAHs in developing avian embryos. Although the LD50 values predicted with the EROD assay were incorrect, we feel that the EROD has potential for screening PAHs for *in ovo* toxic potency.

TCDD and several polychlorinated biphenyls (PCBs) have the capacity to deregulate the heme biosynthetic pathway resulting in the accumulation of a variety of porphyrin precursors. This leads to a conditions similar to an inheritable form of porphyria called porphyria cutanea tarda (Scarlett and Brenner, 1998; Franklin *et al.*, 1997). Unlike TCDD and PCBs, PAHs did not induce the accumulation of porphyrin precursors.

In Chapter 4, we examined the differences in the sensitivity of chicken, Pekin duck, and Greater Scaup hepatocytes to EROD induction by TCDD and 18 PAHs. Chicken hepatocytes were approximately two orders of magnitude more sensitive than Pekin duck and Greater Scaup hepatocytes to EROD induction by TCDD. This is consistent with egg injection studies that showed that chicken embryos are considerably more sensitive than embryos of other avian species to the toxic effects of TCDD. In contrast, all three species of hepatocytes were approximately equally sensitive to EROD induction by PAHs. Although all three species of hepatocytes were approximately equally sensitive to EROD induction by PAHs, in general, the rank order in species sensitivity was chicken > Greater Scaup > Pekin duck. The species

differences in sensitivity to TCDD and PAHs may be due to species specific differences in the concentration and characteristics of the AhR, to other factors downstream of the AhR, or other PAH binding proteins such as glycine N-methyltransferase (GNMT). Overall, interspecies differences in rank order of sensitivity to PAHs are subtle.

We prepared a log-log plot of the ECthr of BkF, DahA, BaP, and BNT versus LD50 of these four PAHs in chicken embryos . Chicken embryo LD50 data was used since no LD50 values were available for duck embryos. Since BkF was four times more toxic in duck embryos than in chick embryos, the LD50 values were divided by four to estimate the embryotoxicity of PAHs in duck embryos. The regression line was used to predict the embryotoxicity of PAHs in duck embryos. The predicted embryotoxicity for Ind, BaP, and Chr was 29, 31, and 59 nMol, respectively.

Over the past twenty years, numerous species of boreal subarctic seaducks, including the Greater Scaup (*Aythya marila*), have been experiencing population declines. Several hypotheses have been proposed to explain the decline in populations: (1) changes in the boreal forest where scaup nest; (2) declines in female survivorship due to changes in migratory patterns; and, (3) decline in female reproductive success due to increased contaminant loads. Several studies indicate that hens feeding on aquatic invertebrates contaminated with polycyclic aromatic hydrocarbons (PAHs) during the period of ovum development (i.e., just prior to laying of eggs) may transfer some of the dietary PAHs to the developing ovum. (Lebedev, *et al.*, 1998; Broman *et al.*, 1990). Chicken embryos are highly sensitive to the embryotoxic effects of PAHs; the LD50 of benzo[k]fluoranthene (BkF) is only about five times

higher than that of PCB 126, the most embryotoxic PCB tested in chicken embryos (Brunström *et al.*, 1991). Consequently, exposure of hens to dietary PAHs during the period of rapid yolk development may result in developmental abnormalities or death in the developing embryo.

Since the arrival of zebra mussels in the Great Lakes several species of diving ducks have altered their migratory/wintering habits to take advantage of zebra mussels (*Dreissena polymorpha*) as an abundant food source (Custer and Custer, 1996; Hamilton *et al.*, 1994; Wormington and Leach, 1992). Mussels serve as a concentrated source of nutrients and an effective conduit for the transfer of non-polar contaminants from sediments to higher trophic levels. Their efficient filter-feeding traps particle-bound contaminants, and their relative inability to metabolize xenobiotics means that contaminants are effectively bioaccumulated in their tissue (Gossiaux *et al.*, 1998; Roper *et al.*, 1996). Consumption of PAHs during the period of follicular development (when the ovum is developing and yolk is being deposited) may lead to the transfer of PAHs to the ovum. Mallard hens feeding on aquatic invertebrates in the highly contaminated Selenga River estuary of Lake Baikal, Russia, were found to contain high levels of PAHs (Lebedev *et al.*, 1998). We thus hypothesized that We Greater Scaup hens feeding on contaminated zebra mussels may transfer some of the ingested PAHs to the developing ovum. However, unlike the mallards in the Selenga River estuary which were feeding on contaminated food while on the breeding site, during the period of rapid yolk development, Greater Scaup hens were feeding on mussels while staging in the Great Lakes prior to migration to the northern breeding grounds. Since follicular development does not begin until arrival at the breeding site, it is unlikely that dietary PAHs will be transferred to the developing ovum.

Thus for species such as Greater Scaup that feed on zebra mussels while staging on the Great Lakes prior to migration to norther breeding grounds, transfer of PAHs to the egg is unlikely since follicular development begins only upon arrival at the breeding grounds. Since dietary PAHs are rapidly metabolized and eliminated, it is unlikely that dietary PAHs ingested while stagging on the Great Lakes will be incorporated into the egg. However, species that feed on zebra mussels, and remain in the Great Lakes during the breeding season may transfer dietary PAHs to the developing ovum. However, results from this study indicate that since scaup hepatocytes are relatively insensitive to EROD induction by zebra mussel extracts, scaup embryos are not likely to experience adverse effects from PAHs transferred to the egg by the hen.

RECOMMENDATIONS FOR FUTURE RESEARCH:

1. The LD50 values for chicken and Pekin duck should be determined for the eight EROD-inducing PAHs. This will provide a stronger statistical baseline against which the ECthr values can be compared. In addition, with more data points, the correlation between *in ovo* lethality and *in vitro* EROD activity will be clearer.

2. The validity of the ECthr is based on the hypothesis that the attenuation of EROD activity is a consequence of the competitive inhibition of the CYP1A enzyme by residual inducer. This will be especially prevalent amongst compounds whose EC50 is higher than that of TCDD. Since CYP1A content and EROD activity may differ, especially at high concentration of inducer, measurement of the amount of CYP1A

protein induced by PAHs will help to determine if PAHs are full or partial agonists. Western blot analysis should be conducted to determine the amount of CYP1A induced upon exposure to PAHs.

3. AhR structure-binding assays could be performed to determine the affinity of PAHs for chicken and Pekin duck AhR, and the relative amounts of AhR in hepatocytes (or other tissues) of each species. This will help in determining whether the differences in sensitivity between chicken and Pekin duck is related to interspecies differences in the affinity of PAHs for the AhR, or to the amount of AhR present in the cells. Lesser Scaup eggs (available from a commercial supplier in Western Canada) could be used as a surrogate if Greater Scaup eggs are unavailable.

4. Bhat *et al.* (1997) recently established that a 4S polycyclic aromatic hydrocarbon binding protein identified as glycine N-methyltransferase (GNMT) mediates the induction of CYP1A through an AhR-independent pathway. For species that appear to have a low or undetectable concentration of Ah receptors, but respond with the induction of CYP1A upon exposure to PAHs, GNMT may represent an alternative pathway for the expression of EROD activity. Partially purified liver preparations prepared from livers from various species, including chicken and Pekin duck, could be examined for the activity of GNMT. If GNMT is detected, binding affinity studies could be conducted to determine the relative affinity of PAHs for GNMT. Such a study may help to explain the interspecies differences in sensitivity to PAHs. In addition, polyclonal antibodies against GNMT could be introduced into chicken and Pekin duck

hepatocytes by either microinjection or by a streptolysin-O permeabilization technique (Raha *et al.*, 1995). The antibodies would irreversibly alter the structure-binding affinity of GNMT for PAHs, effectively eliminating them as a PAH binding protein. Hepatocytes would then be dosed as normal with TCDD or BkF, and CYP1A induction examined with the EROD assay. A decrease in EROD activity of hepatocytes exposed to PAHs, relative to control hepatocytes, would suggest that GNMT is present in the hepatocytes and plays a role in the metabolism of PAHs.

5. To our knowledge, porphyrin accumulation has not been determined in chick embryos exposed to PAHs. While several *in vitro* studies have demonstrated that PAHs do not induce porphyrin accumulation in cultured cells, Francis and Smith (1987) reported that administration of beta-naphthoflavone to iron-loaded mice caused inhibition of hepatic uroporphyrinogen decarboxylase and a porphyria indistinguishable from that previously reported for polyhalogenated aromatic chemicals. Egg injection studies could be conducted to determine whether PAH induce porphyrin accumulation in developing chick embryos. Similar studies should also be conducted with Pekin ducks.

6. Greater Scaup hens are thought to begin their period of follicular development (development of the ovum and laying down of yolk) only after they arrive at the nesting grounds. However, since many Greater Scaup appear to be staging in the Great Lakes for extended periods, and migrating to the northern nesting grounds late in the season, some scaup hens may begin follicular development while still staging in the Great

Lakes. If scaup hens are staging in the Great Lakes (and feeding on zebra mussels) during follicular development, PAHs may be transferred to the egg.

Greater Scaup hens could be collected while staging on the Great Lakes during the spring migration, both during their initial arrival and again when they begin their migration to the northern breeding grounds. Food consumption patterns, and PAH concentrations could be determined in various tissues (gastrointestinal tract, liver, kidney, gall bladder, gonads/ovum) at both staging periods. The reproductive status of the hens could also be determined at this time. Scaup hens showing evidence of follicular development may be transferring PAH to the developing ovum; these ova should be analysed for PAHs. Depending on resources, Greater Scaup hens could be collected at the nesting grounds and the PAH concentrations in the eggs (and other tissues) could be determined at the time of laying.

REFERENCES

Albers, P.H. (1978). The effects of petroleum of different stages of incubation in bird eggs. *Bull. Environ. Contam. Toxicol.*, **19**, 624-630.

Alsharif, N.Z., Lawson, T., and Stohs, S.J. (1994). Oxidative stress induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin is mediated by the aryl hydrocarbon (Ah) receptor complex. *Toxicology*, **92**, 39-51.

Arfsten, D.P., Schaeffer, D.J., and Mulveny, D.C. (1996). The effects of near ultraviolet radiation on the toxic effects of polycyclic aromatic hydrocarbons in animals and plants: a review. *Ecotoxicol. Environ. Saf.*, **33**, 1-24.

Bhat, R., Weaver, J.A., Sterling, K.M., and Bresnick, E. (1996). Nuclear transcription factor Oct-1 binds to the 5'-upstream region of CYP1A1 and negatively regulates its expression. *Int. J. Biochem. Cell Biol.*, **28**, 217-227.

Bhat, R., and Bresnick, E. (1997). Glycine N-methyltransferase is an example of functional diversity. Role as a polycyclic aromatic hydrocarbon-binding receptor. *J Biol. Chem.*, **272**, 21221-21226.

Bigelow, S.W., and Nebert, D.W. (1982). The Ah regulatory gene product. Survey of nineteen polycyclic aromatic compounds' and fifteen benzo[a]pyrene metabolites' capacity to bind to the cytosolic receptor. *Toxicol. Lett.*, **10**, 109-118.

Blumer, M. (1976). Polycyclic aromatic compounds in nature. *Sci. Am.*, **234**, 35-45.

Bock, K.W. (1994). Aryl hydrocarbon or dioxin receptor: biologic and toxic responses. *Rev. Physiol. Biochem. Pharmacol.*, **125**, 1-42.

Bols, N.C., Schirmer, K., Joyce, E.M., Dixon, D.G., Greenberg, B.M., and Whyte, J.J. (1999). Ability of polycyclic aromatic hydrocarbons to induce 7-ethoxyresorufin-*O*-deethylase activity in a trout liver cell line. *Ecotoxicol. Environ. Saf.*, **44**, 118-128.

Bondy, S.C., and Naderi, S. (1994). Contribution of hepatic cytochrome P450 systems to the generation of reactive oxygen species. *Biochem. Pharmacol.*, **48**, 155-159.

Bosveld, A.T.C., Kennedy, S.W., Seinen, W., and Van den Berg, M. (1997). Ethoxyresorufin-*O*-deethylase (EROD) inducing potencies of planar chlorinated aromatic hydrocarbons in primary cultures of hepatocytes from different developmental stages of the chicken. *Arch. Toxicol.*, **71**, 746-750.

Boxall, A.B., and Maltby, L. (1997). The effects of motorway runoff on freshwater ecosystems: 3. Toxicant confirmation. *Arch. Environ. Contam. Toxicol.*, **33**, 9-16.

