POLYCHLORINATED BIPHENYL (PCB) ALTERATIONS OF ANTIOXIDANT DEFENSES AND XENOBIOTIC METABOLISM IN TELEOST FISH:

ROLE OF TISSUE GLUTATHIONE METABOLISM IN XENOBIOTIC DETOXICATION PATHWAYS

By

Diana M.E. Otto

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Title: Polychlorinated Biphenyl (PCB) Alterations of Antioxidant Defenses and Xenobiotic Metabolism in Teleost Fish: Role of tissue glutathione metabolism in xenobiotic detoxication pathways

Author: Diana M.E. Otto, M.Sc. (University of Kuopio, Finland, 1992)

Supervisor: Dr. Thomas W. Moon, Professor, Department of Biology, University of Ottawa

ABSTRACT

Polychlorinated biphenyls (PCBs) are ubiquitously distributed in the aquatic and terrestrial environments, even though their production was stopped more than 20 years ago. The biochemical effects of PCBs are primarily initiated by the induction of drug metabolizing enzymes, especially cytochromes P450. In the first instance, these enzymes detoxify xenobiotics such as PCB congeners to facilitate their excretion from the animal. However, these processes are also known to generate more toxic metabolites, including the production of reactive oxygen species (ROS) which impact on cellular antioxidant defenses. Teleost fish contain an active endogenous antioxidant defense system, which consists of superoxide dismutase, catalase and the glutathione system. Apart from its antioxidant functions, glutathione (GSH) plays a major role in electrophilic conjugation of lipophilic conjugation (initiated by glutathione S-transferase, GST) and in the maintenance of cellular homeostasis.

This thesis investigated the responses of drug metabolizing enzymes and endogenous antioxidant defenses in teleosts exposed to PCBs. The first field study showed that feral brown bullheads, harvested from the St. Lawrence River and contaminated with PCBs, had enhanced activities of ethoxyresorufin O-deethylase (EROD), a cytochrome P4501A (CYP1A) catalytic activity. GST activities were induced several fold in these River bullheads, which suggested active detoxication via GSH conjugation, although at the expense of tissue GSH content. A second field study involved the caging of hatchery reared rainbow trout at a PCB contaminated site. The trout showed significant accumulation of PCB congeners in skeletal muscle in the absence of changes in tissue drug metabolizing enzymes and antioxidant defenses.

Endogenous antioxidant defenses were examined in rainbow trout and black bullheads. These systems were found to underlie age- and/or maturation-dependent changes. In a series of experiments, exogenous GSH was noted to serve as an efficient delivery agent of GSH to tissues in teleosts. This observation suggests that piscine GSH biochemistry differs from that of mammals, which respond poorly to exogenous GSH. This unique characteristic of the piscine GSH system could be important for fish husbandry while providing an interesting tool to study the biochemical effects of PCBs. A series of experiments found that a 3 day exposure of rainbow trout to 3,3',4,4'-tetrachlorobiphenyl (TCB) had little impact upon tissue antioxidant defenses, while a 6 week exposure resulted in significant induction of activities of the conjugation enzyme UDP-glucuronosyltransferase, antioxidant enzymes activities and GSH levels in hepatic and extrahepatic tissues. However in both experiments TCB induced CYP1A metabolism.

Additional experiments attempted to characterize to what extent tissue glutathione status influences basal and TCB induced hepatic CYP1A metabolism. Manipulation of tissue GSH content by either depletion (induced by L-buthionine-[S,R]-sulfoximine, BSO) or supplementation with pro-GSH agents (GSH and lipoate) revealed that glutathione status modified cytochrome P4501A at two stages, the CYP1A mRNA expression and the catalytic activity. This apparent cross-talk between the glutathione and cytochrome P450 systems suggested that glutathione homeostasis plays a major role in PCB induced metabolic alterations.

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LIST OF ABBREVIATIONS

AHH	aryl hydrocarbon hydroxylase
AhR	aromatic hydrocarbon receptor
AhRE	Ah responsive element
AP-1	activator protein-1
ARNT	Ah receptor nuclear translocator
ATCC	American Type Culture Collection
B[a]P	benzo[a]pyrene
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BSO	L-buthionine-[S,R]-sulfoximine
CAT	catalase
Cu,Zn-SOD	cuprozinc SOD
CYP1A	cytochrome P4501A
DHLA	dihydrolipoate
DME	drug metabolizing enzyme
cDNA	complimentary deoxyribonucleic acid
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
EDTA	ethylenediaminetetraacetic acid
EpRE	electrophilic reponsive element
ER	estrogen receptor
EROD	ethoxyresorufin O-deethylase
GCS	γ-glutamylcysteine synthetase
GGT	γ-glutamyl transpeptidase
GPX	glutathione peroxidase
G-6PDH	glucose-6-phosphate dehydrogenase
GR	glutathione disulfide (GSSG) reductase
GS	glutathione synthetase
GSH	reduced glutathione
GSSG	oxidized glutathione or glutathione disulfide
GST	glutathione S-transferases
HMS	hexose monophosphate shunt (also HMP)
H_2O_2	hydrogen peroxide
HSP90	heat shock protein HSP90
IDH	isocitrate dehydrogenase
MC	3-methylcholanthrene
ME	malic enzyme
Mn-SOD	mangano SOD
mRNA	messenger ribonucleic acid
MS 222	3-aminobenzoic acid ethyl ester
MO	monooxygenases
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NFκB	nuclear factor κB
NO·	nitric oxide
O_2^{-}·	superoxide anion
PAGE	polyacrylamide gel electrophoresis
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-p-dioxin
PCDF	polychlorinated dibenzofuran
PR	progesterone receptor
RNA	ribonucleic acid
SDS	sodium dodecylsulfate
SSC	saline sodium citrate
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances
TCB	3,3',4,4'-tetrachlorobiphenyl
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TEF	toxic equivaleny factor
TGSH	total glutathione
UDP	uridine diphosphate
UDPGT	UDPglucuronosultransferases

CHAPTER 1. GENERAL INTRODUCTION

1.1. INTRODUCTION

Production and utilization of synthetic products often result in chemical loading of the aquatic and terrestrial environments. Water covers seventy percent of the earth's surface and ninety percent of the biosphere. Industrial and agricultural activities are primarily responsible for the discharge of thousands of tons of chemicals every year into the aquatic environment. Of major concern is the fate of lipophilic organic substances which are resistent to degradation, accumulate in the food chain and cause serious ecological damage (Johnson & DeRosa 1995). Polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) are quantitatively the major classes of such persistent hazardous chemicals (Safe 1994; Tuvikene 1995).

PCBs have been manufactured and used extensively since 1930. The unique chemical and physical properties of highly chlorinated PCBs are their hydrophobicity and nonvolatility. PCBs are relatively inert towards corrosive materials including acids and alkalies. Temperatures in excess of 1000°C are required for complete combustion of certain PCB congeners (Roberts *et al.* 1978). The production of PCBs ceased more than 20 years ago, but they are still recognized as priority hazardous substances (Johnson & DeRosa 1995). PCBs accumulate in aquatic organisms and bioconcentrate at higher trophic levels of the food chain. The food consumption of fish by fish-eating birds and humans has developed public awareness to these chemicals (Safe *et al.* 1995; Elliot *et al.* 1996).

In order to assess the biological impacts of PCBs in organisms, it is vital to understand the

physiological and biochemical effects initiated in the first instance by these chemicals. These primary alterations occur at the cellular level. Nebert (1994) described the cellular entry of a foreign chemical as being recognized by the cell as a structural mimic of an endogenous substance. In this view the xenobiotic loading of the cell resembles a large amount of "effector molecule". By increasing cellular levels of metabolizing pathways these chemicals are to be degraded and the cell can return this effector level to safe, physiological concentrations. This is a homeostatic principle by which a cell or organism tries to adapt to the xenobiotic exposure. Depending on the chemical structure, the cell or organism will be more or less successful to rid itself of the xenobiotic. The high persistence of certain PCB congeners in the environment (Safe 1984) suggests that such compounds are not easily metabolized and eliminated in exposed organisms. Consequently, PCB may induce biochemical changes which may lead to toxicities in the organism.

The first metabolic steps initiated by toxicant exposure are the induction of drug metabolizing enzymes, which include phase I (cytochromes P450) and phase II (primarily conjugation) enzymes (Nebert *et al.* 1993). The view that such xenobiotics play a potential role in the generation of free radicals which affect cellular antioxidant status has established a new area in toxicology (Kehrer 1993). Glutathione is a major cellular antioxidant, which also serves in electrophilic conjugation and has multiple other homeostatic functions in aerobic organisms (Meister & Anderson 1983). This series of investigations was primarily focussed on the effects of PCB exposure on glutathione homeostasis and drug metabolizing enzymes in teleost fish.

1.2. REVIEW OF THE LITERATURE

1.2.1. Environmental impact of polychlorinated biphenyls

1.2.1.1. Polychlorinated biphenyls in the environment

PCBs have been produced worldwide to serve their purposes in industry as organic diluents, plasticizers, pesticide extenders, dust reducing agents, cutting oils, flame retardants, dielectric fluids for transformers and capacitors, heat transfer fluids, hydraulic lubricants, sealants and in carbonless copy paper (Safe 1994). In the 1960s it was discovered that PCBs spread and accumulate in the environment (Jensen *et al.* 1969). Numerous reports have identified PCBs in most components of the global ecosystem leading to a production stop of these versatile chemicals during the 1970s (for review see Safe 1984; 1994). It has been estimated that 1.5 million metric tons have been produced worldwide and significant quantities of PCBs are at risk to escape to the environment from older electrical equipment and landfill sites (Hargrave *et al.* 1992; Safe 1994).

PCBs comprise a total of 209 possible congeners, which have been identified in different Aroclor and Clophen mixtures. Fig. 1.1 shows the structure of biphenyls. PCBs are differentiated according to substituent positioning in either *para*, *meta* or *ortho* positions. Coplanar PCBs are substituted in both *para* and at least two *meta* positions, while monoortho coplanar PCBs constitute coplanar PCBs with one substituent in the *ortho* position (Safe 1994; Fig. 1.1). These two structural PCB classes are among the most studied PCBs (Harper *et al.* 1993). Amount of substituents and positioning determines the general properties of the congener and contribute to the environmental persistence and stability of PCBs. More highly chlorinated homologs are more

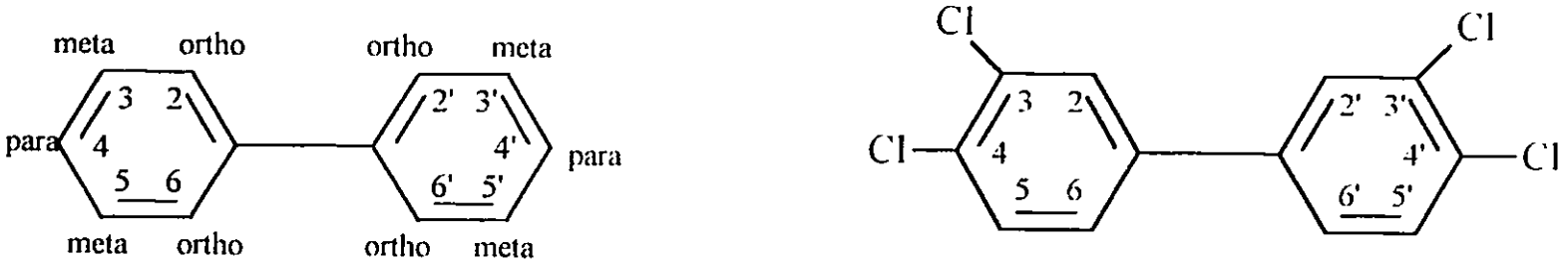


Fig. 1.1. Structure of polychlorinated biphenyls with indication of *para*, *meta* and *ortho* position. and structure of 3,3`,4,4`-tetrachlorobiphenyl.

persistent in the environment which is thought to result from a combination of factors including microbial and metabolic breakdown of lower chlorinated congeners (Safe 1984).