Broman, D., Näf, C., Lundbergh, I., and Zebuhr, Y. (1990). An in situ study on the distribution, biotransformation and flux of polycyclic aromatic hydrocarbons (PAHs) in an aquatic food chain (seston-Mytilus edulus-Somateria mollissima) from the Baltic: and ecotoxicological perspective. *Environ. Toxicol. Chem.*, **9**, 429-442.

Bruner, K.A., Fisher, S.W., and Landrum, P.F. (1994). The role of the zebra mussel, *Dreissena polymorpha*, in contaminant cycling: II. Zebra mussel contaminant accumulation from algae and suspended particles, and transfer to the benthic invertebrate, Gammarus fasciatus. *J. Great Lakes*
Res., **20**, 735-750.

Brunström, B., and Reutergårdh, L. (1986). Differences in sensitivity of some avian species to the embryotoxicity of a PCB, 3,3',4,4'-tetrachlorobiphenyl, injected into the eggs. *Env. Pollut. (Series A)*, **42**, 37-45.

Brunström, B. (1989). Toxicity of coplanar polychlorinated biphenyls in avian embryos. *Chemosphere*, **19**, 765-768.

Brunström, B., Broman, D., and Näf, C. (1990). Embryotoxicity of polycyclic aromatic hydrocarbons (PAHs) in three domestic avian species, and of PAHs and coplanar polychlorinated biphenyls in the common eider. *Env. Pollut.*, **67**, 133-143.

Brunström, B. (1991). Toxicity and EROD-inducing potency of polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) in avian embryos. *Comp. Biochem. Physiol. C*, **100C**, 241-243.

Brunström, B., Broman, D., and Näf, C. (1991). Toxicity and EROD-inducing potency of 24 polycyclic aromatic hydrocarbons (PAHs) in chick embryos. *Arch. Toxicol.*, **65**, 485-489.

Brunström, B. (1992). Embryolethality and induction of 7-ethoxyresorufin *O*-deethylase in chick embryos by polychlorinated biphenyls ans polycyclic aromatic hydrocarbons having Ah receptor affinity. *Chem. Biol. Interact.*, **81**, 69-77.

Brunström, B., Broman, D., and Dencker, L. (1992). Extracts from settling particulate matter collected in the Stockholm archipelago waters: embryolethality, immunotoxicity and EROD-inducing potency of fractions containing aliphatics/monoaromatics, diaromatics or polyaromatics. *Environ. Toxicol. Chem.*, **11**, 1441-1449.

Brunström, B., and Halldin, K. (1998). EROD induction by environmental contaminants in avian embryo livers. *Comp. Biochem. Physiol.C*, **121C**, 213-219.

Brüschweiler, B.J., Würgler, F.E., and Fent, K. (1996). An ELISA assay for cytochrome P4501A in fish liver cells. *Environ. Toxicol. Chem.* **15**, 592-596.

Bucheli, T.D., and Fent, K. (1995). Induction of Cytochrome P450 as a biomarker for Environmental Contamination in Aquatic Ecosystems. *Crit. Rev. Env. Sci. Technol.*, **25**, 201-268.

Burczynski, M.E., Lin, H.K., and Penning, T.M. (1999). Isoform-specific induction of a human aldo-keto reductase by polycyclic aromatic hydrocarbons (PAHs), electrophiles, and oxidative stress: implications for the alternative pathway of PAH activation catalyzed by human dihydrodiol dehydrogenase. *Cancer Res.*, **59**, 607-614.

Burke, M.D., Prough, R.A., and Mayer, R.T. (1977). Characteristics of a microsomal cytochrome P-448-mediated reaction. Ethoxyresorufin O-de-ethylation. *Drug Metab. Dispos.*, **5**, 1-8.

Ciaccio, P.J., Jaiswal, A.K., and Tew, K.D. (1994). Regulation of human dihydrodiol dehydrogenase by Michael acceptor xenobiotics. *J. Biol. Chem.*, **269**, 15558-15562.

Comba, M.E., Metcalfe-Smith, J.L., and Kaiser, K.L.E. (1996). Zebra mussels as biomonitors for organic contaminants in the Lower Great lakes. *Water Qual. Res. J. Canada*, **31**, 411-430.

Couillard, C.M., and Leighton, F.A. (1990). The toxicopathology of Prudhoe Bay crude oil in chicken embryos. *Fundam. Appl. Toxicol.*, **14**, 30-39.

Couillard, C.M., and Leighton, F.A. (1991). Bioassays for the toxicity of petroleum oils in chicken embryo. *Environ. Toxicol. Chem.*, **10**, 533-538.

Custer, C.M., and Custer, T.W. (1996). Food habits of diving ducks in the Great Lakes after the zebra mussel invasion. *J. Field Ornithol.*, **67**, 86-99.

Dalton, T.P., Shertzer, H.G., and Puga, A. (1999). Regulation of gene expression by reactive oxygen. *Annu. Rev. Pharmacol. Toxicol.*, **39**, 67-101.

Davila, D.R., Mounho, B.J., and Burchiel, S.W. (1997). Toxicity of polycyclic aromatic hydrocarbons to the human immune system: models and mechanisms. *TEN*, **4**, 5-9.

de Kock W.C. and C.T. Bowmer (1993). Bioaccumulation, biological effects and food chain transfer of contaminants in the zebra mussel, *Dreissena polymorpha*. In: T. Nalepa and D Schloesser (Eds.), Zebra Mussels Biology, Impacts and Control, Lewis, Ann Arbor, MI, pp. 503-553.

De Matteis, F., and Marks, G.S. (1996). Cytochrome P450 and its interactions with the heme biosynthetic pathway. *Can. J. Physiol. Pharmacol.*, **74**, 1-8.

Dermott, R., and Munawar, M. (1993). Invasion of Lake Erie offshore sediments by *Dreissena*, and its ecological implications. *Can. J. Fish. Aqua. Sci.*, **50**, 2298-2304.

Dipple, A., Khan, Q.A., Page, J.E., Ponten, I., and Szeliga, J. (1999). DNA reactions, mutagenic action and stealth properties of polycyclic aromatic hydrocarbon carcinogens (review). *Int. J. Oncol.*, **14**, 103-111.

Eadie, J.B., (1984). Distribution of polycyclic aromatic hydrocarbons in the Great Lakes. In: J.O. Nriagu and M.S. Simmons (Eds.), Toxic Contaminants in the Great Lakes, Vol. 14, John Wiley and Sons, NY.

Eastbrook, R.W., (1996). Cytochrome P450: From a single protein to a family of proteins - with some personal reflections. In: C. Ioannides (Ed.), Cytochromes P450 Metabolic and Toxicological Aspects, CRC Press, Boca Raton, Florida.

Elder, G.H., and Roberts, A.G. (1995). Uroporphyrinogen decarboxylase. *J. Bioenerg. Biomemb.*, **27**, 207-214.

Ellenton, J.A. (1982). Teratogenic activity of aliphatic and aromatic fractions of Prudhoe Bay crude and fuel oil No. 2 in the chicken embryo. *Toxicol. Appl. Pharmacol.*, **63**, 209-215.

Environment Canada, Department of Fisheries, and Health and Welfare Canada (1991). Toxic Chemicals in the Great Lakes and associated effects: synopsis. National Printers.

Environment Canada (1994). Polycyclic aromatic hydrocarbons. National Printers. Canadian Environmental Protection Act; Priority Substances List Assessment Report.

Fisher, S.W., Gossiaux, D.C., Bruner, K.A., and Landrum, P.F. (1992). Investigations of the toxicokinetics of hydrophobic contaminants in the zebra mussel (*Dreissena polymorpha*). In T.
Nalepa and D. Schloesser (Eds.), Zebra Mussels Biology, Impacts, and Control, Lewis Publishers, Boca Raton, Florida, pp. 465-490.

Flowers-Geary, L., Bleczinki, W., Harvey, R.G., and Penning, T.M. (1996). Cytotoxicity and mutagenicity of polycyclic aromatic hydrocarbon ortho- quinones produced by dihydrodiol dehydrogenase. *Chem. Biol. Interact.*, **99**, 55-72.

Fox, G.A., Collins, B., Hayakawa, E., Weseloh, D.V., Ludwig, J.P., Kubiak, J.T., Erdman, T.C. (1991). Reproductive outcomes in colonial fish-eating birds: A biomarker for developmental toxicants in Great Lakes food chains. *Great Lakes Res.*, **17**, 158-167.

Francis, J.E., and Smith, A.G. (1987). Polycyclic aromatic hydrocarbons cause hepatic porphyria in iron-loaded C57BL/10 mice: comparison of uroporphyrinogen decarboxylase inhibition with induction of alkoxyphenoxazone dealkylations. *Biochem. Biophys. Res. Commun.*, **146**, 13-20.

Franklin, M.R., Phillips, J.D., and Kushner, J.P. (1997). Cytochrome P450 induction, uroporphyrinogen decarboxylase depression, porphyrin accumulation and excretion, and gender

influence in a 3-week rat model of porphyria cutanea tarda. *Toxicol. Appl. Pharmacol.*, **147**, 289-299.

Gannon, M., Gilday, D., and Rifkind, A.B. (2000). TCDD induces CYP1A4 and CYP1A5 in chick liver and kidney and only CYP1A4, an enzyme lacking arachidonic acid epoxygenase activity, in myocardium and vascular endothelium. *Toxicol. Appl. Pharmacol.*, **164**, 24-37.

Gebauer, M.B., and Wesloh, D.V. (1993). Accumulation of organic contaminants in sentinel mallards utilizing confined disposal facilities at Hamilton Harbour, Lake Ontario, Canada. *Arch. Environ. Contam. Toxicol.*, **25**, 234-243.

Giesy, J.P., Ludwig, J.P., and Tillitt, D.E. (1994). Deformities in birds of the Great Lakes region: Assessing causality. *Environ. Sci. Technol.*, **28**, 128-135.

Gilday, D., Gannon, M., Yutzey, K., Bader, D., and Rifkind, A.B. (1996). Molecular cloning and expression of two novel avian cytochrome P450 1A enzymes induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J. Biol. Chem.*, **271**, 33054-33059.

Gilbertson, M., Kubiak, T., Ludwig, J., and Fox, G. (1991). Great Lakes embryo mortality, edema and deformities syndrome (GLEMEDES) in colonial fish-eating birds: similarity to chick-edema disease. *J. Toxicol. Environ. Health*, **33**, 455-520.

Gilek, M., Bjork, M., Broman, D., Kautsky, N., and Näf, C. (1997). The role of the Blue mussel, *Mytilus edulus*, in the cycling of hydrophobic organic contaminants in the Baltic proper. *Ambio*, **26**, 202-209.

Gonzalez, F.J., and Nebert, D.W. (1990). Evolution of the P450 gene superfamily: animal-plant 'warfare', molecular drive and human genetic differences in drug oxidation. *Trends. Genet.*, **6**, 182-186.

Gorman, N., Walton, H.S., Sinclair, J.F., and Sinclair, P.R. (1998). CYP1A-catalyzed uroporphyrinogen oxidation in hepatic microsomes from non-mammalian vertebrates (chick and duck embryos, scup and alligator). *Comp. Biochem. Physiol. C*, **121C**, 405-412.

Gossiaux, D.C., Landrum, P.F., and Fisher, S.W. (1998). The assimilation of contaminants from suspended sediments and algae by the zebra mussel, *Dreissena polymorpha*. *Chemosphere*, **36**, 3181-3197.

Griffiths, R.W., Schloesser, D.W., Leach, J.H., and Kovalak, W.P. (1991). Distribution and dispersal of the zebra mussel (*Dreissena polymorpha*) in the Great Lakes region. *Can. J. Fish. Aqua. Sci.*, **48**, 1381-1388.

Guigal, N., Seree, E., Bourgarel-Rey, V., and Barra, Y. (2000). Induction of CYP1A1 by serum independent of AhR pathway. *Biochem. Biophys. Res. Commun.*, **267**, 572-576.

Hahn, M.E., Lamb, T.M., Schultz, M.E., and Smolowitz, R.M. (1993). Cytochrome P4501A induction and inhibition by 3,3',4,4'-tetrachlorobiphenyl in an Ah receptor- containing fish hepatoma cell line. (PLHC-1). *Aqua. Toxicol.*, **26**, 185-208.

Hahn, M.E., Woodward, B.L., Stegeman, J.J., and Kennedy, S.W. (1996). Rapid assessment of induced cytochrome P4501A protein and catalytic activity in fish hepatoma cells grown in multiwell plates: response to TCDD, TCDF, and two planar PCBs. *Environ. Toxicol. Chem.*, **15**, 582-591.

Hahn, M.E. (1998). The aryl hydrocarbon receptor: a comparative perspective. *Comp. Biochem. Physiol. C*, **121C**, 23-53.

Hamilton, D.J., Ankney, C.D., and Bailey, R.C. (1994). Predation of zebra mussels by diving ducks: an exclosure study. *Ecology*, **75**, 521-531.

Hamilton, J.W., Denison, M.S., and Bloom, S.E. (1983). Development of basal and induced aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity in the chicken embryo in ovo. *Proc. Natl. Acad. Sci. U.S.A*, **80**, 3372-3376.

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

Hansch, C. D., Leo, A.J., Novellino, E., Silipo, C., and Vittoria, A. (1989). Toward a quantitative comparative toxicology of organic compounds. *Crit. Rev. Toxicol.*, **19**, 185-226.

Hara, A., Matsuura, K., Tamada, Y., Sato, K., Deyashiki, Y., and Ishida, N. (1996). Relationship of human liver dihydrodiol dehydrogenases to hepatic bile-acid binding protein and an oxidoreductase of human colon cells. *Biochem. J.*, **313**, 373-376.

Hardin, J.A., Hinoshita, F., and Sherr, D.H. (1992). Mechanisms by which benzo[a]pyrene, an environmental carcinogen, suppresses B cell lymphopoiesis. *Toxicol. Appl. Pharmacol.*, **117**, 155-164.

Harvey, R.G. (1991). Polycyclic aromatic hydrocarbons: Chemistry and carcinogenicity, Cambridge University Press, New York.

Hebert, P.D.N., Muncaster, B.W., and Mackie, G.L. (1989). Ecological and genetic studies on *Dreissena polymorpha* (Pallas): a new mollusc in the Great Lakes. *Can. J. Fish. Aqua. Sci.*, **46**, 1587-1591.

Heinrich-Hirsch, B., Hoffman, D., Webb, J., and Neubert, D. (1990). Activity of aldrinepoxidase, 7-ethoxycoumarin-*O*-deethylase and 7- ethoxyresorufin-*O*-deethylase during the development of chick embryos in ovo. *Arch. Toxicol.*, **64**, 128-134.

Hestermann, E.V., Stegeman, J.J., and Hahn, M.E. Relative contributions of affinity and intrinsic efficacy to aryl hydrocarbon receptor ligand potency. *Toxicol. Appl.*, **168**, 160-172.

Hinson, J.A., and Roberts, D.W. (1992). Role of covalent and noncovalent interactions in cell toxicity: effects on proteins. *Annu. Rev. Pharmacol. Toxicol.*, **32**, 471-510.

Hoffman, D.J. (1979). Embryotoxic and teratogenic effects of petroleum hydrocarbons in mallards (Anas platyrhynchos). *J. Toxicol. Environ. Health*, **5**, 835-844.
Hoffman, D.J., and Gay, M.L. (1981). Embryotoxic effects of benzo[a]pyrene, chrysene, and 7,12-dimethylbenz[a]anthrancene in petroleum hydrocarbon mixtures in mallard ducks. *J. Toxicol. Environ. Health.*, **7**, 775-787.

Honour, S.M., Trudeau, S., Kennedy, S.W., and Wobeser, G. (1995). Experimental vitamin A deficiency in mallards (Anas platyrhynchos): lesions and tissue vitamin A levels. *J. Wildl. Dis.*, **31**, 277-288.

Hoyer, D., and Boddeke, H.W. (1993). Partial agonists, full agonists, antagonists: dilemmas of definition. *Trends Pharmacol. Sci.*, **14**, 270-275.

Huuskonen, S.E., Tuvikene, A., Trapido, M., Fent, K., and Hahn, M.E. (2000). Cytochrome P4501A induction and porphyrin accumulation in PLHC-1 fish cells exposed to sediment and oil shale extracts. *Arch. Environ. Contam. Toxicol.*, **38**, 59-69.

Jackson, J.A., Dilberto, J.J., and Birnbaum, L.S. (1993). Estimation of Octanol-water partition coefficients and correlation with dermal absorption for several polyhalogenated aromatic hydrocarbons. *Fund. Appl. Toxicol.*, **21**, 334-344.

Jerina, D.M., and Daly, J.W. (1974). Arene oxides: a new aspect of drug metabolism. *Science*, **185**, 573-582.

Kaminsky, L.S., and Fasco, M.J. (1991). Small intestinal cytochromes P450. *Crit. Rev. Toxicol.*, **21**, 407-422.

Kamps, C., and S. Safe (1987). Binding of polynuclear aromatic hydrocarbons to the rat 4S cytosolic binding protein: structure-activity relationships. *Cancer Lett.*, **34**,129-137.

Kappas, A., and Alvares, A.P. (1975). How the liver metabolizes foreign substances. *Sci. Am.*, **232**, 22-31.

Karchner, S.I., Kennedy, S.W., Trudeau, S., and Hahn, M. E. (2000). Towards molecular understanding of species differences in dioxin sensitivity: initial characterization of Ah Receptor cDNAs in birds and an amphibian. *Mar. Env. Res.*, **50**, 51-56.

Kennaway, E. (1955). The identification of a carcinogenic compound in coal-tar. *British Medical Journal*, **24**, 749-752.

Kennedy, S.W., Lorenzen, A., James, C.A., and Collins, B.T. (1993). Ethoxyresorufin-*O*-deethylase and porphyrin analysis in chicken embryo hepatocyte cultures with a fluorescence multiwell plate reader. *Anal. Biochem.*, **211**, 102-112.

Kennedy, S.W., & Jones, S.P. (1994). Simultaneous measurement of cytochrome P4501A catalytic activity and total protein concentration with a fluorescence plate reader. *Anal. Biochem.*, **222**, 217-223.

Kennedy, S.W., Jones, S.P., and Bastien, L.J. (1995). Efficient analysis of cytochrome P4501A catalytic activity, porphyrins, and total proteins in chicken embryo hepatocyte cultures with a fluorescence plate reader. *Anal. Biochem.*, **226**, 362-370.

Kennedy, S.W., Lorenzen, A., Jones, S.P., Hahn, M.E., and Stegeman, J.J. (1996a). Cytochrome P4501A induction in avian hepatocyte cultures: a promising approach for predicting the sensitivity of avian species to toxic effects of halogenated aromatic hydrocarbons. *Toxicol. Appl. Pharmacol.*, **141**, 214-230.

Kennedy, S.W., Lorenzen, A., and Norstrom, R.J. (1996b). Chicken embryo hepatocyte bioassay for measuring cytochrome P4501A-based 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent concentrations in environmental samples. *Environ. Sci. Technol.*, **30**, 706-715.

Kim, P.M., DeBoni, U., and Wells, P.G. (1997). Peroxidase-dependent bioactivation and oxidation of DNA and protein in benzo[a]pyrene-initiated micronucleus formation. *Free Radic. Biol. Med.*, **23**, 579-596.

Leach, J.H. (1993). Impacts of the zebra mussel (*Dreissena polymorpha*) on water quality and fish spawning reefs in western Lake Erie. In: T. Nalepa and D Schloesser (Eds.), Zebra Mussels Biology, Impacts and Control, Lewis Publishers, Boca Raton, Florida, pp. 381-398.

Lebedev, A.T., Poliakova, O.V., Karakhanova, N.K., Petrosyan, V.S., and Renzoni, A. (1998). The contamination of birds with organic pollutants in the Lake Baikal region. *Sci. Total Environ.*, **212**, 153-162.

Leo, J.L (1993). Calculating P_{oct} from structures. *Chem. Rev.*, **93**, 1281-1306.

Levin, W., Wood, A., Chang, R., Ryan, D., Thomas, P., Yagi, H., Thakker, D., Vyas, K., Boyd, C., Chu, S.Y., Conney, A., and Jerina, D. (1982). Oxidative metabolism of polycyclic aromatic hydrocarbons to ultimate carcinogens. *Drug Metab. Rev.*, **13**, 555-580.

Lorenzen, A., Kennedy, S.W., Bastien, L.J., and Hahn, M.E. (1997). Halogenated aromatic hydrocarbon-mediated porphyrin accumulation and induction of cytochrome P4501A in chicken embryo hepatocytes. *Biochem. Pharmacol.*, **53**, 373-384.

Lusimbo, W.S., and Leighton, F.A. (1996). Effects of Prudhoe Bay crude oil on hatching success and associated changes in pipping muscles in embryos of domestic chickens (*Gallus gallus*). *J. Wildl. Dis.*, **32**, 209-215.

Machala, M., Matlova, L., and Svoboda, I. (1996). Induction effects of polychlorinated biphenyls, polycyclic aromatic hydrocarbons and other widespread aromatic environmental pollutants on microsomal monooxygenase activities in chick embryo liver. *Arch. Toxicol.*, **70**, 362-367.

Mackay, D., Shiu, W.Y., and Ma, K.C. (1992). Illustrated handbook of physical-chemical properties and environmental fate for organic chemicals (2). Lewis Publishers, Chelsea, MI.

MacKenzie, K.M., and Angevine, D.M. (1981). Infertility in mice exposed *in utero* to benzo(a)pyrene. *Biol. Reprod.*, **24**, 183-191.

Mahajan, S.S., and Rifkind, A.B. (1999). Transcriptional activation of avian CYP1A4 and CYP1A5 by 2,3,7, 8- tetrachlorodibenzo-*p*-dioxin: differences in gene expression and regulation compared to mammalian CYP1A1 and CYP1A2. *Toxicol. Appl. Pharmacol.*, **155**, 96-106.

Mann, K.K., Matulka, R.A., Hahn, M.E., Trombino, A.F., Lawrence, B.P., Kerkvliet, N.I., and Sherr, D.H. (1999). The role of polycyclic aromatic hydrocarbon metabolism in dimethylbenz[a]anthracene-induced pre-B lymphocyte apoptosis. *Toxicol. Appl. Pharmacol.*, **161**, 10-22.

Mansuy, D. (1998). The great diversity of reactions catalyzed by cytochromes P450. *Comp. Biochem. Physiol.C*, **121C**, 5-14.

Marks, G.S., McCluskey, S.A., Mackie, J.E., Riddick, D.S., and James, C.A. (1989). Interaction of chemicals with cytochrome P-450: implications for the porphyrinogenicity of drugs. *Clin. Biochem.*, **22**, 169-175.

Marks, G.S. (1985). Exposure to toxic agents: the heme biosynthetic pathway and hemoproteins as indicator. *Crit. Rev. Toxicol.*, **15**, 151-179.

Marks, G.S., McCluskey, S.A., Mackie, J.E., Riddick, D.S., and James, C.A. (1988). Disruption of hepatic heme biosynthesis after interaction of xenobiotics with cytochrome P-450. *FASEB J*, **2**, 2774-2783.

Mattison, D.R., Shiromizu, K., and Nightingale, M.S. (1983). Oocyte destruction by polycyclic aromatic hydrocarbons. *Am. J. Ind. Med.*, **4**, 191-202.

McGowan, J.A., Cayan, D.R., and Dorman, L.M. (1998). Climate-Ocean Variability and Ecosystem Response in the Northeast Pacific. *Science*, **281**, 210-216.

Meador, J.P., Stein, J.E., Reichert, W.L., and Varanasi, U. (1995). Bioaccumulation of polycyclic aromatic hydrocarbons by marine organisms. *Rev. Environ. Contam. Toxicol.*, **143**, 79-165.

Meyers, P.A. (1984). Petroleum contaminants in the Great Lakes. In: J.O. Nriagu and M.S. Simmons (Eds.), Toxic Contaminants in the Great Lakes, Vol. 14, John Wiley and Sons, NY.

Mills, E.L., Leach, J.L., Carleton, J.T., and Secor, C.L. (1994). Exotic species and the integrity of the Great Lakes: Lessons from the past. *Bio Science*, **44**, 666-676.

Morgan, E.T., and Coon, M.J. (1984). Effects of cytochrome b5 on cytochrome P-450 catalyzed reactions: studies with manganese substituted cytochrome b5. *Drug. Metab. Disp.*, **12**, 358-364.

Mumtaz, M.M., George, J.D., Gold, K.W., Cibulas, W., and DeRosa, C.T. (1996). ATSDR evaluation of health effects of chemicals. IV. Polycyclic aromatic hydrocarbons (PAHs): understanding a complex problem. *Toxicol. Ind. Health*, **12**, 742-971.