PCBs share many properties with two other groups of stable environmental pollutants, polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). PCBs, like PCDDs and PCDFs, are lipophilic and preferentially bioaccumulate and biomagnify in higher trophic levels of the food chain. PCBs are still considered major aquatic pollutants and accumulate in the distinct trophic levels of various ecosystems (Bush *et al.* 1989; Elskus *et al.* 1989; Hargrave *et al.* 1992; Rodríguez-Ariza *et al.* 1993; Elliot *et al.* 1996). Thus PCBs fall into the list of top ten priority substances for assessment of public health and remediation plans (Johnson & DeRosa 1995).

1.2.1.2. Toxic effects of PCBs

With the exception of some microbial species which are capable of biodegrading various PCB congeners (Damaj & Ahmad 1996), PCBs have accumulated in the environment and exert a series of health effects on most organisms. Toxicities of commercial PCBs and individual congeners depend largely on factors such as chlorine content, animal species, age and sex of species and the route and duration of exposure to the PCB mixture or congener (Safe 1994). PCBs are known to be neurotoxic and elicit changes in physical behavior of mammals (Kodavanti *et al.* 1993; Bernhoft *et al.* 1994). Various PCBs are reported to cause alterations in the heme biosynthesis, which leads to the development of porphyria, and disrupted thyroid hormone homeostasis of various vertebrates (Miranda *et al.* 1992; Den Besten *et al.* 1993; Darnerud *et al.* 1996a; Hahn & Chandran 1996). Porphyria is characterized by the hepatic accumulation and urinary excretion of

uroporphyrin, an oxidation product of the heme precursor uroporphyrinogen (Hahn & Chandran 1996). The immunosuppressive effects of PCBs and related compounds have been amply described (Harper *et al.* 1993; 1994a; 1995).

It has been hypothesized that high levels of PCBs in fish and wildlife populations are responsible for reproductive failure among these species (Darnerud *et al.* 1986; Safe & Krishnan 1995). Particularly fish-eating birds in the Great Lakes region and other areas with known PCB contamination have shown poor breeding success (Elliot *et al.* 1996). Fish consumption from PCB contaminated sites has often been associated with high concentration of PCB congeners in human plasma (Asplund *et al.* 1994; Safe *et al.* 1995). Of major concern is the uptake of PCBs and metabolites observed in rodent foetuses (Darnerud *et al.* 1986; 1996b; Borlakoglu *et al.* 1993a). The lipophilicity of such compounds promotes their sequestration in the adipose tissue of the breast and concentration in the milk during lactation. Thus a preferred uptake route for PCBs in nursing mammals appears to be via lactational transfer (Borlakoglu *et al.* 1993a,b; Sinjari *et al.* 1996). Loss of PCB congeners through lactation may be beneficial for mothers, but these distribution routes put neonates at elevated risks.

In order to predict the fate of PCBs and related compounds, several studies have been undertaken to assess the absorption, tissue distribution and elimination of these toxicants. Liver, adipose tissue, uterus and lung appear to be prime target sites in toxicant exposed rodents (Narasimhan *et al.* 1994; Diliberto *et al.* 1996; Pillai *et al.* 1996). Depending on the uptake route and the congener, the distribution, metabolism and elimination are affected. For example, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) appears to be slowly metabolized and elimination occurs also at a slow rate (Diliberto *et al.* 1996). Studies in fish have not yet looked at PCB distribution,

but at pesticide uptake in various tissues. Liver, stomach, intestine and gills of rainbow trout showed rapid absorption of a carbamate pesticide. On the other hand, the relatively high water solubility of this pesticide enabled rapid elimination from tissues, identifying the elimination via the gills and bile as important organs for xenobiotic elimination (Schlenk *et al.* 1992).

1.2.1.3. Biochemical mode of action

Biochemical effects of PCBs are thought to be primarily initiated by ligand binding to the cytosolic aryl hydrocarbon receptor (Ah receptor, AhR) and subsequent genetic expression of cytochrome P450s and conjugation enzymes (Safe 1994) (see section 2.2). The widely characterized dioxin TCDD, among other PCDDs and PCDFs, polycyclic aromatic hydrocarbons (PAHs) and PCBs are well described Ah receptor ligands (Safe 1984; 1993). Coplanar PCBs and monoortho coplanar PCBs of the various PCB congeners are the most active Ah receptor agonists (Safe 1994). Cytochrome P450 catalyzed PCB metabolites include chlorinated catechols and hydroxyquinones (Amaro *et al.* 1996), and these metabolites are likely to trigger biochemical responses of their own (see 1.2.3.1.). Recently it has been suggested that the toxicant modulated disruption of heme biosynthesis leading to hepatic porphyria involves the Ah receptor. A fish hepatoma cell line showed porphyrin accumulation after exposure to various PCB congeners, which was closely related to AhR dependent cytochrome P450 induction (Hahn & Chandran 1996).

Interestingly, several PCBs, PCDDs and PCDFs elicit antiestrogenic effects in mammals (Dickerson *et al.* 1995; Safe & Krishnan 1995). PCDFs and PCDDs have been observed to inhibit cytosolic and nuclear progesterone and estrogen receptor (PR and ER) ligand binding (Harper *et*

al. 1994b; Dickerson *et al.* 1995). Similar antiestrogenic effects are reported for pentachlorophenol in rainbow trout (Flouriot *et al.* 1995). Safe and coworkers have worked extensively on cell lines which are ER positive and negative (ER^+/ER^-) in combination with AhR^+/AhR^- to illustrate a crosstalk between the AhR and estrogen receptor signal transduction pathways. TCDD alone was not able to induce cytochrome P4501A in ER^-AhR^+ cells (Wang *et al.* 1996). Moreover, ER^+AhR^- cell variants showed, like the wild type, decreased estrogen responsiveness after exposure to TCDD (Moore *et al.* 1996). These studies suggested that this type of crosstalk between AhR and ER may be due to altered responsiveness of the receptors to respective ligand binding. However, recently it was shown that the AhR-TCDD complex interferes with the ability of liganded ER to activate transcription. This was shown to result from a loss of ER binding to an estrogen response element in the presence of TCDD (Khara & Saatcioglu 1996). These results indicate that the crosstalk between ER and AhR affect molecular mechanisms as well as receptor ligand binding in response to TCDD.

PCBs, like PAHs, PCDDs and PCDFs show biological and biochemical similarities with TCDD, the most toxic halogenated aryl hydrocarbon. Toxic equivalency factors (TEFs) have been implicated to compare the relative potencies or fractional toxicity of such congeners with TCDD (Safe 1993). Such an approach can be generally employed, but is subjected to limitations with some PCB mixtures. PCB mixtures often exert lower metabolic effects than based upon the content of individual congeners within the mixture and thus show antagonistic interactions (Aarts *et al.* 1995; Harper *et al.* 1995). A TEF approach overestimates the toxicity of these mixtures due to these antagonistic interactions of the PCBs (Harper *et al.* 1995). In contrast, PAH mixtures appear to exert opposite effects with several hundred fold higher potencies than expected upon

their content of benzo[a]pyrene (B[a]P), a well studied Ah receptor agonist (Chaloupka *et al.* 1993). This suggests some non-additive, synergistic effects among PAHs.

Apart from carcinogenicity resulting from Ah receptor mediated processes and increased expression of various cytochromes P450, PCBs induce protooncogenes such as c-Has-ras and C-raf (Jenke *et al.* 1994; Borlak *et al.* 1996). Also a very novel pathway for gene expression has been recently proposed, as PCBs stimulated the phosphorylation of c-Jun (Tanno & Aoki 1996). The well described transcription factor activator protein-1 (AP-1) consists of heterodimers of c-Jun and c-Fos. It has been previously shown that dioxin induces *c-fos* and *c-jun* expression in an AhR independent process and increases the levels of AP-1 (Puga *et al.* 1992a).

1.2.2. Xenobiotic metabolism

1.2.2.1. Definitions

Xenobiotic (Greek: χένος foreign, βίος life) metabolism summarizes to a large extent the actions of drug metabolizing enzymes (DMEs) which metabolize drugs, carcinogens and other environmental pollutants. These enzymes are often divided into two broad classes: phase I and II enzymes. Phase I represent almost exclusively cytochromes P450 (>95%), which function by inserting one atom of atmospheric oxygen into a relatively inert substrate. P450 enzymes use a large number of substrates (thus polysubstrate) and the number of individual, distinct P450 genes in mammals is estimated to range between 60 and perhaps several hundred (Nebert *et al.* 1993, 1996). Phase II enzymes act on oxygenated substrates, including phase I metabolites, usually by conjugating them with various endogenous substrates (glucuronide, glutathione, sulfate) to produce highly hydrophilic products and facilitate the excretion from the cell (Nebert *et al.* 1990).

DMEs are regarded as "detoxication systems" by eliminating lipophilic substrates that would otherwise accumulate within the cell. However, there is ample evidence that phase I intermediates and conjugated products can themselves be toxic, mutagenic or carcinogenic (Nebert *et al.* 1996). *I.e.* toxic effects of PCDDs, PCDFs, coplanar and monoortho coplanar PCBs are thought to be primarily Ah receptor mediated (Safe 1994), so activation of these enzymes may pose a high risk factor on the organism by attempting to metabolize and excrete the xenobiotic.

Certain DMEs, including many subsets of cytochromes P450, might have existed 1 to 2 billion years ago. The early roles in prokaryotes of perhaps insertion of oxygen, splitting of a molecule have diversified in a large number of complex signal transduction pathways in eukaryotes today (Nebert *et al.* 1993). Certain DME subsets are inducible by the Ah receptor. The murine *Ah* gene battery consists of at least six genes which are coordinately induced by xenobiotics (Nebert 1994). These genes of two cytochrome P450s (CYP1A1 and CYP1A2) and four phase II genes (a UDPglucuronsyltransferase, a glutathione S-transferase, a 'class 3' aldehyde dehydrogenase and DT diaphorase), are the most characterized (Nebert *et al.* 1993) and are reviewed in the following sections.