Näf, C., Broman, D., and Brunström, B. (1992). Distribution and metabolism of Polycyclic aromatic hydrocarbons (PAHs) injected into eggs of chicken (Gallus domesticus) and common eider (*Somateria mollissma*). *Environ. Toxicol. Chem.*, **11**, 1653-1660.

Nakai, K., Ward, A.M., Gannon, M., and Rifkind, A.B. (1991). β-Naphthoflavone induction of a cytochrome P450 arachidonic acid epoxygenase in chick embryo liver distinct from the arly hydrocarbon hydroxylase and from phenobarbitol-induced arachidonate epoxygenase. *J. Biol. Chem.*, **267**, 19503-19512.

Neff, J.M. (1979). Polycyclic aromatic hydrocarbons in the aquatic environment: sources, fates, and biological effects. London: Applied Science.

Nelson, S.D., and Pearson, P.G. (1990). Covalent and noncovalent interactions in acute lethal cell injury caused by chemicals. *Annu. Rev. Pharmacol. Toxicol.*, **30**, 169-195.

Nernst, W. (1891). Vertherlungeines stoffes zwischen zwei Losungsnitteln und zwischer Losungsmittel und Dampfraum. *Z. Phys. Chem.*, **8**, 110.

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. Lett.*, **70**, 1-22.

Olson, J.R., McGarrigle, B.P., Gigliotti, P.J., Kumar, S., and McReynolds, J.H. (1994). Hepatic uptake and metabolism of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 2,3,7,8-tetrachlorodibenzofuran. *Fundam. Appl. Toxicol.*, **22**, 631-640.

Ortiz de Montellano, P.R.O., and Correia, M.A. (1983). Suicidal destruction of cytochrome P450 during oxidative drug metabolism. *Ann. Rev. Pharmacol. Toxicol.*, **23**, 481-503.

Overton, E. (1901). Studien Uber Die Narkose, Zugleich eion Beirag zur Allgemeine Pharmakologie, Fisher, Jenna.

Palmer, R.S. (1976). Greater Scaup. In: Handbook of North American Birds, Vol. 3, Yale University Press, New Haven and London.

Pathirana, S., Connell, D.W., and Vowles, P.D. (1994). Distribution of polycyclic aromatic hydrocarbons (PAHs) in an urban roadway system. *Ecotoxicol. Environ. Saf.*, **28**, 256-269.

Patil, P.N. (1996). Pharmacologic quantitation. *Indian J Exp. Biol.*, **34**, 615-633.

Penning, T.M., Ohnishi, S.T., Ohnishi, T., and Harvey, R.G. (1996). Generation of reactive oxygen species during the enzymatic oxidation of polycyclic aromatic hydrocarbon *trans*-dihydrodiols catalyzed by dihydrodiol dehydrogenase. *Chem. Res. Toxicol.*, **9**, 84-92.

Piechocki, M.P., and Hines, R.N. (1998). Functional characterization of the human CYP1A1 negative regulatory element: modulation of Ah receptor mediated transcriptional activity. *Carcinogenesis*, **19**, 771-780.

Perkins, C.R., and Barclay, J.S. (1997). Accumulation and mobilization of organochlorine contaminants in wintering Greater Scaup. *J. Wildl. Manage.*, **61**, 444-449.

Petrulis, J.R. and Bunce, N.J. (1999). Competitive inhibition by inducer as a confounding factor in the use of the ethoxyresorufin-O-deethylase (EROD) assay to estimate exposure to dioxin-like compounds. *Toxicol. Lett.* **105**, 251-260.

Piskorska-Pliszczynska, J., Keys, B., Safe, S., and Newman, M.S. (1986). The cytosolic receptor binding affinities and AHH induction potencies of 29 polynuclear aromatic hydrocarbons. *Toxicol. Lett.*, **34**, 67-74.

Pliska, V. (1999). Partial agonism: mechanisms based on ligand-receptor interactions and on stimulus-response coupling. *J. Recept. Signal Transduct. Res.*, **19**, 597-629.

Pohl, R.J., and Fouts, J.R. (1980). A rapid method for assaying the metabolism of 7-ethoxyresorufin by microsomal subcellular fractions. *Anal. Biochem.*, **107**, 150-155.

Poland, A, and Glover, E. (1973). Chlorinated dibenzo-*p*-dioxins: Potent inducers of δ-aminolevulinic acid synthetase and aryl hydrocarbon hydroxylase. II. A study of the structure-activity relationships. *Mol. Pharmacol.*, **9**, 736-747.

Poland, A., and Glover, E. (1977). Chlorinated biphenyl induction of aryl hydrocarbon hydroxylase activity: a study of the structure-activity relationship. *Mol. Pharmacol.*, **13**, 924-938.

Poland, A., and Knutson, J.C. (1982). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related aromatic hydrocarbons: Examination of the mechanism of toxicity. *Annu. Rev. Pharmacol. Toxicol.*, **22**, 517-554.

Poland, A., and Bradfield, C. (1992). A brief review of the Ah locus. *Tohoku. J. Exp. Med.*, **168**, 83-87.

Pollenz, R.S. (1996). The aryl-hydrocarbon receptor, but not the aryl-hydrocarbon receptor nuclear translocator protein, is rapidly depleted in hepatic and nonhepatic culture cells exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Mol. Pharmacol.*, **49**, 391-398.

Ponting, C.P., and Aravind, L. (1997). PAS: a multifunctional domain family comes to light [letter]. Curr. Biol., **7**, R674-R677.

Powis, G., Drummond, A.H., Macintyre, D.E., and Jondorf, W.R. (1976). Development of liver microsomal oxidations in the chick. *Xenobiotica*, **6**, 69-81.

Prough, R.A. (1996). Introduction: basal and inducible expression of cytochromes P450 and related enzymes. *FASEB J.*, **10**, 807-808.

Raha, A., Wagner, C., MacDonald, R.G., Bresnick, E. (1994). Rat liver cytosolic 4 S polycyclic aromatic hydrocarbon-binding protein is glycine N-methyltransferase. *J. Biol. Chem.*, **269**, 5750-5756.

Raha, A., Joyce, T., Gusky, S., and Bresnick, E. (1995). Glycine N-methyltransferase is a mediator of cytochrome P4501A1 gene expression. *Arch. Biochem. Biophys.*, **322**, 395-404.

Ramana, K.V., and Kohli, K.K. (1998). Gene regulation of cytochrome P450--an overview. *Indian J. Exp. Biol.*, **36**, 437-446.

Rattner, B.A. (1981). Tolerance of adult mallards to subacute ingestion of crude petroleum oil. *Toxicol. Lett.* , **8**, 337-342.

Readman, J.W., Mantoura, R.F., and Rhead, M.M. (1987). A record of polycyclic aromatic hydrocarbon (PAH) pollution obtained from accreting sediments of the Tamar Estuary, U.K.: evidence for non- equilibrium behaviour of PAH. *Sci. Total. Environ.*, **66**, 73-94.

Reeders, H.H., Bij deVaate, A., and Slim, F.J. (1989). The filtration of *Dreissena polymorpha* (Bivalva) in three Dutch lakes with reference to biological water quality management. *Freshwater Biol.*, **22**, 133-141.

Richet, M.C. (1893). Note sur le rapport entre la toxiatie et les properties physiques des corps. *Soc. Biol. Mem.*, **45**, 775.

Riddick, D.S., Huang, Y., Harper, P.A., and Okey, A.B. (1994). 2,3,7,8-Tetrachlorodibenzo-p-dioxin versus 3-methylcholanthrene: comparative studies of Ah receptor binding, transformation, and induction of CYP1A1. *J. Biol. Chem.* **269**, 12118-12128.

Rifkind, A.B., Kanetoshi, A., Orlinick, J., Capdevila, J.H., and Lee, C. (1994). Purification and biochemical characterization of two major cytochrome P- 450 isoforms induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in chick embryo liver. *J. Biol. Chem*, **269**, 3387-3396.

Roe, S.L., and MacIssac, H.J. (1998). Temporal variation of organochlorine contaminants in the zebra mussel, *Dreissena polymorpha*, in Lake Erie. *Aqua. Toxicol.*, **41**, 125-140.

Roper, J.M., Cherry, D.S., Simmers, J.W., and Tatem, H.E. (1996). Bioaccumulation of toxicants in the zebra mussel, *Dreissena polymorpha*, at the Times Beach confined disposal facility, Buffalo, New York. *Environ. Pollut.*, **94**, 117-129.

Ross, J.A., & Nesnow, S. (1999). Polycyclic aromatic hydrocarbons: correlations between DNA adducts and ras oncogene mutations. *Mutat. Res.*, **424**, 155-166.

Rowlands, J.C., and Gustafsson, J.A. (1997). Aryl hydrocarbon receptor-mediated signal transduction. *Crit. Rev. Toxicol.*, **27**, 109-134.

Safe, S. (1990). Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and related compounds: Environmental and mechanistic considerations which support the development of toxic equivalency factors (TRFs). *Crit. Rev. Toxicol.*, **51**, 51-88.

Safe, S.H. (1994). Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. *Crit. Rev. Toxicol.*, **24**, 87-149.

Sanderson, J.T., Kennedy, S.W., and Giesy, J.P. (1998). In vitro induction of ethoxyresorufin-*O*-deethylase and porphyrins by halogenated aromatic hydrocarbons in avian primary hepatocytes. *Environ. Toxicol. Chem.*, **17**, 2006-2018.

Sanderson, J.T., and Bellward, G.D. (1995). Hepatic microsomal ethoxyresorufin O-deethylase-inducing potency in ovo and cytosolic Ah receptor binding affinity of 2,3,7,8-tetrachlorodibenzo-p-dioxin: comparison of four avian species. *Toxicol. Appl. Pharmacol.*, **132**, 131-145.

Santodonato, J. (1997). Review of the estrogenic and antiestrogenic activity of polycyclic aromatic hydrocarbons: relationship to carcinogenicity. *Chemosphere*, **34**, 835-848.

Scarlett, Y.V., and Brenner, D.A. (1998). Porphyrias. *J. Clin. Gastroenterol.*, **27**, 192-198.

Schmidt, J.V. and, Bradfield, C.A. (1996). Ah receptor signaling pathways. *Ann. Rev. Cell Dev. Biol.*, **12**, 55-89.

Secor, C.L., Mills, E.L., Harshbarger, J., Kuntz, H.T., Gutenmann, W.H., and Lisk, D.J. (1993). Bioaccumulation of toxicants, element and nutrient composition, and soft tissue histology of zebra mussels (*Dreissena polymorpha*) from New York State waters. *Chemosphere*, **26**, 1559-1575.

Shou, M., Krausz, K.W., Gonzalez, F.J., and Gelboin, H.V. (1996). Metabolic activation of the potent carcinogen dibenzo[a,l]pyrene by human recombinant cytochromes P450, lung and liver microsomes. *Carcinogenesis*, **17**, 2429-2433.

Silkworth, J.B., Lipinskas, T., and Stoner, C.R. (1995). Immunosuppressive potential of several polycyclic aromatic hydrocarbons (PAHs) found at a Superfund site: new model used to evaluate additive interactions between benzo[a]pyrene and TCDD. *Toxicology*, **105**, 375-386.

Sinclair, P.R., Bement, W.J., Bonkovsky, H.L., and Sinclair, J.F. (1984). Inhibition of uroporphyrinogen decarboxylase by halogenated biphenyls in chick hepatocyte cultures. Essential role for induction of cytochrome P-448. *Biochem. J.*, **222**, 737-748.

Sinclair, P.R., Gorman, N., Walton, H.S., Sinclair, J.F., Lee, C.A., and Rifkind, A.B. (1997a). Identification of CYP1A5 as the CYP1A enzyme mainly responsible for uroporphyrinogen oxidation induced by Ah receptor ligands on chicken liver and kidney. *Drug Metab. Disp.*, **25**, 79-783.

Sinclair, P.R., Walton, H.S., Gorman, N., Jacobs, M.M., and Sinclair, J.F. (1997b). Multiple roles of polyhalogenated biphenyls in causing increases in cytochrome P445 and uroporphyrin accumulation in cultured hepatocytes. *Toxicol. Appl. Pharmacol.* **147**, 171-179.

Sinclair, P.R., Gorman, N., Walton, H.S., Bement, W.J., Dalton, T.P., Sinclair, J.F., Smith, A.G., and Nebert, D.W. (2000). CYP1A2 is essential in murine uroporphyria caused by hexachlorobenzene and iron. *Toxicol.Appl.Pharmacol.*, **162**, 60-67.

Sinning, A.R. (1998). Role of Vitamin A in the formation of congenital heart defects. *Anat. Rec.*, **253**:147-153.

Smithgall, T.E., Harvey, R.G., and Penning, T.M. (1988). Spectroscopic identification of *ortho*-quinones as the products of polycyclic aromatic *trans*-dihydrodiol oxidation catalyzed by dihydrodiol dehydrogenase. *J. Biol. Chem.*, **263**, 1814-1820.

Spacie, A. and, Hamelink, J.L. (1985). Bioaccumulation. In: G.M. Rand and S.R. Petrocelli (Eds.), Fundamentals of aquatic toxicology: methods and application, Hemisphere Pub. Corp., New York, pp. 495-525.

Stagg, R.M., Rusin, J., McPhail, M.E., McIntosh, A.D., Moffat, C.F., and Craft, J.A. (2000). Effects of polycyclic aromatic hydrocarbons on expression of CYP1A in salmon (*Salmo salar*) following experimental exposure and after the *Braer* oil spill. *Environ. Toxicol. Chem.*, **19**, 2797-2805.

Stanczykowska, A. (1977). Ecology of *Dreissena polymorpha* (Pallas) (Bivalva) in lakes. *Pol. Arch. Hydrobiol.*, **46**, 461-530.

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: D.C. Malins, G.K.Ostrander (Eds.), Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives, CRC Press, Boca Raton, Florida, pp. 87-209.

Stronkhorst, J., Ysebaert, T.J., Smedes, F., Meiningr, P.L., Dirksen, S., and Boudewijn, T.J. (1993). Contaminants in eggs of some waterbirds species form the Scheldt Estuary, SW Netherlands. *Marine Pollution Bulletin*, **26**, 572-578.

Sturkie, P.D. (1976). Reproduction in the female and egg production. In: P.D. Sturkie, (Ed.), Avian Physiology, Springer-Verlag, New York, pp 302-326.

Stryer, L. (1988). Biosynthesis of lipids, page 566-567. In: Biochemistry, W.H. Freeman and Company, New York.

Suess, M.J. (1976). The environmental load and cycle of polycyclic aromatic hydrocarbons. *Sci.Total Environ.*, **6**, 239-250.

Swackhamer, D.L., and Skoglund, R.S. (1993). Bioaccumulation of PCBs by algae: kinetics versus equilibrium. *Environ. Toxicol. Chem.*, **12**, 831-838.

Toftgård, R., Franzén, B., Gustafsson, J.-Å., and Löfroth, G. (1985). Characterization of the TCDD-receptor ligands present in extracts of urban particulate matter. *Environ. Int.*, **11**, 369-374.

Taylor, B.L., and Zhulin, I.B. (1999). PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiol. Mol. Biol. Rev.*, **63**, 479-506.

Tukey, R.H. and E.F. Johnson (1990). Molecular aspects of the regulation and structures of the drug-metabolizing enzymes. In: W.B. Pratt and P. Taylor (Eds.), Principles of drug action: the basis of pharmacology, Churchill Livingstone Inc., New York, pp 423-467.

Tysklind, M., Bosveld, A.T.C., Andersson, P., Verhallen, E., Sinnige, T., Seinen, W., Rappe, C., and Van den Berg, M. (1995). Inhibition of ethoxyresorufin-*O*-deethylase (EROD) activity in mixtures of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and polychlorinated biphenyls. *Environ. Sci. Pollut.*, **2**, 211-216.

van Birgelen, A.P., Fase, K.M., van der Kolk, J., Poiger, H., Brouwer, A., Seinen, W., and Van den Berg, M. (1996). Synergistic effect of 2,2',4,4',5,5'-hexachlorobiphenyl and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on hepatic porphyrin levels in the rat. *Environ. Health Perspect.*, **104**, 550-557.

Vanglider, L.D., and Peterle, T.J. (1981). South Louisiana crude oil or DDE in the diet of mallard hen-effects on egg quality. *Bull. Env. Contam. Toxicol.*, **26**, 328-336.

Van Veld, P.A., Vetter, R.D., Lee, R.F., and Patton, J.S. (1987). Dietary fat inhibits the intestinal metabolism of the carcinogen benzo[a]pyrene in fish. *J. Lipid Res.*, **28**, 810-817.

Van Veld, P.A., Vogelbein, W.K., Cochran, M.K., Goksoyr, A., and Stegeman, J.J. (1997). Route-specific cellular expression of cytochrome P4501A (CYP1A) in fish (*Fundulus heteroclitus*) following exposure to aqueous and dietary benzo[a]pyrene. *Toxicol. Appl. Pharmacol.*, **142**, 348-359.

Vermeulen, N.P.E., (1996). Role of Metabolism in Chemical Toxicity. In: C. Ioannides (Ed.), Cytochromes P450 Metabolic and Toxicological Aspects, CRC Press, Boca Raton, Florida.

Vetter, R.D., Carey, M.C., and Patton, J.S. (1985). Coassimilation of dietary fat and benzo(a)pyrene in the small intestine: an absorption model using the killifish. *J. Lipid Res.*, **26**, 428-434.

Villeneuve, D.L., R.L. Crunkilton, and W.M. DeVita (1995). Identification of Cytochrome P4501A inducers in Complex Mixtures of Polycyclic Aromatic Hydrocarbons (PAHs). Society of Environmental Toxicology and Chemistry, Second World Congress, Vancouver, Canada, Abstract No. PT215.

Villeneuve, D.L., Blankenship, A.L., and Giesy, J.P. (2000). Relative potency estimates on In Vitro bioassay results: response equivalence regression (RER) approach and a framework to guide method selection. Environ. Toxicol. Chem. (In Press).

Waid, J.S. (1986). PCBs and the environment. V. 2. CRC Press, Boca Raton, Florida.

Walker, M.K., Heid, S.E., Smith, S.M., and Swanson, H.I. (8-30-0). Molecular characterization and developmental expression of the aryl hydrocarbon receptor from the chick embryo. *comp. Biochem. Physiol. C. Pharmacol. Toxicol. Endocrinol.*, **126**, 305-319.

Walker, M.K., Pollenz, R.S., and Smith, S.M. (997). Expression of the aryl hydrocarbon receptor (AhR) and AhR nuclear translocator during chick cardiogenesis is consistent with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced heart defects. *Toxicol. Appl. Pharmacol.*, **143**, 407-419.

Wells, P.G., Kim, P.M., Laposa, R.R., Nicol, C.J., Parman, T., and Winn, L.M. (1997). Oxidative damage in chemical teratogenesis. *Mutat. Res.*, **396**, 65-78.

Willett, K.L., Gardinali, P.R., Sericano, J.L., Wade, T.L., and Safe, S. (1997). Characterization of the H4IIE rat hepatoma cell bioassay for evaluation of environmental samples containing polynuclear aromatic hydrocarbons (PAHs). *Arch. Environ. Contam. Toxicol.*, **32**, 442-448.

Whitlock, J.P.J. (1990). Genetic and molecular aspects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin action. *Annu. Rev. Pharmacol. Toxicol.*, **30**, 251-277.

Whitlock, J.P.J., Okino, S.T., Dong, L., Ko, H.P., Clarke-Katzenberg, R., Ma, Q., and Li, H. (1996). Cytochromes P450 5: induction of cytochrome P4501A1: a model for analyzing mammalian gene transcription. *FASEB J.*, **10**, 809-818.

Whitlock, J.P.J. (1999). Induction of cytochrome P4501A1. *Annu. Rev. Pharmacol. Toxicol.*, **39**, 130-125.

Wilson, C.L., and Safe, S.H. (1998). Mechanisms of ligand-induced aryl hydrocarbon receptor-mediated biochemical and toxic responses. *Toxicol. Pathol.*, **26**, 657-671.

Wormington, A., and Leach, J.H. (1992). Concentrations of migrant diving ducks at Point Pelee National Park, Ontario, in response to invasion of zebra mussels, *Dreissena polymorpha*. *Canadian Field-Naturalist*, **106**, 376-380.

Zhu, B.T. (1996). Rational design of receptor partial agonists and possible mechanisms of receptor partial activation: a theory. *J. Theor. Biol.*, **181**, 273-291.

Zile, M.H. (1998). Vitamin A and embryonic development: an overview. *J. Nutr.*, **128**,455S-458S.

Appendix 1. Preparation of Dilutions of PAHs.

PAHs were dissolved in DMSO to give stock solutions of 1.0 mg/ml. Solubilization was generally slow and required shaking overnight, followed by sonication. Stock solutions were serially diluted in DMSO to give working solutions 200 times the final desired experimental concentration (*addition of 2.5 µl of DMSO to 500 µl of culture medium results in a 200 times dilution). Working concentrations were ranging from 10,000 nM to 0.3 nM were prepared in DMSO. The working solutions were stored at room temperature in the dark. Serial dilutions were prepared as described below.

(A) Prepare stock solution in DMSO at x mg/ml (Stock '[A]') [g/L]

(B) Highest concentration of working solution = (C)(αM)(MW)

Where: (C) = Concentration Factor = 200

(αM) = Highest molarity of PAH working solution (e.g., 1.0×10^{-5} M) [mol/L]

(MW) = Molecular weight of PAH [g/mol]

(C) Determine dilution factor (F) to prepare highest working concentration from Stock [A]:

$$F = \frac{\text{Stock [A]}}{(C)(\alpha M)(MW)}, \quad \frac{[g/L]}{[g/mol] \bullet [mol/L]} \Rightarrow F = \frac{x}{(C)(\alpha M)(MW)} \qquad \text{[unitless]}$$

(D) Preparation of highest concentration working solution form Stock [A]: prepare (Q) µl of each sample (e.g., 1000 µl) by diluting (V) µl of Stock [A] in DMSO

$$\mu l \text{ Stock [A]} = V = \frac{Q}{F} \ [\mu l] \Rightarrow V = \frac{Q}{(x)[(C)(\alpha M)(MW)]^{-1}}$$

Therefore Volume of Stock [A] to make 1.0×10^{-5} M working solution:

$V = (C)(Q)(\alpha M)(MW)(x)^{-1}$ Make up volume to 1000 µl with (Q-V) µl DMSO

EXAMPLE:

For benzo[a]pyrene: C = 200; Q = 1000 µl; αM = 1×10^{-5} M; MW = 252.3; x = 1.0 g/L

Therefore: Volume of Stock [A] to make 10,000 nM working solution:

$V = (200)(1000)(10^{-5})(252.3)(1)^{-1}$

V = 504.6 µl Stock [A] + (1000 - 504.6 µl) DMSO

Thus, the schedule for preparing dilutions of B[a]P working solutions:

10,000 nM = 504.6 µl Stock [A] + 495.4 µl DMSO;
3,000 nM = 151.38 µl Stock [A] + 848.62 µl DMSO;
1,000 nM = 50.46 µl Stock [A] + 949.54 µl DMSO;
300 nM = 30 µl 10,000 nM + 970 µl DMSO; 100 nM = 10 µl 10,000 nM + 990 µl DMSO;
30 nM = 30 µl 1,000 nM + 970 µl DMSO; 10 nM = 10 µl 1,000 nM + 990 µl DMSO;
3 nM = 30 µl 100 nM + 970 µl DMSO; 1 nM = 10 µl 100 nM + 990 µl DMSO;
0.3 nM = 30 µl 10 nM + 970 µl DMSO; 0.1 nM= 10 µl 10 nM + 990 µl DMSO.

Appendix 2. Catalogue numbers of chemicals and reagents used in this study.