1.2.2.2. Ah receptor transduction pathway

The first discovered inducers for CYP1A1 were carcinogenic PAHs, such as 3-methylcholanthrene (MC). Later TCDD was shown to be the most potent inducer, as it binds with high affinity (in picomolar range) to the Ah receptor (Whitlock *et al.* 1996). However, also low toxicity compounds such as plant indoles and flavones are inducers of CYP1A1 (Okey 1990). The Ah receptor and induction of several P450s have been the subjects of several recent reviews

(Okey 1990; Okey *et al.* 1994 a,b; Denison & Whitlock 1995; Whitlock *et al.* 1996). The Ah receptor has been identified in multiple species, tissues and cells and has been widely characterized using [^{3}H]TCDD or polyclonal antibodies (Okey *et al.* 1994a; Giannone *et al.* 1995). In fish an AhR was initially not detected, but was identified by Lorenzen & Okey (1990) in a trout hepatoma cell line. Characterization of the AhR in fish species has been further pursued by Hahn and coworkers (Hahn *et al.* 1994; Hahn & Karchner 1995).

Although most research on AhR has focused on toxicants such as TCDD, MC and PCBs, it has been postulated that an endogenous substrate for AhR may function in embryonic development (Nebert 1994). This has been further supported with the construction of an AhR deficient ($AhR^{-/-}$) mouse line. These mice showed decreased survival rates, decreased accumulation of lymphocytes and reduced size of livers, suggesting that AhR plays an important role in the development of the liver and the immune system (Fernandez-Salguero *et al.* 1995). These AhR deficient mice and also cell lines are considered interesting models in the elucidation of AhR functions (Gonzalez *et al.* 1995; Ma & Whitlock 1996).

The AhR functions as a ligand activated transcription factor, which resides in its unliganded form in the cytoplasm. The inactive AhR is complexed to the heat shock protein HSP90 and possibly other proteins (Okey *et al.* 1994b). Although it has been presumed that AhR resembles steroid receptors, the identification of the Ah-receptor-nuclear-translocator (ARNT) protein, a functional partner to AhR, shows a distinct transduction pathway for AhR (Hoffman *et al.* 1991). AhR and ARNT have similar sequence domains (referred to as PAS) to two Drosophila proteins (Per and Sim), which are basic helix-loop-helix DNA binding proteins. The DNA binding domain of AhR is therefore distinct from steroid receptors, that have 'zinc-fingers' as DNA binding

domains (Okey *et al.* 1994b). After ligand binding, AhR dissociates from HSP90, translocates to the nucleus and heterodimerizes with ARNT. AhR and ARNT bind both to respective DNA sequences of the Ah-responsive elements (AhREs), also termed dioxin-responsive (DREs) or xenobiotic-responsive elements (XREs) (Fukunaga & Hankinson 1996). Genes containing AhRE in their 5' regulatory sequence are not only *Ah* battery genes, but also include a large variety of other genes such as *c-fos* and *c-jun* (Lai *et al.* 1996).

Induction of the *Ah* gene battery is positively controlled by AhR and involves all six genes. The upstream regulatory region of the murine CYP1A1 and CYP1A2 contain multiple AhREs, whereas the phase II genes contain at least one AhRE and one electrophilic responsive element (EpRE), also termed antioxidant responsive element (ARE) (Nebert 1994). EpRE further contains AP-1 binding sites (Friling *et al.* 1992). A similar system to the murine *Ah* gene battery has been proposed for lower vertebrates, including fish, and has been tested primarily in environmental pollution studies (Stegeman & Hahn 1994).

1.2.2.3. Cytochromes P450 in different vertebrates

Induction of CYP1A1 gene expression and catalytic activity is a common and sensitive indicator of exposure to xenobiotics. Catalytic activity of CYP1A1 is determined as aryl hydrocarbon hydroxylase (AHH) or ethoxyresorufin-O-deethylase (EROD), both of which are used for aquatic organisms in environmental monitoring studies (Gooch *et al.* 1989; Tuvikene 1995). Induction of cytochromes P450 in mammals, other vertebrates and invertebrates has been extensively reviewed by Okey (1990). Within the fish field, different forms of cytochromes P450s in various fish species have been characterized by Stegeman's group (Stegeman & Kloepper-

Sams, 1987; Stegeman & Hahn 1994; Morrison *et al.* 1995), Buhler's laboratory (Schlenk *et al.* 1993; Miranda *et al.* 1993, 1994; Buhler *et al.* 1994) and by Goksøyr and coworkers (Husøy *et al.* 1994). Xenobiotic inducible cytochrome P450 genes, whether AhR mediated or not, have been recently summarized for mammalian species (Denison & Whitlock 1995). Related genes appear to be induced in fish. In mammals phenobarbital induces genes of the cytochrome P4502B family. Such a response mechanism is thought to be absent in fish species (Kleinow *et al.* 1987; Miranda *et al.* 1994). However, phenobarbital has been recently shown to induce rainbow trout CYP1A in primary culture (Sadar *et al.* 1996). Multiple piscine P450 genes show marked sequence homology to their mammalian counterparts, with CYP1A genes the most characterized. Fish CYP1A genes are orthologous to the mammalian CYP1A1 and CYP1A2 subfamilies; *e.g.* the rainbow trout CYP1A protein shares 51-59% amino acid identity with the mammalian CYP1A1 and CYP1A2 forms (Stegeman & Hahn 1994). It has thus been proposed that a gene duplication event with CYP1A has occurred after the phylogenetic split between fish and the line leading to mammals (Morrison *et al.* 1995). Two CYP1A genes, termed CYP1A1 and CYP1A2 have been identified in rainbow trout, but sequences of both genes differ by only 4%, in comparison to mammalian CYP1A1 and CYP1A2 which differ by 27-30% (Berndtson & Chen 1994). However, these designated trout CYP1A1 and CYP1A2, like other fish CYP1A forms are more closely related to mammalian CYP1A1 than to CYP1A2 (Morrison *et al.* 1995).

1.2.2.4. Xenobiotics and P450 expression

An important aspect for CYP1A induction is the structure-activity relationship for each class of AhR ligands, although cross-class comparisons show differences. TCDD in Hepa-1 cells binds

the AhR with approximately 3-4-fold greater affinity than MC. But TCDD in these cells is 1,000-fold more potent than MC as an inducer of CYP1A1 catalytic activity (Riddick *et al.* 1994). This difference was mainly attributed to an increased metabolism of MC over time. TCDD is resistant to oxygenation by DMEs, which appears to be a key element in its biological potency and toxicity (Riddick *et al.* 1994). Affinity for the AhR alone may thus not determine an overall effect on metabolic activity, which is an important consideration for xenobiotics such as PCB congeners. Non-ortho coplanar PCBs are marked inducers of CYP1A in vertebrates, while monoortho analogs possess diminished but significant inducing potencies (Gooch *et al.* 1989; Safe 1994). Certain PCDDs and PCBs are known to antagonize TCDD mediated induction of CYP1A most likely by interference at the TCDD-AhR complex (Merchant & Safe 1995). Even well-described AhR agonists such as 3,3',4,4'-tetrachlorobiphenyl (TCB) suppress CYP1A catalytic EROD activity at higher exposure concentrations in different vertebrates. This suppression of EROD occurs in the presence of highly elevated CYP1A mRNA and/or CYP1A protein (Gooch *et al.* 1989; Hahn *et al.* 1993).

Ames and coworkers (Park *et al.* 1996) have recently shown that TCDD induced CYP1A1 activity is associated with oxidative DNA damage, apparently caused by a leak of oxygen radicals. They suggested further that the poor metabolism of TCDD may uncouple CYP1A1 and generate activated oxygen from the catalytic site (Park *et al.* 1996). Interference of several factors can play a vital role for cellular metabolism of a xenobiotic and modulate its toxicity.

1.2.2.5. Factors influencing cytochrome P450 activity

Although P450 induction primarily occurs by transcriptional regulation, some post-

transcriptional events influence the induction mechanism (Okey 1990). Stability of mRNA and the availability of heme are critical factors for P450 induction. Xenobiotics may still be able to induce P450 apoprotein, which is catalytically inactive without the prosthetic heme group (Okey 1990). But also other cellular parameters may play an important role for P450 induction. CYP1A catalytic activity was inhibited by immunostimulants, which stimulate nitric oxide (NO·) production. Immunostimulant-induced NO· synthesis was reported to be positively correlated with the severity of P450 inhibition (Khatsenko *et al.* 1993). On the other hand, there is some evidence that some cytochromes P450 such as CYP3A positively influence rat hepatocyte NO· synthesis (Kuo & Abe 1995). The inhibition of CYP1A by NO·-induction has been demonstrated in several reports (Stadler *et al.* 1994; Kim *et al.* 1995; Osawa *et al.* 1995). A suggested mechanism for NO· impairment of CYP1A is enhanced heme degradation and intracellular heme loss. This effect could be directly mediated by NO· on intracellular heme content (i.e. through ferrochelatase) or indirectly by activation of heme oxygenase (Kim *et al.* 1995). The involvement of heme oxygenase in CYP1A activity is moreover interesting, as heme oxygenase is induced by oxidative stress and is thought to enhance the antioxidant potential of the cell (Keyse & Tyrell 1989).

1.2.2.6. Phase II enzymes

Conjugation enzymes play vital functions for the fate of a xenobiotic. Major phase II pathways are *i.e.* the conjugation with glutathione by glutathione S-transferases (GST), UDP-glucuronic acid by UDPglucuronosyltransferases (UDPGT), or with sulfate by sulfotransferases. Most of these conjugation pathways involve multiple gene families and establish vast research fields of their own. A GST form and an UDPGT form are part of the *Ah* gene battery and their induction

by xenobiotics is a major research topic (Nebert 1994). In addition to AhRE, phase II battery genes are controlled by EpRE. The influence of oxidative stress on phase II induction has long been proposed (Nebert *et al.* 1990). Glutathione (GSH) deficiency has been shown to induce phase II battery genes and is suggested to be mediated by EpRE (Shertzer *et al.* 1995). The presence of AP-1 binding sites at the EpRE stimulated investigation of Jun and Fos regulation of phase II battery genes (Friling *et al.* 1992; Jaiswal 1994).

In fish, *Ah* battery phase II enzymes have been extensively studied, most prominently GST and UDPGT (George 1994). Because of the involvement in protection against oxidative stress DT diaphorase has been increasingly investigated (Hasspieler & Di Giulio 1992, 1994). GST and UDPGT activities are inducible by aquatic pollutants such as PAHs and PCBs, however, there appear to be species differences. Qualitatively GST and UDPGT function similarly as in mammals and the various genes often share extensive sequence homologies with mammalian species (Scott *et al.* 1992; George 1994; Pirrit *et al.* 1995). It has been suggested that glucuronidation in fish is quantitatively the most important conjugation pathway (Clarke *et al.* 1991). Induction of phase II enzymes displayed increased UDPGT activities in PCB exposed rainbow trout, while changes in GST activities were less pronounced (Huuskonen *et al.* 1996). Basal GST activity in channel catfish and brown bullhead is remarkably high (Hasspieler *et al.* 1994a,b). It has been suggested that the expression of GSTs may play a contributing role in the resistance of channel catfish to environmental carcinogens (Gallagher *et al.* 1996). Similar importance for GSH conjugation has been proposed for white suckers, which are known to efficiently eliminate B[a]P. Loss of GST-mediated protection was shown to favour liver neoplasms in suckers repeatedly exposed to carcinogenic PAHs (Kirby *et al.* 1995).

1.2.2.7. Environmental implications

The effects of aquatic pollution on AhR regulated enzymes in various fish species has been amply described (Lindström-Seppä 1990, Tuvikene 1995). The role of plants, as occupants of a crucial niche in the aquatic system, in uptake, bioconcentration and elimination of various pollutants and responses of xenobiotic metabolism is well reported (Roy 1994). Accumulation of chemicals in fish tissues has been frequently used to assess the health hazard on aquatic organisms. Also various bioindicators, which used to be primarily phase I and II enzymes, assess biochemical or physiological changes in response to pollutant loaded environments. These are extensively employed as indicators of fish health and possibly pollution driven changes in fish populations and entire ecosystems (George 1994; Stegeman & Hahn 1994). However, with the complex cellular changes triggered by single xenobiotics (see above), individual bioindicators alone may not give a clear picture of the pollution impact, which normally consists of a multitude of different classes of chemicals. A vast number of bioindicators are now employed in aquatic pollution studies (Pandrangi *et al.* 1995; McCain *et al.* 1996). The use of multiple bioindicators as a multimarker approach for environmental pollution studies has been proposed to generate better knowledge of pollutant impact on aquatic species especially for long-term monitoring (Burgeot *et al.* 1996). Within the past decade, antioxidant defenses have received recognition in the fish field as potential bioindicators and vital components of fish health.

1.2.3. Roles of Antioxidant defenses

1.2.3.1. Reactive oxygen species

A free radical is any species capable of independent existence (hence "free") that contains an unpaired electron in the valence shell ("radical"). Pairing of oppositely spinning electrons renders stability to these electrons and the instability makes radicals sometimes highly reactive. Unlike radicals, reactive oxygen species (ROS) include also non-radical derivatives of oxygen (i.e. H_2O_2, peroxides, singlet oxygen, etc.). During metabolic processes harmful radicals and ROS can be generated from oxygen and other elements. Oxygen radicals and ROS have been implicated in oxidative tissue damage and pathological states of diseases (Kappus 1986; Halliwell & Gutteridge 1989). Radicals and ROS generated in the first instance in biological processes are mostly superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$). Superoxide is a weak oxidizing agent able to oxidize ascorbic acid, thiols and other molecules. Superoxide rapidly dismutates into hydrogen peroxide (1), a reaction greatly accelerated by superoxide dismutase (SOD),

$$2\ O_2^{\cdot-} + 2\ H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

H_2O_2 is removed by the action of catalase and glutathione peroxidases.

The hydroxyl radical is the most reactive oxygen species and may be formed through either the Fenton reaction (2) or the Haber -Weiss reaction (3).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + OH^- \qquad (2)$$

$$O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + HO^{\cdot} + OH^- \qquad (3)$$

Transition metals, particularly iron serve as catalysts for significant hydroxyl radical production. Half-life differs vastly between ROS, whereby the hydroxyl radical has one of the shortest half-

lives (10^{-9} sec) and so its reactions take place at the site of generation (Sies 1993). The hydroxyl radical is an extremely aggressive oxidant and can attack proteins, carbohydrates, DNA and lipids (Halliwell & Gutteridge 1989).

Several enzymes produce H_2O_2 or superoxide, which include the peroxisomal glycolate oxidase and L-α-hydroxyacid oxidase, the cytosolic aldehyde oxidase and xanthine oxidase among several others (Sen 1994). Moreover, uncoupling side reactions of cytochromes P450 may generate H_2O_2 and superoxide (Mueller *et al.* 1995). Activation of cytochromes P450 by *e.g.* xenobiotics can thus increase intracellular ROS levels. CYP1A metabolic activation has been previously shown to be accompanied by oxidative DNA damage (Park *et al.* 1996). However, the generation of ROS by P450s may itself suicide-inactivate the P450 protein (Karuzina & Archakov 1994). Apart from P450 uncoupled side reactions, xenobiotics themselves may form ROS (for review see Kappus 1986; Aust *et al.* 1993; Fig. 1.2). Several lines of evidence have shown that various PCB congeners stimulate the production of ROS (Toborek *et al.* 1995; Amaro *et al.* 1996; Tithof *et al.* 1996). Whether this type of ROS generation always involves Ah receptor mediation remains a controversial point. Hydroxylation of PCBs to redox-cycling quinones is catalyzed by cytochrome P450. However, it was recently shown that PCB activated superoxide production by neutrophils in an Ah receptor independent pattern (Tithof *et al.* 1996). The implications of xenobiotic derived ROS production in aquatic organisms continuously exposed to polluted environments have been subject to some review (Winston & Di Giulio 1991; Winston 1991).

Apart from deleterious effects of ROS, they themselves can serve useful functions *in vivo*. Nitric oxide is a free radical which is synthesized in endothelial cells and phagocytes and plays

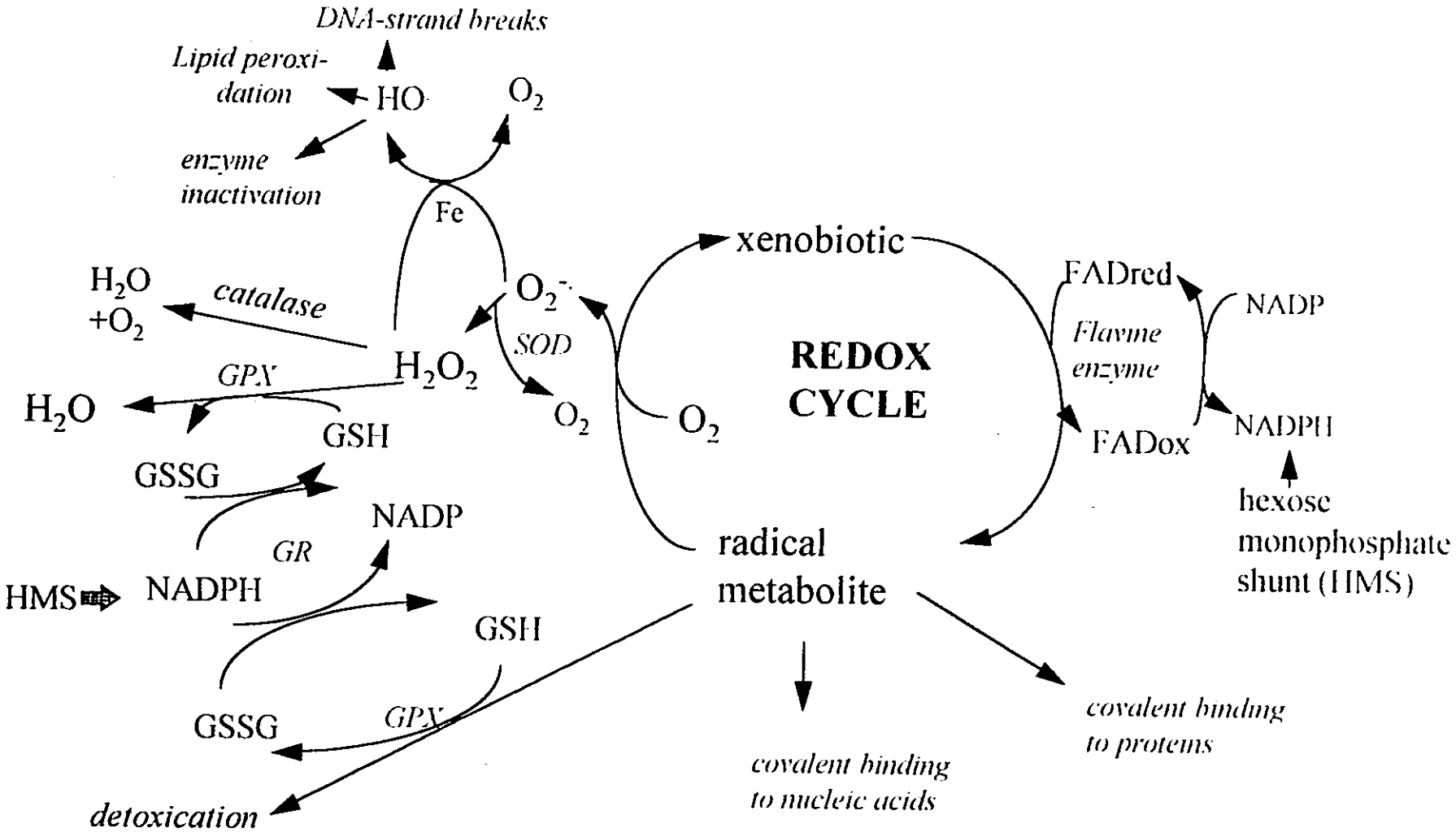


Fig. 1.2. Redox cycling xenobiotic and involved detoxication systems. SOD: superoxide dismutase, GPX: glutathione peroxidase, GR: glutahione reductase, GSH: reduced glutathione, GSSG: glutathione disulfide (adapted from Kappus 1986).

important roles as a vasodilator and a neurotransmitter (Halliwell 1996). Superoxide is produced by phagocytic cells (neutrophils, macrophages) to inactivate viruses and bacteria. ROS are known to increase expression of *c-fos*, *c-jun* and *c-myc* which encode transcription factors involved in cell proliferation. Such a mechanism in early inflammation can also contribute to wound healing (Burdon 1995). ROS are known to activate nuclear factor κB (NFκB) (Meyer *et al.* 1993). NFκB controls the transcription of a number of immunologically important genes such as interleukin-2 and tumor necrosis factor-α (Dröge *et al.* 1994; Sen & Packer 1996).

The actions of ROS are opposed by a balanced system of antioxidant defenses. Disturbance of the pro- and anti-oxidant balance in favour of prooxidant forces results in oxidative stress.

1.2.3.2. Endogenous antioxidants

Halliwell and Gutteridge (1989) define antioxidants as any substance that, when present in low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate. Oxidizable substrate can be virtually most components of a cell including proteins, lipids, DNA, etc. Physiological antioxidants serve aerobic life forms in the circumvention of oxygen toxicity. Primary components of these endogenous antioxidants include superoxide dismutases (EC 1.15.1.1) and catalase (EC 1.11.1.6) and the glutathione system. Several auxiliary systems such as phase II enzymes (GST, UDPGT, DT diaphorase), NADPH supplying systems (hexose monophosphate shunt) and oxidative damage repair systems (DNA repair, oxidized protein turnover, etc.) contribute to the antioxidant defense capacity. In a further sense several regulons including EpRE, and bacterial oxyR, oxoR, soxR and soxS may serve an alternate line of defense against ROS attack (Harris 1992; Sen 1994).

1.2.3.2.1. Superoxide dismutases (SOD). McCord and Fridovich (1969) discovered SOD which functions in the dismutation of superoxide anion radical and eliminates toxic effects of $O_2^{-\cdot}$ and derived radicals (Fig. 1.2). There are two cellular types: cytosolic cuprozinc SOD (Cu,Zn-SOD) and mitochondrial mangano SOD (Mn-SOD). 'Extracellular SOD' is also a Cu,Zn enzyme, but largely distinct from the normal Cu,Zn-SOD (Harris 1992). The physiological importance of SOD has been extensively described in exercise (Sen 1995), as well as aging and degenerative diseases (Warner 1994). The involvement of SOD in detoxication reactions of redox cycling xenobiotics has been suggested (Winston & Di Giulio 1991; Aust *et al.* 1993). However, as SOD is only specific for superoxide, this enzyme along with catalase may be of limited use for toxicological purposes.