1-methylpyrene	Fluka	69025
anthracene	Sigma	A-3885
benzo[a]pyrene	Sigma	B-1760
benzo[b]naphtho[2,3-d]thiophene	EQ laboratories	206050
benzo[e]pyrene	Sigma	B-8382
benzo[k]fluoranthene	Fluka	12488
benz[a]anthracene	Sigma	B-2750
benzo[ghi]perylene	Sigma	B-6511
Bovine Serum Albumin:		
-for cell culture	Sigma	A-4503
-for EROD standard	Sigma	A-7030
chrysene	Sigma	C-6898
collagenase	Sigma	C-0130
coronene	Aldrich	33,535-5
dibenzothiophene	Aldrich	34,783-3
dibenz[ah]anthracene	Sigma	D-8534
DNAse	Boehringer Manheim	1284 932
ethoxyresorufin	Sigma	E-3763
fluoranthene	Sigma	F-4418
fluorene	Sigma	F-6379
fluorescamine	Sigma	F-9015
indeno[1,2,3-cd]pyrene	Supelco	48499
insulin	Sigma	I-5500
L-thyroxine	Sigma	T-2501
NADPH	Sigma	N-1630
penicillin G sodium salt	Sigma	PEN-NA
Percoll	Pharmacia Biotech	17-0891-01
perylene	Sigma	P-8625
phenanthrene	Sigma	P-2528
porphyrins standards	Porphyrin Products	--
pyrene	Sigma	P-2146
resorufin	Sigma	R-3257
streptomycin sulfate	Sigma	S-6501
sucrose	BDH	7654-36
Waymouth medium	GIBCO	MB 752/1

Appendix 3. Tissue extraction procedure.
Tissue was extracted from shell and homogenized in a 25 ml stainless steel Sorvall cup Sorvall (Omni Mixer #17105, Dupont, Newtown, CT)

(A) Tissue Preparation: (all glass ware was pre-cleaned with dichloromethane (DCM):hexane (1:1) before use)

7. Weigh sample; 5 to 10 g
8. Add 30 g pre-cleaned anhydrous Na_2SO_4 to tissue in a mortar and pestle
9. Grind tissue in Na_2SO_4 till all tissue dispersed in Na_2SO_4; allow to dry 20 to 30
10. Add approximately 50 ml DCM:hexane to column
11. Add Na_2SO_4/tissue to column; all tissue/Na_2SO_4 should be submerged
12. Wash mortal and pestle 3x with DCM:hexane into column
13. Allow to sit for 30 to 60 minutes
14. Elute supernatant to just above tissue into a collection flask
15. Add 250 ml of DCM:hexane and elute tissue to dryness into two 250 ml collection flasks
16. Evaporate (Rotavapour, Büchi, Switzerland) at 37°C until total volume of both flasks has been reduced to approx. 5 ml
17. Filter eluant into a graduated TT with a pre-cleaned 0.22 μm filter in a glass syringe
18. Bring to a known volume (e.g., 25 ml) with DCM:hexane
19. Remove a 1 ml sample (with a pre-cleaned 2 ml pipette) to a pre-weighed tin weigh boat, allow solvent to evaporate, and re-weigh to determine lipid percent (may dry for 1 hr in 105°C oven) for calculation of percent lipid, see bottom
20. Transfer eluate to small rotovap flask (rinse TT 3x with DCM:hexane into flask) and rotovap to approx. 1 ml.

(B) Preparation Of GPC: (Column packed with fluorsil washed with DCM:hexane)

1. Volume solvent = (flow rate) x (# samples +1) x (time of extraction)
   where: flow rate = 5 ml/min
   time of extraction = dump time + collect time = approx. 60 min
2. Bring column in line for 10 to 15 min to re-wet column
3. Transfer sample from flask to glass syringe (about 1 ml) and inject sample into GPC
4. Wash flask 3x with 1 ml DCM:hexane
5. Inject final volume of 1.5 ml DCM:hexane to "chase" sample from dead volume of GPC port into sample loop (Max. Volume of sample + wash + chase = 605 ml)
6. Set pressure gauge to: Dump = 6 psi
   Load = 4-5 psi
   Run = 4 psi
7. Set dump and Collect times to 30 minutes each

(C) Calculation Of % Lipid:

$$\% \text{ Lipid} = \frac{W_L}{W_T} \times \frac{V_T}{V_e} \times 100$$

Where: W_L = Weight of lipid (see A15 of tissue extraction procedure)
W_T = Total (wet) weight of sample
V_T = Total volume of extract (e.g., 50 ml)
V_e = Volume of extract used in lipid determination (e.g., 2 ml)

(D) *Preparation Of DSMO-Extracts*:

After running samples through GPC:
- rotovap PAH fraction to approx. 5 ml
- during this process a green precipitate formed; this is probably pigments (chlorophyl?), extract was washed several times to remove as much precipitate as possible
- transfer to 25 ml volumetric flask (wash rotovap flask and syringe 3x hexane)
- make up volume to 25 ml with hexane
- transfer a volume equivalent to 10 g of tissue was of each sample to a 50 ml rotovap flask
- with rotovap apparatus at 90° to water bath, rotovap sample to small volume (i.e., 1mL)
- add 200 µl DMSO directly to sample
- rotovap off remaining solvent
- DMSO-extract was transferred to sample jars, and serial dilutions were prepared in DMSO as outline in the dilution schedule below:

Note: the 200 µl extract-DMSO represents a 1x (stock) for bioassay

1	(1X)	Stock 200 µl	→ stock **A**
0.3	(0.3)	60 µl '**A**' + 140 µl DMSO	
0.1	(0.1)	20 µl '**A**' + 180 µl DMSO	→ stock **B**
0.03	($3X10^{-2}$)	60 µl '**B**' + 140 µl DMSO	
0.01	($1X10^{-2}$)	20 µl '**B**' + 180 µl DMSO	→ stock **C**
0.003	($3X10^{-3}$)	60 µl '**C**' + 140 µl DMSO	
0.001	($1X10^{-3}$)	20 µl '**C**' + 180 µl DMSO	→ stock **D**
0.0003	($3X10^{-4}$)	60 µl '**D**' + 140 µl DMSO	
0.0001	($1X10^{-4}$)	20 µl '**D**' + 180 µl DMSO	→ stock **E**
0.00003	($3X10^{-5}$)	60 µl '**E**' + 140 µl DMSO	

Appendix 4. Solutions for hepatocyte isolation and cell culture. From Fischer, P.W.F. and Marks, G.S. (1976). Tissue Cult. Assoc. Manual, 2: 449-452.

(A) Kreb-Ringers Buffer (KRB):

Salt	g	mM
$NaHCO_3$	2.1	25.0
NaCl	0.279	4.77
KCl	8.97	120.32
KH_2PO_4	0.1575	1.157
$CaCl_2 \cdot H_2O$	0.1403	1.088
$MgSO_4 \cdot (7H_2O)$	0.285	1.156

Make up to 1 L with ddH_2O, and filter sterilize (0.22 μm pore).

(B) Percoll\Sucrose

sucrose	10 ml of a 2.5 M solution
Percoll	90 ml

Dissolve 64.2 g sucrose in 75 ml ddH_2O in a 100 ml bottle and autoclave sterilize. Add 10 ml of 2.5 M sucrose solution to 90 ml Percoll, mix thoroughly, and store in 24 ml aliquots.

(C) Waymouth's Cell Culture Medium

Waymouth's	13.78 g/L
$NaHCO_3$	2.24 g/L

Make up to 1L with ddH_2O, and filter sterilize (0.22 μm pore). Store in the dark, and just prior to use prepare and add 2 ml of each of the following per liter of Waymouth's:

1. Penicillin/Streptomycin: dissolve 300 mg penicillin and 500 mg streptomycin in 10 ml ddH_2O; filter sterilize.
2. L-thyroxine: dissolve 5 mg in 1 ml of 0.1 N NaOH and bring volume to 10 ml with ddH_2O; filter sterilize.
3. Insulin: dissolve 5 mg in 1 ml of 1.0 N HCl and bring volume to 10 ml with saline (8.76 g NaCl in ddH_2O); filter sterilize.

(D) Sodium Phosphate Buffer (for EROD assay)

Salt	g	Volume	mM
$NaH_2PO_4 \cdot H_2O$ (monobasic)	3.45	0.5 L	50
Na_2HPO_4 anhyd. (dibasic)	7.1	1 L	50

Make monobasic sodium phosphate up to 500 ml with ddH_2O. Make dibasic sodium phosphate up to 1 L with ddH_2O. Warm both solutions to 37°C, and bring pH of dibasic sodium phosphate solution to 8.0 with monobasic solution.

(E) Phosphate Buffered Saline (PBS) (for rinsing cells)

Salt	g	mM
KCl	0.2	2.7
KH_2PO_4	0.2	1.47
NaCl	8.0	137
Na_2HPO_4	2.16	8
D-glucose	1.0	5.6
EDTA	0.37	1.0

Bring to 1 L with ddH_2O; pH to 7.4 with 3 N NaOH

Appendix 5. Cell culture protocol for chicken, Pekin duck, and Greater Scaup.

(1) Solutions & Culture Medium:

- Collagenase:
  (a) chicken: 50 mg/100 ml KRB; filter sterilize (0.45 μm pore); prewarm to 37°C
  (b) Pekin duck and Greater Scaup:100 mg/90 ml KRB + 10 ml DNAse; filter sterilize (0.45 μm pore); prewarm to 37°C
- BSA: 2g/100 ml KRB; filter sterilize (0.45 μm pore); prewarm to 37°C
- Medium: Waymouth/bicarbonate; add pen/strep, insulin, L-thyroxine; prewarm to 37°C
- DNAse 1:·0.2 mg/10 ml KRB; filter sterilize (0.45 μm pore)

(2) Dissection:

- Spray eggs with 70% ethanol
- Kill embryos by decapitation, dissect livers in groups of three; rinse 2x in KRB, and transfer to clean petri dish with fresh KRB
- After dissections, transfer livers to clean, dry petri dish and mince thoroughly
- Transfer minced tissue to a 250 ml Erlenmeyer flask; wash petri dish with collagenase, and transfer washes to Erlenmeyer flask
- Total volume of collagenase in Erlenmeyer flask: 75 ml for chicken; 50 ml for Pekin duck and Greater Scaup

(3) Digestion Protocol:

(A) Chicken:

- Digest with shaking for 45 minutes, or until all tissue digested, at 37°C
- After digestion, filter through nylon filtration apparatus into 80 ml BSA solution
- Pass remaining BSA solution through filter to wash through single cells remaining in filter

(B) Pekin duck and Greater Scaup:

- First Digestion: 10 minutes with shaking at 37°C
- Remove 25 ml digest and filter through nylon screen into 80 ml warm BSA solution
- Replace with 25 ml of fresh collagenase/DNAse
- Second digestion: 10 minutes with shaking at 37°C
- Remove 25 ml digest and filter into BSA solution
- Replace with remaining 25 ml of collagenase/DNAse
- Third digestion: 20 minutes with shaking at 37°C
- Filter remaining digest into BSA solution with first two digests
- Pass remaining BSA solution through filter to wash through single cells remaining in filter

(4) Cell Purification:

- Transfer digests into four 50 ml plastic centrifuge tubes
- Centrifuge 5 minutes at 300 x g
- Discard supernatant
- Combine pellets and bring volume to 25 ml with Waymouth's medium
- Triturate gently with large mouth pipette to disperse tissue chunks
- Add 24 ml Percoll/Sucrose solution, invert tube several times to mix
- Centrifuge 10 minutes at 50 x g

- Transfer upper cell layer (hepatocytes) to a pre-weighed 15 ml plastic centrifuge tube
- Resuspend in 10 ml DNAse, and agitate gently for 5 to 10 minutes (or until clumps disappear)
- Centrifuge 5 minutes at 50 x g (repeat DNAse treatment if necessary)
- Discard supernatant and rinse cells with fresh medium
- Centrifuge 5 minutes at 50 x g. Repeat
- Discard supernatant and weigh pellet
- Resuspend 1 part cells to 32 parts medium (assume density is 1 g/ml); keep cells (gently) suspended with a magnetic stir bar
- Add 25 µl cell suspension to 36 wells of a 48 well plate (12 wells are set aside for standards)

Appendix 6. Arrangement of 48 well plate for cell culture and dosing.

Table A6.1. Arrangement of 48 well plate for dosing.

	1	2	3	4	5	6	7	8
A			DMSO blank	Lowest Dose (1)	-3	-5	-7	-9
B			DMSO blank	1	-3	-5	-7	-9
C			DMSO blank	-1	-3	-5	-7	-9
D			DMSO zero	-2	-4	-6	-8	Highest Dose (10)
E			DMSO zero	-2	-4	-6	-8	-10
F			DMSO zero	-2	-4	-6	-8	-10

Waymouth media (500 µl/well) was added to all wells of a 48 well tissue culture plate, and pre-warmed to 37°C. Hepatocytes (25 µl/well) were added to wells in columns 3 to 8; columns 1 and 2 were reserved for standards, and were not plated with cells (Table A6.1). After 24 hours incubation, the cells were dosed as follows: (a) column 3 was dosed with DMSO only, and represent the blank and zero controls; (b) the remaining 30 wells (columns 4 to 8) were dosed with DMSO plus the test compound. Ten concentration the test compound, dosed in triplicate, were tested per plate. Volume of DMSO with test compound added was 2.5 µl per well. Plates were incubated for a further 24 hours, after which the medium was removed, cells rinsed with PBS, and flash frozen on dry ice and stored at -80°C until analysed. Note: To protect the environment, all used media containing TCDD, PAHs, PCBs and other toxic compounds were filtered twice through activated charcoal to remove these compounds prior to disposal down the drain. Contaminated charcoal was disposed of in accordance with federal regulations.