1.2.3.2.2. Catalase. Catalase is a heme protein using H_2O_2 as substrate and found in tissues of all species. It is primarily located in the peroxisomes, but can also be found in the cytosol (Eriksson *et al.* 1992). Two H_2O_2 are split into two H_2O and O_2. Catalase, like SOD is inducible by oxidative stress conditions. Particularly a combination of added catalase, SOD and glutathione peroxidases can efficiently improve cellular protection against insult from oxidative stress (Michiels *et al.* 1994).

1.2.3.2.3. The glutathione system. The discovery of glutathione (GSH) dates back to 1888, when de Rey-Pailhade observed that yeast cells and a number of different tissues (e.g. fish and beef muscle, sheep blood, fresh asparagus tips) contain a substance which spontaneously reacts with elemental sulfur to yield hydrogen sulfide (for review see Meister 1988). This substance was thought to be a dipeptide consisting of glutamate and cysteine and named philothion (Greek: φίλεο to love, θήον sulphur). By 1935 philothion had been renamed to glutathione, recognized

as a tripeptide, L-γ-glutamyl-L-cysteinylglycine, through the research of Hopkins and other pioneers. Much of the biochemistry of this low molecular weight thiol which is ubiquitously distributed in living cells has been contributed by Meister and coworkers (Meister and Anderson 1983; Meister 1988; 1991; 1994; 1995; Fig. 1.3).

GSH serves in a diverse series of critical cellular defensive functions which include free radical scavenging, detoxication of electrophiles, maintenance of thiol-disulfide status and signal transduction (Meister & Anderson 1983; Deneke & Fanburg 1989; DeLeve & Kaplowitz 1991, Dröge *et al.* 1994; Sen & Packer 1996). Glutathione peroxidase (GPX, EC 1.11.1.9) is specific for its hydrogen donor GSH, but uses a wide range of substrates extending from H_2O_2 to organic hydroperoxides. GPX is primarily a cytosolic selenoprotein, but there exist other GPXs, i.e. some isoenzymes of glutathione S-transferase, some glycosylated GPX in the plasma and a phospholipid hydroperoxide GPX associated with membranes (PHGPX) (Michiels *et al.* 1994). As a consequence of GPX action, GSH is oxidized to glutathione disulfide (GSSG), which is rapidly reduced back to GSH by glutathione disulfide reductase (GSSG reductase, GR, EC 1.6.4.2). This reaction requires reducing equivalents derived from the NADP-dehydrogenases glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase (6-PGDH), isocitrate dehydrogenase and malic enzyme (Reed 1986). GSH also functions in a second detoxication pathway initiated by GST (EC 2.5.1.18) catalyzing the conjugation between the sulfhydryl group of GSH and the electrophilic atom of a xenobiotic.

Synthesis of GSH is a two step process which are both ATP dependent. γ-

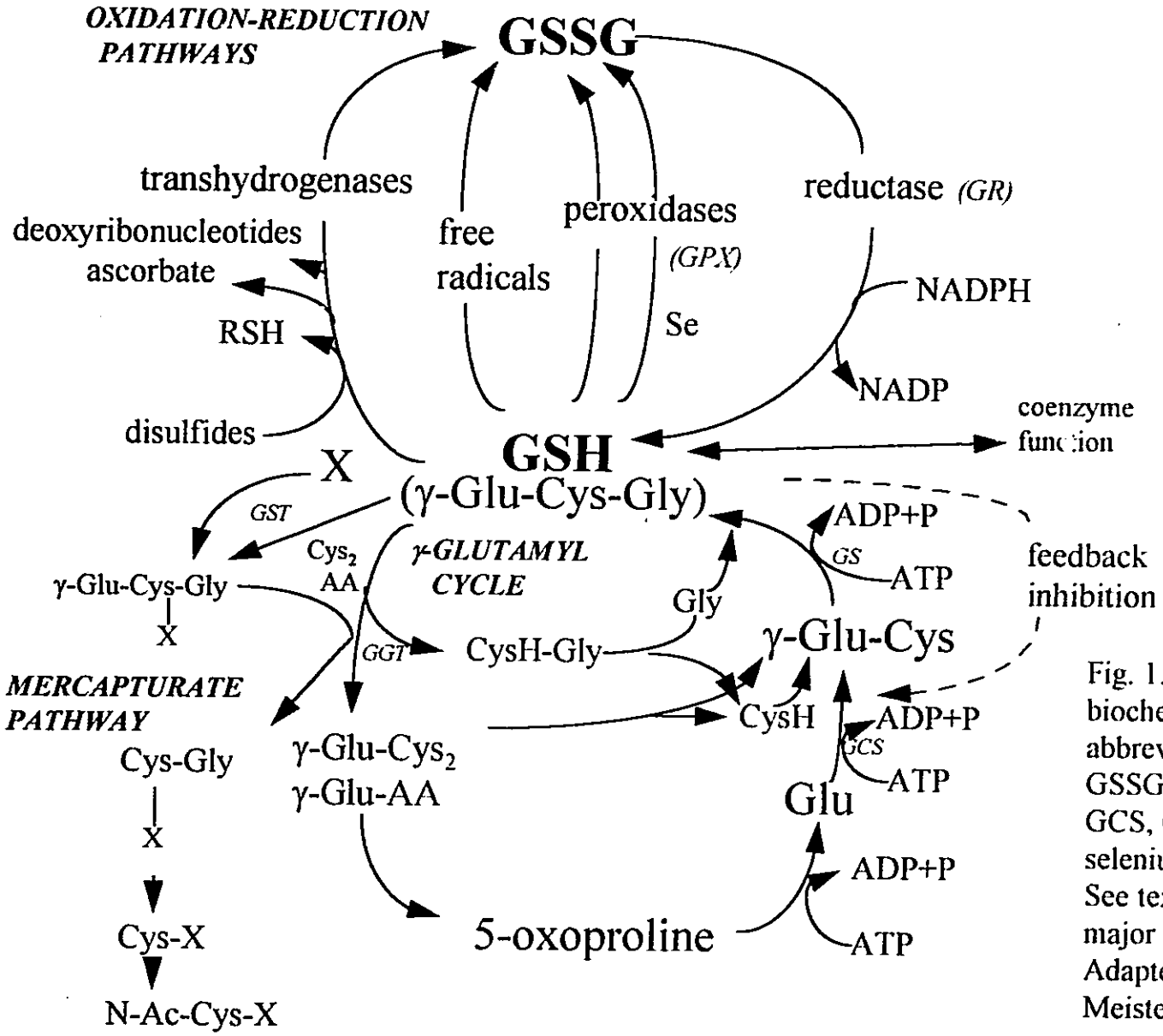


Fig. 1.3. Outline of GSH biochemistry. See list of abbreviations for GSH, GSSG, GR, GPX, GST, GCS, GS. Se-GPX, selenium dependent GPX. See text for description of major pathways. Adapted from Meister (1991).

Glutamylcysteine synthetase (GCS, EC 6.3.2.2) forms the dipeptide γ-glutamylcysteine, to which glycine is subsequently added by glutathione synthetase (GS, EC 6.3.2.3). GCS is subjected to feedback inhibition by GSH. The membrane bound γ-glutamyl transpeptidase (GGT, EC 2.3.2.2) couples the γ-glutamyl moiety to a suitable amino acid acceptor for transport into the cell. GGT aside from direct amino acid transport supplies the substrates for GSH synthesis. In view of Fig. 1.3, GSH, GPX, GR, GST, GGT, GCS and GS are collectively critical determinants of glutathione homeostasis of a tissue. At the same time GSH and its enzymes determine interorgan flows of GSH. The liver is largely regarded as a synthesizer and user of GSH and exporting large quantities of GSH into the blood. Kidney, lung and intestine have high activities of GGT and are thought to be primarily importers of GSH in mammals (Deneke & Fanburg 1989). In mammals liver supplies 90% of the circulating GSH. It has been suggested that GSH from liver is mobilized to other organs, *e.g.* skeletal muscle under oxidative stress (Ji 1995).

GSH serves also as a storage and transport form of cysteine moieties and thus protects the thiol group of cysteine from otherwise rapid metabolic utilization (Meister 1991). Most of the intracellular glutathione exists in the thiol form, while mixed disulfides, thioesters and GSSG are contributors to the total cellular pool of glutathione. Intracellular GSSG content is low (approximately 5 %) compared with GSH levels (Anderson 1985). Under severe oxidative stress the ability of the cell to reduce GSSG via GR may be overcome, leading to intracellular GSSG accumulation. In circumvention of toxicity of high cellular GSSG levels, GSSG are actively transported out of the cell (DeLeve & Kaplowitz 1991; Meister 1991).

It has been known that lymphocytes have a demand for thiols for their functional performance. Cysteine is the limiting substrate for GSH synthesis and GSH is vital for T cell functions (Dröge *et*

al. 1995). With the discovery that humans infected with the human immunodeficiency virus (HIV) show cysteine and GSH deficiency (Dröge *et al.* 1995), the immunological and immunopathological functions of GSH are increasingly investigated (Dröge *et al.* 1994). In order to overcome this GSH deficiency in HIV infected patients, cysteine derivatives such as N-acetylcysteine (NAC) have been considered for clinical trials (Dröge *et al.* 1995; McLellan *et al.* 1995) to boost GSH synthesis.

1.2.3.3. Exogenous antioxidants and supplementations

The most widely studied protective antioxidant agents are nutrients including the vitamins E, C and A, carotenoids and numerous phenolic compounds (for reviews see Liebler 1993; Meister 1994; Winkler *et al.* 1994; Sies & Stahl 1995). Many of these are presumed to have therapeutic potential in biomedical research. A concise listing of antioxidants for therapeutic treatment is given in Sen (1994).

Vitamin E, a lipid soluble vitamin, is a poor antioxidant outside the membrane bilayer, but very effective when incorporated into the membrane (Gutteridge 1994). It is regarded as the most important radical scavenger within membranes and lipoproteins. Its antioxidant properties are crucial for the prevention and interception of lipid peroxidation. However, the radical scavenging ability of vitamin E relies on either ascorbate (vitamin C) or GSH for regeneration (Liebler 1993). Vitamin C is considered the most important antioxidant in extracellular fluids as well as serving as an intracellular antioxidant. Vitamin C is reported to scavenge superoxide, H_2O_2, hydroxyl radical and peroxyl radicals (Sies & Stahl 1995). Vitamin C acts like vitamin E as a chain breaking antioxidant. Vitamin C and glutathione are shown to form an active redox couple in a cell, which

has been amply described (Meister 1994; Winkler *et al.* 1994). Carotenoids are lipophilic compounds that mostly contain conjugated double bonds which are responsible for their antioxidant action. β-Carotene has often been employed for cancer treatment (Sies & Stahl 1995). Selenium, a cofactor for GPX and ebselen, which mimics GPX action, are well described antioxidants (Sies 1993). Also phenolic antioxidants, including butylated hydroxytoluene and butylated hydroxyanisole were used as food antioxidants, but were restricted as they may be metabolized to potentially reactive intermediates (Sies 1993).

Several pro-glutathione agents are used in clinical studies in the prevention of numerous oxidant-related diseases. N-Acetylcysteine promotes GSH synthesis, reacts with ROS and is a potential drug for palliative treatment of HIV infection (McLellan *et al.* 1995). α-Lipoic acid is a thiol antioxidant that scavenges hydroxyl radicals, hypochlorous acid and singlet oxygen. Lipoate is rapidly converted in many tissues into its reduced form dehydrolipoic acid (DHLA), which regenerates the radical forms of ascorbate and vitamin E (Packer *et al.* 1995). The DHLA/lipoate couple has a redox potential of -32 V and is thus able to reduce GSSG to GSH, as the redox potential of the GSH/GSSG couple is -24 V in comparison. Lipoate efficiently increases cellular GSH concentrations (Busse *et al.* 1992) and has potential applications for many aspects of the pathologies of various diseases (Packer *et al.* 1995).

Vitamin E, C, DHLA and GSH thus act synergistically in the form of an antioxidant chain reaction (Sen 1995). When ROS production is excessive, the antioxidant defense of an organism can be hampered by nutritional deficiencies, pharmacological intervention, exposure to xenobiotics, prolonged exercise or cold exposure and ROS are likely to overwhelm the inadequate defense causing insult by oxidative stress. Nutritional supplementation of antioxidants can thus aid

in the circumvention of oxidative stress states of an organism. Such preventive measures have been discussed for mammals (Ji 1995), but also for lower vertebrates such as fish with applications in the aquacultural industry (Sigurgisladottir *et al.* 1994a). However, in fish these supplementation are mainly based on lipid and water soluble vitamins. Efficient elevation of tissue GSH content in mammals has been mostly achieved indirectly by pro-GSH agents or by GSH monoesters (Meister 1991). GSH *per se* is not efficiently transported into most mammalian cell types.

1.2.3.4. Antioxidant defenses in fish

The aquatic environment is a sink for numerous contaminants, which often produce ROS. Aquatic organisms are thus continuously exposed to ROS in waters polluted with organic contaminants (Winston & Di Giulio 1991). Multiple studies have used antioxidant defenses as sensitive markers in pollution monitoring (Mather-Mihaich & Di Giulio 1991; Rodríguez-Ariza *et al.* 1993; Thomas & Wofford 1993; Otto *et al.* 1994, 1996b; Förlin *et al.* 1995; Roy *et al.* 1995b). Only little work has been conducted to assess the mechanism of antioxidant defense systems in fish. Piscine glutathione biochemistry and associated antioxidant defenses are largely described to resemble those of mammals (for review see Otto 1992). Much of the current knowledge about physiological antioxidant systems in different fish species has been contributed by Di Giulio and coworkers (Gallagher *et al.* 1992; Hasspieler *et al.* 1994a,b; Di Giulio *et al.* 1995). Most of these studies assessed GSH content and antioxidant enzymes activities in different fish species exposed to ROS. Overall the studies executed in fish contribute only a small percentage to the vast literature on endogenous antioxidants presently available. The basic nature

of antioxidant defenses, particularly glutathione homeostasis and possibly differences to the mammalian system remain to be elucidated.

1.3. AIMS OF THE PRESENT STUDY

The purpose of the present series of investigations was to evaluate the functions of the endogenous antioxidant machinery in polychlorinated biphenyl (PCB) exposed teleost species. Emphasis in the first instance was to test antioxidant defenses along with commonly used xenobiotic detoxication parameters in teleost fish species exposed to PCBs. A second major objective was to determine the extent to which thiol status interferes with the various detoxication pathways particularly those mediated by cytochrome P4501A.

The specific aims of the studies included in this thesis were to:

* assess alterations in xenobiotic detoxication parameters and tissue antioxidant defenses in feral brown bullheads exposed to organic pollution in the St. Lawrence River compared with a non-polluted lake

* determine the effect of continuous PCB exposure on tissue PCB accumulation as well as xenobiotic and antioxidant responses in caged rainbow trout in the St. Lawrence River

* evaluate the basic features of endogenous antioxidant defenses, especially the GSH system in tissues of rainbow trout and black bullhead and their alteration in mature individuals

* characterize the long-term effects of a single dose of 3,3',4,4'-tetrachlorobiphenyl (TCB) on drug metabolizing enzyme activities and antioxidant defenses in hepatic and extrahepatic tissues of rainbow trout, and especially the role of skeletal muscle along with liver and kidney in the

detoxication of TCB

* test the efficiency of exogenous GSH as a delivery agent of GSH to tissues in rainbow trout and American eel and elucidate whether such thiol manipulation increases antioxidant defenses in TCB exposed trout

* reveal whether manipulation of thiol status regulates TCB induced cytochrome P4501A metabolism in rainbow trout

* examine the nature of regulatory effects of thiol manipulation on basal and TCB induced cytochrome P4501A function

In summary of these objectives, it will be shown that teleost fish rely on active endogenous antioxidant systems which participate in detoxication reactions when exposed to PCBs. Whole body glutathione homeostasis resembles that of mammals in many aspects, but shows differences in tissue distributions and response to exogenous GSH. Glutathione status plays a vital role in basal and TCB induced cytochrome P450 metabolism.

CHAPTER 2.

PHASE I AND II ENZYMES AND ANTIOXIDANT RESPONSES IN DIFFERENT TISSUES OF BROWN BULLHEADS FROM RELATIVELY POLLUTED AND NON-POLLUTED SYSTEMS

Introduction

The brown bullhead (*Ameiurus nebulosus*) is a desired food fish which has been used extensively in environmental pollution studies (Grady *et al.* 1992; Folmar *et al.* 1993; Hasspieler *et al.* 1994a). As bottom dwellers, brown bullheads are in close contact with contaminated sediments in polluted waters. They are reported to tolerate suboptimal water quality (Scott and Crossman 1973) and maintain a responsive endogenous defense against exogenously added toxicants (Hasspieler *et al.* 1994a). Related species of the same order including carp and channel catfish have been shown to accumulate in liver and skeletal muscle chlorinated dibenzo-p-dioxins and dibenzofurans, constituents of bleached kraft mill effluents (Schell *et al.* 1993; Ahokas *et al.* 1994).

Hepatic cytochrome P450-dependent monooxygenases (MO), especially ethoxyresorufin O-deethylase (EROD), are widely accepted indicators of exposure to common organic pollutants and these have been studied in catfish and related species (Mather-Mihaich and Di Giulio 1991; Munkittrick *et al.* 1993; Ahokas *et al.* 1994). Although polychlorinated dibenzofurans and dibenzo-p-dioxins are key MO inducers, MO induction has been reported in fish after exposure to unbleached pulp mill effluents containing no chlorinated compounds (Otto *et al.* 1994). MO

induction is often associated with changes in cellular antioxidant defenses (Winston 1991; Rodriguez-Ariza *et al.* 1993; Otto *et al.* 1994).

The intracellular tripeptide glutathione (L-γ-glutamyl-L-cysteinylglycine; GSH) is a major component of cellular antioxidant defenses by its involvement in two major detoxication pathways (Meister and Anderson 1983). The conjugation of a toxicant with GSH catalyzed by glutathione S-transferase (GST) is an important phase II pathway, rendering a toxicant more water soluble and facilitating its excretion. Glutathione peroxidase (GPX) binds GSH with high affinity and oxidizes it to glutathione disulfide (GSSG). GPX can detoxify H_2O_2 and organic hydroperoxides which are formed during tissue oxidative stress. These peroxides arise from various xenobiotics that can undergo redox cycling and generate reactive oxygen species such as superoxide ($O_2^{\cdot}$) and H_2O_2 or other harmful radicals (Kappus 1986). Related antioxidant enzymes that play an important role in detoxication of $O_2^{\cdot}$ and H_2O_2 are superoxide dismutase (SOD) and catalase (CAT). These antioxidant defenses have been shown to change in channel catfish and brown bullhead exposed to organic pollutants (Mather-Mihaich and Di Giulio 1991; Hasspieler *et al.* 1994a).

The St. Lawrence River is derived from the Great Lakes, which supply much of its water flow. Even at the estuary portion of the St. Lawrence River, more than 50% of its water originates from the Great Lakes. The St. Lawrence River contains a multitude of toxic compounds which arise from both the Great Lakes and from the highly industrialized areas (paper mill, car manufacture and chemical industry) along its shoreline (Government of Canada 1991). A large fish community is found in the St. Lawrence River and brown bullheads are a major component of this community that is economically important for fishery and recreational activities (Eckersley and McCullough

1990). In an attempt to evaluate the long-term effects of these waters on feral fish species, we harvested brown bullheads from the St. Lawrence River, south of Ottawa, Ontario (Lake St. Francis) and from a relatively non-polluted lake, Lac La Pêche, Gatineau Park, Qué. It was our primary objective to select a water system that is largely free from industrial or domestic effluents or agricultural runoff, but under similar climatic conditions as is the St. Lawrence River. Lac La Pêche is located north of Ottawa in a non-developed park area and receives pollutants primarily from atmospheric sources.

The comparison of fish from polluted and less polluted areas is a standard paradigm in environmental toxicology (Munkittrick *et al.* 1992, 1993; Rodriguez-Ariza *et al.* 1993). Organic contaminants in the St. Lawrence River are thought to be primarily polychlorinated biphenyls (PCBs) (Ontario Ministry of Environment and Energy 1993). Therefore, we determined PCB concentrations in white muscle of bullheads from both sites. We attempted to correlate tissue PCB contents with phase I and II enzymes and antioxidant status in tissues of these feral bullheads. These parameters are used biomarkers for aquatic pollution studies (Förlin *et al.* 1995) and their analysis is meant to clarify the role of these defense mechanisms in the whole body detoxication of pollutants by this species. To do so, we have estimated activities of EROD and of the conjugation enzymes UDP-glucuronosyltransferase (UDPGT) and GST in liver and other tissues. We have reported that white skeletal muscle of rainbow trout is an important component of the detoxication system (Chapter 5), but whether this is a general piscine-characteristic is not known. Antioxidant defenses were monitored in bullheads collected from both the St. Lawrence River and Lac La Pêche by determining the antioxidant enzymes GPX, SOD and CAT and total glutathione concentrations (TGSH; GSH + GSSG) in liver, kidney, red and white muscles.

Materials and Methods

Chemicals

Ethoxyresorufin, uridine 5'-diphosphoglucuronic acid (UDPGA), NADPH, 5,5-dithio-bis(2-nitrobenzoic acid), 1-chloro-2,4-dinitrobenzene (CDNB), GSH, Bakers yeast glutathione reductase, xanthine, xanthine oxidase (XOD) and p-nitrophenol were obtained from Sigma Chemical Co. All other reagents were analytical grade.