Appendix 7. Arrangement of 48 well plate for EROD, protein, and porphyrin assay.

Solutions

(a) $NaPO_4$: 50 nM sodium phosphate buffer.
(b) Resorufin: Concentrated stock solution prepared in methanol; dilute to 7 μM working solution by addition of sodium phosphate buffer.
(c) Ethoxyresorufin: Concentrated stock solution prepared in methanol; dilute to 35 μM working solution by addition of sodium phosphate buffer.
(d) BSA: 2 mg/ml working solution prepared in sodium phosphate buffer.
(e) NADPH: 13.4 nM (1.2 mg/ml) solution prepared in sodium phosphate buffer
(f) Acetonitrile/fluorescamine: 150 μg/ml fluorescamine dissolved in acetonitrile; chill to 4°C.
(g) 3 N HCl
(h) Porphyrin standard: dissolve a few grains of URO porphyrin in 12 N HCl, dilute with 1 N HCl until absorbance (at 380-420 nm) is 0.7 to 0.9; dilute to 1 μM with ddH_2O.

EROD Assay

Plates were remove from freezer, and allowed to thaw for 10 minutes. While thawing, BSA and resorufin standards were prepared in columns 1 and 2. Sodium phosphate buffer was added to all wells containing hepatocytes (150 μl to three "blank" wells, and 125 μl to all remaining wells). Ethoxyresorufin (50 μl/well) was added to all wells (see Table A7.1 for details). Plates were pre-warmed on a block heater (37°C) for 15 minutes. The assays were carried out directly in the 48 well plates, and concentrations of the fluorescent reaction products, (total protein, resorufin and porphyrins; Figure A7.1) were determined with a fluorescent plate reader. The EROD reaction was started by the addition of 25 μl NADPH (added to all wells except "blank"wells). The reaction was stopped after 7 minutes by the addition of cold acetonitrile/fluorescamine (150 μl/well). Plates were removed from the plate warmer, and allowed to come to room temperature (about 15 minutes) before placing in the fluorescence plate reader. Resorufin production was measured with a 530 nm excitation filter and a 590 nm emission filter, and total protein was measured with a 400 nm excitation filter and 460 nm emission filter. After EROD data was collected, 3 N HCl and porphyrin standards were added to the standard wells, and 3 N HCl (400 μl/well) was added to the wells with hepatocytes (see Table A7.2 for details), and were allowed to sit for one hour at room temperature. Porphyrins were measured with a 400nm excitation filter and a 645 nm emission filter. Data was imported into Sigma Plot for analysis.

Table A7.1. Concentrations of reagents for EROD assay.

	Well #	$NaPO_4$ (µl)	Ethoxyresorufin Final [ER] (µl)	(µM)	NADPH Final [NADPH] (µl)	(µM)	BSA Final [BSA] (µl)	(µM)	Resorufin (Res) Final [Res] (µl)	(µM)	Acetonitrile + Fluorescamine (µl)
Standard Curves	1 AB	125	50	5	1.0	0.1	0	0	0	0	150
	1 CD	113.75	50	5	1.0	0.1	10	57	1.25	0.025	150
	1 EF	101.25	50	5	1.0	0.1	20	114	3.75	0.075	150
	2 AB	82.5	50	5	1.0	0.1	30	171	12.5	0.25	150
	2 CD	66.25	50	5	1.0	0.1	40	229	18.75	0.375	150
	2 EF	50	50	5	1.0	0.1	50	285	25	0.5	150
Dosed Cells	3 ABC	150	50	5	1.0	0					150
	3 DEF	125	50	5	1.0	0.1					150
	4 ABC	125	50	5	1.0	0.1					150
	4 DEF	125	50	5	1.0	0.1					150
	5 ABC	125	50	5	1.0	0.1					150
	5 DEF	125	50	5	1.0	0.1					150
	6 ABC	125	50	5	1.0	0.1					150
	6 DEF	125	50	5	1.0	0.1					150
	7 ABC	125	50	5	1.0	0.1					150
	7 DEF	125	50	5	1.0	0.1					150
	8 ABC	125	50	5	1.0	0.1					150
	8 DEF	125	50	5	1.0	0.1					150

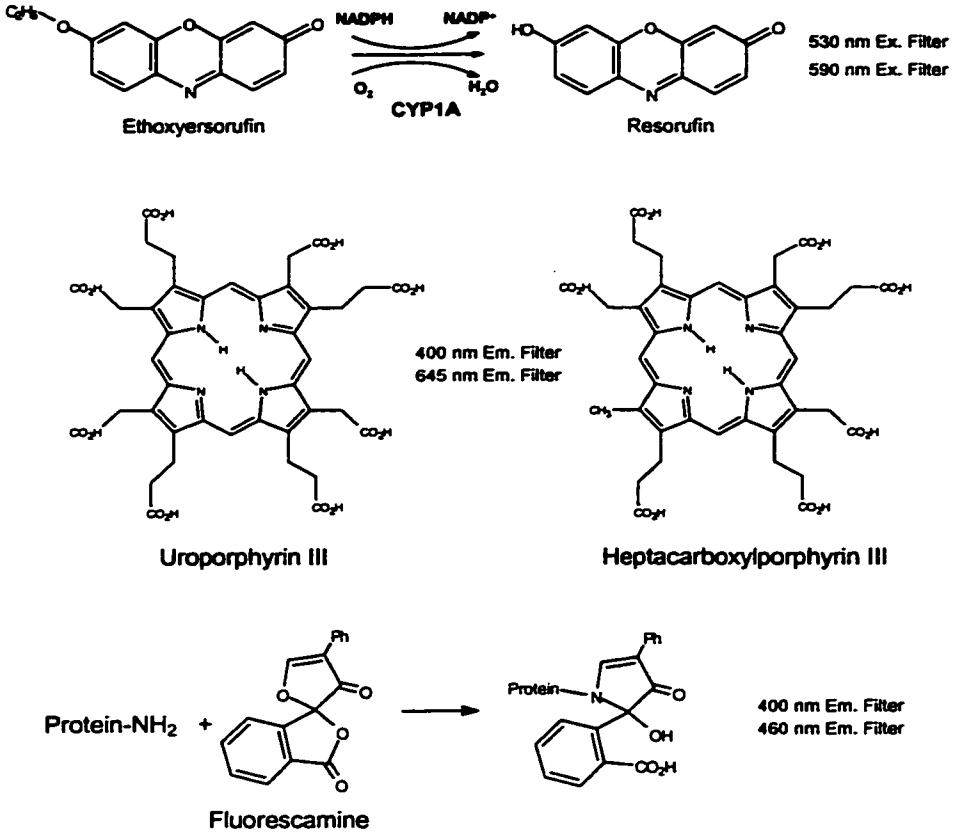

Figure A7.1. Conversion of ethoxyresorufin to resorufin by CYP1A; structures of uroporphyrin III and heptacarboxylporphyrin III; and reaction of protein with fluorescamine.

Table A7.2. Concentrations of reagents for porphyrin analysis.

	Well #	1000 nM URO III Standard Final [URO] (µl)	(µM)	3 N HCl (µl)
Standard Curves	1 AB	0	0	400
	1 CD	15	20	385
	1 EF	30	40	370
	2 AB	45	60	355
	2 CD	60	80	340
	2 EF	75	100	325
Dosed Cells	3 ABC			400
	3 DEF			400
	4 ABC			400
	4 DEF			400
	5 ABC			400
	5 DEF			400
	6 ABC			400
	6 DEF			400
	7 ABC			400
	7 DEF			400
	8 ABC			400
	8 DEF			400

Appendix 8. EROD, protein and porphyrin analysis, and calculation of relative potencies.

(A) Calculation of EC50 Dose-Response:

(i) EROD.

Data obtained from the EROD assay were normalized against total protein and fitted to a modified Gaussian curve:

$$y(d) = Y_b + (Y_m - Y_b)\exp\{-C[\ln(d) - \ln(d_m)]^2\},$$

Equation 1

$$\text{where} \qquad C = \frac{\ln(2)}{[\ln(EC_{50}) - \ln(d_m)]^2},$$

where:

$y(d)$ = EROD activity at dose d

Y_b = basal EROD activity

Y_m = maximal EROD activity

d_m = dose where EROD activity is maximal

EC50 = lower dose where EROD activity is half way between maximal and basal activity

(ii) Porphyrins.

Porphyrin data obtained from the plate reader was imported into Sigma Plot and fitted to a logistic curve:

$$Y(d) = Y_b + (Y_m - Y_b)\{1 + \exp[-g(\ln(d) - \ln(EC_{50}))]\}^{-1},$$

Equation 2

Where:

$y(d)$ = porphyrin concentration at inducer concentration d

Y_b = basal porphyrin concentration

Y_m = maximal porphyrin concentration

g = a slope parameter

EC50 = the dose at which porphyrin concentration is one-half maximal

(B) Derivation of the Threshold Dose-Response (ECthr) Equation:

Substances eliciting, at high concentrations, a lower maximal response relative to a standard that achieves a "full maximal" response (e.g., TCDD) are defined as partial agonists (Pliska, 1999; Patil, 1996). Various mechanisms may act synchronously to cause substances to act as partial agonists: the receptor-agonist complex may exist in several conformational states that is intermediate between the conformations induced by agonists and antagonists; a partial agonist may only partially shift the receptor state between active and inactive; or the efficacy of the agonist may be based on the efficacy of the cell signal transduction pathway (see Hoyer and Boddeke, 1993, Zhu, 1996, and Hesterman *et al.*, 2000 for reviews on receptor theory, agonism and antagonism). In addition, the maximal activity may reflect the rate at which the agonist is metabolized. TCDD, for example, is not only the one of the most avid AhR agonists, but it is also resistant to metabolism. Since TCDD is poorly metabolized and eliminated slowly, it is a persistent AhR agonist. Consequently, TCDD maintains the AhR

signal transduction pathway in a continuous state of activation, with CYP1A gene being continually expressed.

The EROD assay estimates inducer potency indirectly by measuring the catalytic activity of the induced CYP1A enzyme on a surrogate substrate, ethoxyresorufin (ER). TCDD is one of the most potent EROD inducers, and is considered the prototypical EROD inducer, and is used as a standard against which other compounds are compared. While many other chemicals are known agonists to the AhR and induce CYP1A activity (e.g., PCBs and PAHs), few have the same potency or efficacy as TCDD. Compounds exhibiting lower maximal EROD activity than TCDD may be acting as partial agonists. However, other mechanisms may contribute to the lower maximal EROD activities observed with some AhR agonists. For example, high concentrations of some inducers have been shown to competitively inhibit CYP1A catalytic activity resulting in biphasic concentration-response curves. Since a biphasic EROD concentration-response curve may underestimate CYP1A content at high doses, measurement of both EROD activity and immuno-detectable CYP1A protein is recommended.

A variety of alternative methods have been proposed for estimating relative potencies, including parallel line, slope-ratio, response equivalent regression (Villeneuve, 1999), and threshold methods. Regardless of the method used to estimate potency, the EROD assay relies on the assumption that the activity of the induced enzyme accurately reflect the amount of enzyme induced. However, inhibition of the enzyme by residual inducer may interfere with the catalytic activity of the enzyme reducing the maximal EROD activity and erroneously suggesting that the compound is a partial agonist. Hahn and co-workers (1996) reported that while PCBs 126 and 77 had maximal EROD activities that were approximately 40% that of TCDD, measurement of immuno-detectable CYP1A protein indicated that PCBs 126 and 77, and TCDD induced similar amounts of CYP1A protein, indicating that PCBs 126 and 77 were full agonists. These observations are supported by Petrulis and Bunce (1999) who reported similar biphasic curves in an enzyme kinetic study. Consequently, EC50 based potency estimates for compounds exhibiting non-ideal concentration-response curves may overestimate potency.