Animals

Brown bullheads were collected in the month of August. St. Lawrence River bullheads were obtained from a local fisherman at South Lancaster, located south of Ottawa and 50 km east of Cornwall, ON. After trapping, the fish were caged at place in the River for 2 days until their transport in tanks of oxygenated river water to Ottawa (approximately 2 h). Lac La Pêche is located approximately 25 km north of Ottawa in Gatineau Park. Lake bullheads were captured with gill nets set in the afternoon and removed 8 h later, and transported in tanks of aerated lake water to Ottawa (1 h). All fish were killed immediately upon arrival in the laboratory by a blow to the head. Tissues were excised, cleaned and freeze-clamped in liquid nitrogen. Frozen tissues were stored at -80°C until homogenized. Pectoral spines were removed, cleaned and air-dried. Annuli were counted microscopically in sliced sections to estimate fish age. Frozen muscle samples were analyzed for PCB concentrations by capillary gas chromatography by the EPA approved laboratory of Dr. Brian Bush, Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201-0509, USA. Samples were homogenized with sodium sulfate and hexane and analyzed on a Hewlett-Packard 5840A gas chromatograph equipped with a Ni-63 electron capture detector according to Bush *et al.* (1989).

Glutathione measurements

Frozen (< 2 months) tissue samples were homogenized in ice-cold 5% sulfosalicylic acid and centrifuged at 4,000g (Sorvall RC 28S) at 4°C for 10 min to remove the precipitate. Supernatants were stored at 0-4°C until total glutathione (TGSH) concentrations were determined according to Sen *et al.* (1992), in which the rate of reduction of DTNB is correlated to the sum of reduced and oxidized glutathione (GSH and GSSG) using a standard curve.

Preparation of microsomal and cytosolic tissue fractions

Samples of frozen (< 1 week) tissues were rapidly weighed and homogenized in 0.25 M sucrose with a Potter-Elvehjem-type homogenizer for liver and kidney and with a Polytron PCU-2 homogenizer (Brinkmann Instruments, Rexdale, ON) for muscles. Homogenates were centrifuged at 10,000g (Sorvall RC 28S) for 15 min at 4°C. The resulting supernatant fractions were centrifuged at 105,000g (Beckman L8-70M Ultracentrifuge, 50Ti rotor) at 4°C for 1 h. Microsomes were resuspended in 0.25 M sucrose containing 60 mM Tris, 5 mM EDTA, and 20% glycerol (pH 7.4) by homogenization with a Potter-Elvehjem-type homogenizer. Microsomes and supernatants were stored at -80°C until analyzed for enzyme activities.

Cytosolic enzyme activity estimates

Enzymes were assayed at 18°C, a temperature which is within the range experienced by this species at the time of harvest (August). Only SOD and CAT were assayed at the temperatures described in the original methods (25°C and 0-2°C, respectively). Substrate concentrations were those of the original assay and a protein content was chosen to give linear changes in enzyme activity. Previous studies from our laboratory have found that tissue freezing does not alter the bullhead enzyme activities assayed in this study.

Glutathione peroxidase (GPX, EC 1.11.1.9) activity in the different tissues was determined by the method of Tappel (1978), using original GSH concentrations (0.25 mM) and cumene hydroperoxide (0.05mg/ml) in 50 mM Tris buffer, pH7.6. The data are expressed as nmol NADPH oxidized per min per mg of protein.. Glutathione S-transferase (GST, EC 2.5.1.18) activity was monitored according to Habig *et al.* (1974) by measuring conjugate derivative formation as a function of time, with final concentrations of 1 mM GSH and 1 mM CDNB. Specific activities are expressed a nmol GSH conjugated per min per mg protein using an extinction coefficient at 340 nm of 9.6 x 10^3/mol per cm^2. Superoxide dismutase (SOD, EC 1.15.1.1) activity was by Beauchamp and Fridovich (1971) procedure, in which xanthine-xanthine oxidase is the superoxide generating system and nitro blue tetrazolium is the indicator of superoxide production. By inhibition of Cu,Zn-SOD with cyanide, the activity of Mn-SOD was determined. Catalase (CAT, EC 1.11.1.6) activity was determined according to Cohen *et al.* (1970). In short, supernatants were treated with ethanol and Triton X-100 to increase observable catalase levels. The enzyme-catalyzed decomposition of H_2O_2 is carried out in an ice-bath, residual H_2O_2 is determined by its reaction with permanganate. Residual permanganate concentrations are determined spectrophotometrically. Catalase activity is calculated by estimating residual H_2O_2 concentrations by linear regression from a standard curve.

Microsomal enzyme activities

Cytochrome P450-dependent monooxygenase (MO) was detected as ethoxyresorufin O-deethylase (EROD) activities as described by Burke and Mayer (1974), and UDP-glucuronosyltransferase (UDPGT, EC 2.4.1.17) activity was measured according to Hänninen (1968) using p-nitrophenol as a substrate.

All enzyme activities are expressed as units per mg protein. Cytosolic and microsomal protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Data analysis

Significant differences between Lac La Pêche and St. Lawrence River bullheads were tested with a Student's t-test for unpaired sample means. With nonhomogeneous variance in many variables, determined by the test for equal variances, data were transformed into their common logarithm and analyzed with the Student's t-test. A level of $p<0.05$ was accepted as significant.

Results

The physical characteristics of the bullheads used in this study are summarized in Table 2.1. The two groups did not differ in length, but the weight of the St. Lawrence River bullheads was significantly different from those of Lac La Pêche. This weight difference was also reflected in the calculated condition factor (CF), as the CF was significantly higher in the St. Lawrence River fish. On the other hand, liver somatic index (LSI) did not differ between the groups. All fish were females and non-spawning except for two individuals from Lac La Pêche and one from the St. Lawrence River, which retained eggs in their gonads. Lac La Pêche bullheads were significantly older than those from the St. Lawrence River (Table 2.1), although six of the nine bullheads from the Lac La Pêche group overlapped the age profile of the St. Lawrence River group.

Total PCB content and concentrations of various congeners in bullhead white muscle are reported in Table 2.2. Total PCB content is 22-fold higher in bullheads from the St. Lawrence River than from Lac La Pêche ($p<0.01$), even though the percentage of fat was not significantly

Table 2.1. Physical characteristics of bullheads collected from Lac La Pêche and the St. Lawrence River.

	Lac La Pêche (9)	St. Lawrence River (10)
Weight (g)	249 ± 16	324 ± 16*
Length (cm)	27.6 ± 0.60	28.7 ± 0.50
LSI	1.9 ± 0.12	1.9 ± 0.12
CF	1.2 ± 0.05	1.4 ± 0.03#
Age (y)	4.3 ± 0.5	3.2 ± 0.13*

Number of fish (n) analyzed are given in parentheses. Data represent means ± SE; LSI (liver somatic index: 100 x liver weight/body weight); CF (condition factor: body weight/length^3). *, #: significantly different from Lac La Pêche bullheads ($p<0.05$; $p<0.01$, respectively).

Table 2.2. Amounts of various PCB congeners detected in white muscle tissue of bullheads from Lac La Pêche and the St. Lawrence River

PCB congener	La Pêche	St. Lawrence
2,5,2',5'	ND	0.272±0.046
2,4,2',5'	ND	0.253±0.059
2,4,2',4'+2,4,5,2'[c]	ND	0.243±0.066
2,3,2',5'	ND	0.185±0.033
2,4,3',4'	ND	0.282±0.118
2,3,4,4'+3,4,2',3'[c]	ND	0.318±0.134
3,4,3',4'+2,3,6,3',4'[c]	0.095[a]	0.538±0.201
2,4,5,2',5'	ND	0.377±0.086
2,4,5,3',4'	0.05[a]	0.407±0.106
2,4,5,2',4',5'+ 2,3,4,2',3',6[c]	0.062±0.031	0.458±0.114*
2,3,5,6,2',4',5'+ 2,3,4,5,2',4',6[c]	0.198±0.170	0.352±0.114
2,3,4,5,2',4',5'	0.08-0.11[b]	0.327±0.116
DDE	0.060±0.018	0.505±0.187**
Mirex	ND	0.113±0.045
total PCB	0.726±0.435	16.04±8.66**
% fat	0.188±0.066	0.552±0.183

Data represent mean ± SE (ng/g wet weight); n=5-6, with one sample of each group was a pooled sample from two individuals. ND: Not detectable, values less than 0.062 ng/g. [a]: detected in 1 sample of 5 assayed. [b]: range of 2 sample of 5 assayed. [c]: mixtures of congeners that appear in same peak. *, **: significantly different from Lac La Pêche bullheads ($p<0.05$; $p<0.01$, respectively).

different, despite a higher weight and condition factor in the bullheads from the St. Lawrence River than Lac La Pêche. From the total PCB congeners analyzed individual congeners with highest concentrations in most samples were also summarized in the same table. Scanning of these congeners revealed that tetrachlorobiphenyls and mirex were found in significant concentrations in all fish analyzed from the St. Lawrence River, but were non-detectable in almost all fish analyzed from Lac La Pêche. Higher chlorinated biphenyls (penta-, hexa- and heptachlorobiphenyl mixtures), as well as DDE could be detected in both groups, but tended to be higher in bullheads from the St. Lawrence River than Lac La Pêche. Some congeners could only be found in one or two of the five fish samples from Lac La Pêche (Table 2.2).

Hepatic EROD activities were significantly higher (2.8-fold) in bullheads collected from the St. Lawrence River as compared with Lac La Pêche (Table 2.3). There were no differences in kidney EROD activities between the two groups. Both groups demonstrated higher hepatic than renal EROD activities.

Phase II enzymes related to the conjugation of toxicants with UDP-glucuronic acid (UDPG) were also estimated. UDPGT activities assayed in liver and kidney of both groups did not differ (Table 2.3). On the other hand, GST activities in liver, kidney, red and white muscles were significantly different in St. Lawrence River bullheads as compared with the Lac La Pêche group (Table 2.4). Liver GST activities were 3-fold higher, while a similar trend was also noted in kidney and white muscle with 1.5- and 1.2-times higher activities, respectively. In contrast, GST activity was significantly lower in the red muscle of St. Lawrence (0.92-times) as compared with Lac La Pêche bullheads.

Liver and kidney samples were also analyzed for SOD and CAT activities (Table 2.3). SOD

Table 2.3. Ethoxyresorufin O-deethylase (EROD), UDPglucuronosyltransferase (UDPGT), superoxide dismutase (SOD) and catalase (CAT) activities in liver and kidney of bullheads from the St. Lawrence River and Lac La Pêche.

	LIVER	KIDNEY
EROD		
La Pêche	3.45±0.45	0.86±0.11
St Lawrence	9.82±0.57$^{\#}$	1.23±0.16
UDPGT		
La Pêche	170 ± 10.6	21.2 ± 1.2
St. Lawrence	164 ± 11.9	17.0 ± 2.8
SOD		
La Pêche	198 ± 23.7	123 ± 12.8
St. Lawrence	303 ± 23.3**	127 ± 11.2
CAT		
La Pêche	21.7 ± 2.7	1.9 ± 0.38
St. Lawrence	16.8 ± 1.3	1.0 ± 0.10*

Data represent mean activity ± SE [units/(min*mg protein) (SOD), µmol H_2O_2 decomposed/(min*mg protein) (CAT) and pmol/(min*mg protein) (EROD, UDPGT)]; n=9-10. *, **, $^{\#}$: significantly different from Lac La Pêche bullheads ($p<0.05$; $p<0.01$; $p<0.001$, respectively).