Since competitive inhibition of the CYP1A enzyme by residual inducer will be minimal at the lower concentrations of inducer, potencies estimated at low concentrations of inducer where inhibition of catalytic activity is minimal should provide a more reliable estimate of potency. The approach described here, the TCDD-threshold method (ECthr), is based on estimating the concentration of inducer producing a response equivalent to 10% of the maximal response induced by TCDD (a full agonist). Compounds with concentration-response curves shifted to the right relative to TCDD typically exhibit decreased maximum activities relative to TCDD. Figure A8.1 shows idealized concentration-response curves for TCDD and a hypothetical PAH. The PAH concentration-response curve is shifted to the right relative to TCDD, and possesses a maximal activity approximately 60% that of TCDD. The lower EROD activity of the PAH is due in part to the rapid metabolism of the PAH, inactivating it an a CTP1A inducer. However, the lower activity may also be due to the fact that since the PAH doses were 1000 times higher than TCDD, inhibition of catalytic activity at high doses is more likely to occur for PAHs than for TCDD.

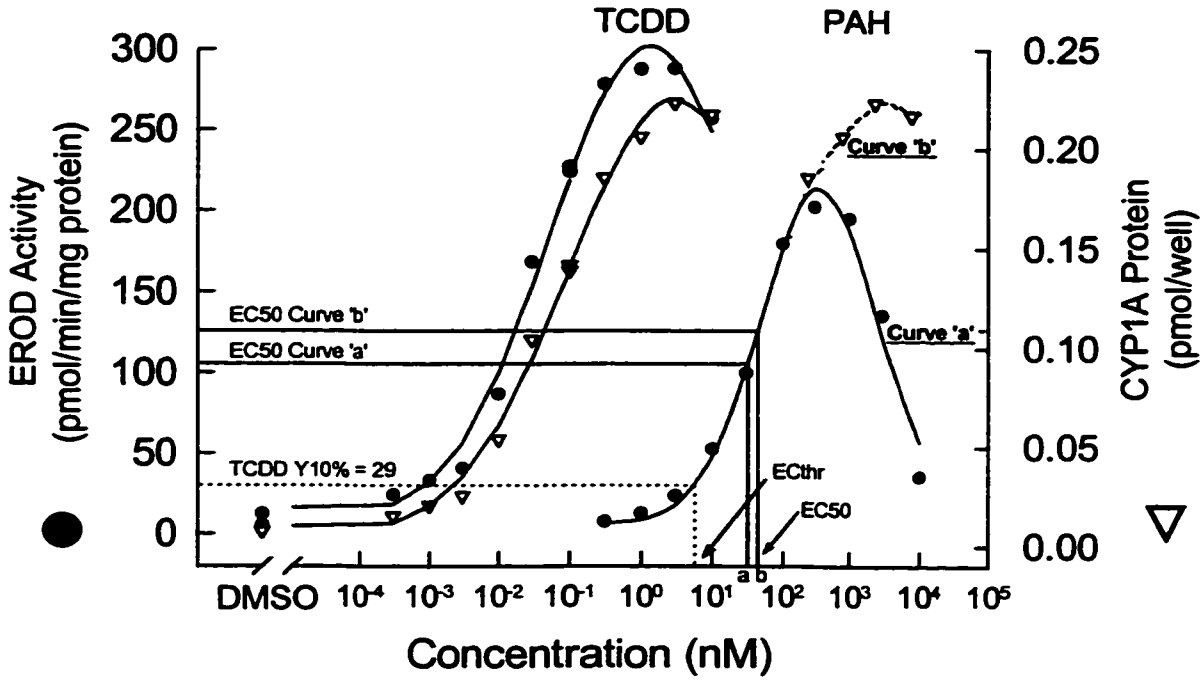

Figure A8.1. Idealized EROD (closed circles) and CYP1A protein (open triangles) concentration-response curves for TCDD and a hypothetical PAH in chicken embryo hepatocytes (CEH). Both TCDD and the PAH exhibit biphasic concentration-response EROD curves. However, Hahn *et al.* (1996) reported that, in contrast to EROD activity, which exhibits attenuated maximal EROD responses at high concentrations of inducer, immuno detectable CYP1A protein increases monotonically in CEH cells upon exposure to TCDD and several PCBs (TCDD curves). Similar results were reported by Brüschweiler *et al.* (1996) for 3-methylcholanthrene (a potent EROD inducing PAH) in salmon microsomes. Thus, the most potent EROD inducing PAHs (e.g., BkF and DahA) are likely full agonists, and the biphasic concentration-response curves are likely caused by inhibition of CYP1A catalytic activity. Estimation of PAH potency by measurement of EROD activity is complicated by the biphasic nature of the concentration-response curve. Inspection of Figure 8.1 shows that the EC50 estimate based on EROD activity (closed circles) results in a lower potency estimate than the estimate based on the amount of CYP1A protein (open triangles). In the absence of CYP1A inhibition, the maximal EROD activity of the PAHs curve would be similar to that of curve b. The attenuation of maximal EROD activity results in a shift of the EC50 to the left, overestimating the potency of the PAH. Thus, inhibition of CYP1A catalytic activity results in attenuation of maximal EROD activity, and can an overestimate potency. Since the ECthr is estimated at a lower concentration where inhibition is less likely to occur, the ECthr provides a more reliable estimate of potency.

The hypothesis behind the ECthr is based on the observation that although inhibition or inactivation of CYP1A catalytic activity may result in biphasic EROD concentration-response curves, measurement of CYP1A protein has shown that several compounds (e.g., PCBs 77 and 126) are in fact full agonists relative to TCDD. The concentration of ethoxyresorufin (ER, the substrate in the EROD assay) is 10 μM and the highest concentration of the PAH is also 10 μM. Assuming that after discarding the medium and rinsing the cells to remove residual PAH, 1% of the PAH originally present in the medium remains in the hepatocytes during the EROD assay, the concentration of residual PAH in the assay at the highest dose (1% of 10^4 nM (10 μM)) will be 0.1 μM or about 1% of the concentration of ER. This residual concentration may

be high enough to cause inhibition of CYP1A activity. In contrast, the concentration where ECthr is estimated is approximately 1000 times lower (10 nM or 10^{-2} μM), and residual PAHs will be present at 1% of 10^{-2} μM or 10^{-4} μM, or 0.001% of the concentration of ER where inhibition is less likely to occur.

To calculate the ECthr dose , the variable 'd' is in Equation (1) is isolated:

let $\{-C[\ln(d)-\ln(d_m)]^2\} = \alpha$ → $y(d) = Y_b + (Y_m - Y_b)\exp^{\alpha}$

$$\exp^{\alpha} = \frac{y(d)-Y_b}{Y_m - Y_b} \rightarrow \alpha = \ln\left(\frac{y(d)-Y_b}{Y_m - Y_b}\right)$$

$$\{-C[\ln(d)-\ln(d_m)]^2\} = \ln\left(\frac{y(d)-Y_b}{Y_m - Y_b}\right) \rightarrow \ln(d)-\ln(d_m) = \pm\sqrt{\frac{\ln\left(\frac{y(d)-Y_b}{Y_m - Y_b}\right)}{-C}}$$

$$\ln(d)-\ln(d_m) = -\sqrt{\frac{\ln\left(\frac{y(d)-Y_b}{Y_m - Y_b}\right)}{-C}}$$

NOTE: Since we are selecting for the lower concentration within a biphasic response, the '±' is replaced with '-'

$$\text{thus, } d = \exp\left(\ln(d_m) - \sqrt{\frac{\ln\left(\frac{y(d)-Y_b}{Y_m - Y_b}\right)}{-C}}\right) \qquad \textbf{Equation (3)}$$

(C) Calculation of ECthr dose for TCDD:

(i) Since the ratio $\left(\frac{y(d)-Y_b}{Y_m - Y_b}\right)$ (the "threshold factor") in Equation (3) describes the threshold activity (e.g. 10% of the activity of the TCDD curve), Equation (3) can be rewritten:

$$d_{(\text{TCDD-ECthr})} = \exp\left(\ln(d_m) - \sqrt{\frac{\ln(0.1)}{-C}}\right) \qquad \textbf{Equation (4)}$$

where the threshold factor has been replaced with '(0.1)'. Various ECthr values can be easily calculated by assigning the appropriate value to the threshold factor. For example, to calculate the EC5%, the threshold factor in Equation 3 would be (0.05). The importance of setting the basal activity to zero is illustrated in Figure A8.2. In Figure A8.2, the actual (non-adjusted) TCDD data (Curve B) has basal activity of approximately 50, while the adjusted TCDD data (Curve A) has a basal activity of zero; the corresponding TCDD Y10% values are 68 activity

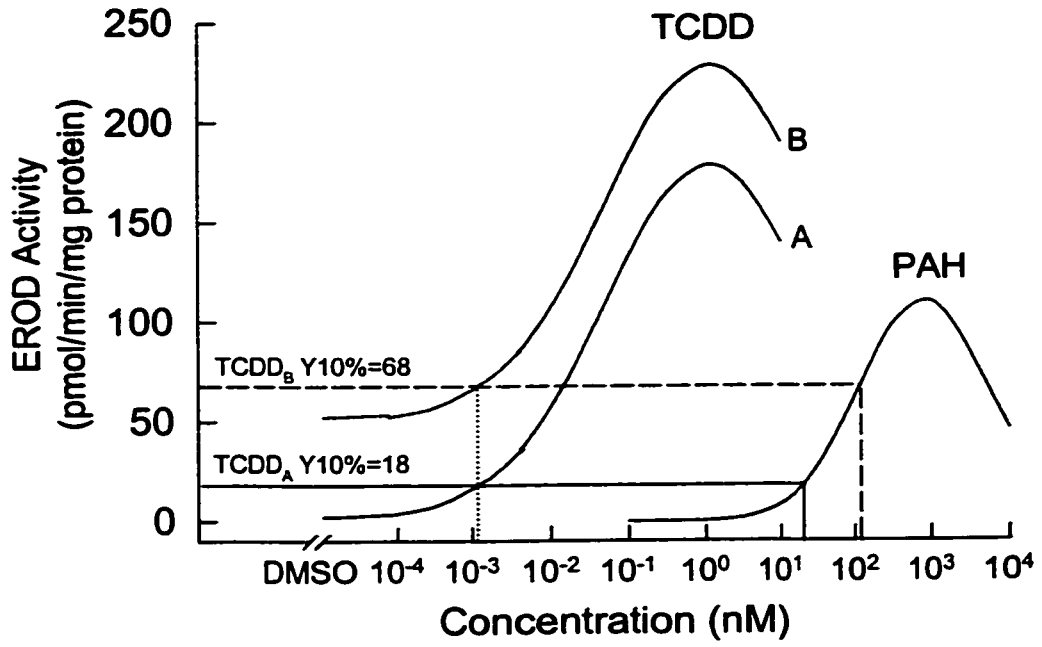

Figure A8.2. Effect of basal EROD activity on ECthr values. See text for details.

Although the effect of the basal activity of the TCDD curve does not effect its ECthr value, it has a dramatic effect on the ECthr of DahA. Using the Y10% of TCDD Curve B (dashed line; 68 activity units) would underestimate the ECthr value of DahA by approximately one order of magnitude. For example, in Figure A8.2, if the basal activity of DahA was a non-zero value (e.g., 25 activity units), the Y10% of TCDD Curve A (solid line; 18 activity units)would pass under the DahA curve, and an ECthr value could not be estimated. Since basal activities are usually non-zero values, and are commonly different for different compounds, adjusting the curves such that the basal activities are zero provides a common reference point for calculating the ECthr, and eliminates errors when comparing ECthr values between compounds.

(ii) Since the threshold factor in Equation (4) has a value of 0.1, the EROD activity at dose $Y(d)$ for the TCDD curve can be determined by rearranging the threshold factor:

$$y(d) = 0.1(Y_m - Y_b) + Y_b$$

However, in order to ensure that ECthr values are independent of non-zero basal activities, it is necessary to ensure that the value returned is the absolute Y10% and not the Y10% relative to a non-zero baseline value:

Thus, $y(d) = 0.1(Y_m - Y_b)$ **Equation (5)**

(D) Calculation for ECthr dose for compound:

$$d_{ECthr} = \exp\left(\ln(d_m) - \sqrt{\frac{\ln\left(\frac{y(d) - Y_b}{Y_m - Y_b}\right)}{-C}} \right) \qquad \textbf{Equation (6)}$$

Again, it is necessary to ensure that the PAH basal activity is set to zero, thus the equation becomes:

$$d_{ECthr} = \exp\left(\ln(d_m) - \sqrt{\frac{\ln\left(\frac{y(d)}{Y_m - Y_b}\right)}{-C}} \right) \qquad \textbf{Equation (7)}$$

Where y(d) = 10% of the TCDD maximal activity as defined by equation 5.