Table 2.4. Glutathione dependent enzyme activities and total glutathione (TGSH) concentrations in different tissues of bullheads from Lac La Pêche and the St. Lawrence River.

	LIVER	KIDNEY	RED MUSCLE	WHITE MUSCLE
GST				
La Pêche	904±99	278±26	163±3.6	121±6.7
St. Lawrence	2815±273**	412±17**	151±2.9*	144±6.1*
GPX				
La Pêche	78±15.5	55.3±7.0	10.8±1.0	3.0±0.24
St. Lawrence	120±17.7	60.9±6.0	5.0±0.7**	2.1±0.27*
TGSH				
La Pêche	782±58	807±45	337±20	230±15
St. Lawrence	629±33*	680±32*	341±14	150±10**

Data represent mean ± SE [nmol/(min*mg protein) (glutathione S-transferase, GST and glutathione peroxidase, GPX) and nmol/g wet tissue weight (TGSH)]; n=9-10. *, **: significantly different from Lac La Pêche bullheads ($p<0.05$; $p<0.001$, respectively).

activities were significantly higher (1.5-fold) in bullhead livers from the St. Lawrence River than from Lac La Pêche. SOD activities reflected Cu,Zn-SOD activities, as complete inhibition of SOD activities was achieved with cyanide, indicating no detectable cytosolic Mn-SOD activity in either organ. Cytosolic CAT activities in liver were not significantly different between groups, but kidney CAT activities were significantly lower in St. Lawrence River as compared with Lac La Pêche fish. The third antioxidant enzyme, GPX, was monitored in four tissues, but no significant differences were noted in liver and kidney. In contrast, red and white muscle GPX activities were significantly lower in St. Lawrence River fish as compared with those from Lac La Pêche.

Total glutathione (TGSH) concentrations in liver, kidney and white muscle were significantly lower in St. Lawrence River bullheads (0.80-, 0.84- and 0.65-times, respectively) as compared with Lac La Pêche bullheads, but TGSH concentrations were not different in the red muscle (Table 2.4).

Discussion

Feral fish species living in the St. Lawrence River are exposed to a high and a multiple content of pollutants (Government of Canada 1991). Uptake and accumulation studies are hampered as many constituents in these aquatic systems, e.g. various compounds in paper mill effluents, are yet to be identified (Mather-Mihaich and Di Giulio 1991). However, it is the ultimate impact of all constituents which affect the physiology of these species.

The St. Lawrence River ecosystem is known to contain inorganic (heavy metals) and organic toxicants, which are primarily PCBs, but also polycyclic aromatic hydrocarbons, pesticides and other substances from municipal, agricultural and industrial wastewaters (Government of Canada

1991; Ontario Ministry of Environment and Energy 1993). Whether the increased pollutant load in the river system did not negatively affect physical characteristics of bullheads (Table 2.1) could not be determined, as the biotic and abiotic environments differed between both systems. Weight and condition factor were significantly higher in St. Lawrence River bullheads as compared to the fish from Lac La Pêche. Slower growth and lower condition factors have been reported in sturgeon populations in fluvial lakes on the Ottawa River in comparison to lakes on the St. Lawrence River (Fortin *et al.* 1993). This is related to the less mineralized, less productive waters of the Ottawa River versus the green waters with high mineral content of the St. Lawrence River. But slower growth may also be related to the availability of prey items, as fish are known to respond more positively to abundance of food than to physical habitat conditions (Weisberg and Burton, 1993). Food limitation may be a partial explanation of our results of smaller, but older lake versus river bullheads (Table 2.1). On the other hand, liver somatic index did not differ between lake and river bullheads. LSI has often been referred to as a non-specific indicator of subacute exposure of fish to pollutants (Andersson *et al.* 1988), but laboratory exposure of rainbow trout to environmental pollutants did not result in significant changes of LSI (Otto *et al.* 1994).

Several studies have monitored the tissue content of major aquatic contaminants and effects on phase I enzyme activities (Ahokas *et al.* 1994; George *et al.* 1995). Although antioxidant defenses have been analysed in brown bullheads in the laboratory (Hasspieler *et al.* 1994a), they combined with phase I and II enzymes have not been analyzed in feral bullheads and related to tissue contaminant load. Analyses of these parameters should be evaluated in this species regarding its potential to tolerate suboptimal water qualities and its economical importance (Scott and

Grossman 1973; Eckersley and McCullough 1990). Although bullheads from the St. Lawrence River tended to be younger than from Lac La Pêche, accumulated PCB congeners were significantly higher in the St. Lawrence River fish, particularly tetrachlorobiphenyls (Table 2.2). Accumulation of PCB congeners determined by the same method have been reported to be several hundred times higher in striped bass from highly contaminated areas (Bush *et al.* 1989), but present results indicate that higher PCB concentrations in tissues can be correlated to detoxication enzymes and TGSH concentrations in feral bullheads. Also 3,4,3',4'-tetrachlorobihenyl (TCB) which was detected as a mixture with 2,3,6,3',4'-pentachlorobiphenyl was found in significant amounts in bullheads from the St. Lawrence River, but were only detected in one out of five fish from Lac La Pêche. TCB is known to be a potent inducer of phase I and II enzymes and antioxidant defenses in rainbow trout (Chapter 5). Despite higher concentrations of PCB congeners, and the presence of not analyzed contaminants in the St. Lawrence River, brown bullheads are not restricted for food consumption in comparison to other species that have higher contaminant concentrations and are not recommended for eating (Ontario Ministry of Environment and Energy, 1993).

Alteration in cytochrome P450-dependent enzymes and antioxidant defenses are early indicators of exposure to environmental contaminants (Winston 1991; Munkittrick *et al.* 1993; Rodríguez-Ariza *et al.* 1993). The St. Lawrence River bullheads examined in this study showed significantly higher hepatic EROD activities than those from Lac La Pêche, indicating an activation of cytochrome P450-dependent processes in these River fish. Various studies have monitored EROD activities in feral fish at different distances from paper mill effluents (Munkittrick *et al.* 1992; Ahokas *et al.* 1994) and have shown the highest EROD induction at the

closest proximity to the paper mill (Munkittrick *et al.* 1992). EROD activity has been described as a reliable indicator in the assessment of polyaromatic hydrocarbon spills being more economical and showing a more persistent effect than contaminant content in muscle tissues (George *et al.* 1995). Present data indicate positive hepatic MO response to contaminant load in muscle.

Activation of MO activities is generally the first step in detoxication and excretion of toxicants, followed by conjugation by phase II enzymes. Analysis of two major conjugation enzymes revealed that UDPGT activities were unchanged, but significantly higher GST activities in liver, kidney and white muscle of St. Lawrence River bullheads (Tables 2.3 and 2.4). This response is consistent in major body tissues, as liver, kidney and white muscle all showed significant increases and appear all to be involved in the detoxication process by responding to muscle contaminant load. Also the GST increase in white muscle was only 1.2-fold, but white muscle represents 70-80% of whole body weight, thus the detoxication potential of this tissue may be a significant contributor to whole body detoxication processes. We had shown previously that i.p. injection of TCB in rainbow trout induces hepatic and renal UDPGT activities to much greater extent (5- and 2-fold, respectively), while GST was only induced in liver (2-fold), but not in kidney or muscle (Chapter 5). Also exposure studies of perch and rainbow trout to industrial effluents caused changes in UDPGT activities (Andersson *et al.* 1988; Otto *et al.* 1994), while GST activities remained unaffected (Otto *et al.* 1994). It has been suggested that glucuronidation may be the quantitatively more important pathway for toxicants in fish (Clarke *et al.* 1991). Gallagher and Di Giulio (1992) reported that chlorothalonil detoxication occurred through a GST catalyzed conjugation with GSH in gills of channel catfish. In the present study only enzyme activities were examined and not genetic expression, but the data do not exclude the possibility that feral

bullheads with a higher contaminant load favour conjugation with GSH over UDPglucuronic acid, indicating that GST may act as an important detoxication enzyme in this species.

SOD, CAT and GPX are classic antioxidant enzymes involved in the scavenging of oxyradicals and byproducts. There has been a major effort to characterize these enzyme activities in different fish species (Filho *et al.* 1993; Lemaire *et al.* 1993). In the present study, cytosolic SOD activities in liver were higher in St. Lawrence River bullheads, while CAT in kidney and GPX in muscle tissues were lower than in Lac La Pêche fish (Tables 2.3 and 2.4). These results indicate an involvement of these antioxidant enzymes in liver, but possibly not in other tissues. Exposure of channel catfish to bleached kraft mill effluents increased hepatic CAT activity, but not SOD or GPX activities (Mather-Mihaich and Di Giulio 1991). Rainbow trout exposed to unbleached paper mill effluents did not activate SOD or CAT activities (Otto *et al.* 1994). Both studies could not clarify how paper mill effluents impose oxidative stress on fish. However, Rodríguez *et al.* (1993) reported increased activities of GPX, CAT and SOD in mullet harvested from polluted waters and proposed their usage in the biomonitoring of environmental pollution. Our study does not resolve this controversy, although the increase observed in hepatic SOD activities implies some involvement of antioxidant enzymes in the detoxication processes in bullhead.

Glutathione is a major cellular antioxidant (Meister and Anderson 1983) and it has been implicated in the detoxication of organic pollutants in different fish species (Mather-Mihaich and Di Giulio 1991; Gallagher and Di Giulio 1992; Rodríguez-Ariza *et al.* 1993; Otto *et al.* 1994; Chapter 5). TGSH, which is the sum of GSH and GSSG concentrations, is a reliable indicator in exposures to reactive oxygen species (Sen *et al.* 1992). The significantly lower TGSH levels in liver, kidney and white muscle of St. Lawrence River bullheads support the involvement of GSH in the detoxication process. These lower concentrations also suggest that the overall antioxidant

capacity in these fish has been compromised. GSH is known to play multiple functional roles in the cell, including an involvement in DNA and protein synthesis, coenzyme function and overall cellular protection (Meister and Anderson 1983). As GSH may be used by other detoxication processes (*i.e.* conjugation catalyzed by GST), the low GSH levels may impair other metabolic processes and thus weaken the physiological status of the organism. The white muscle of St. Lawrence River bullheads has a particularly low TGSH concentration in comparison with Lac La Pêche fish and given the large white muscle mass relative to total body weight, a weakened antioxidant status appears to hold for the entire organism. The present results support an involvement of white muscle in the detoxication process and thus confirm our previous findings characterizing this tissue as an important detoxication organ in rainbow trout (Chapter 5).

In summary, the present study has shown that detoxication processes are affected in feral bullheads collected from the St. Lawrence River compared with Lac La Pêche. Although the biotic and abiotic conditions differ between the two systems, the higher contaminant load in the St. Lawrence River bullheads may account for the differences of biochemical responses observed in the present study. PCB concentrations in white muscles are severalfold higher in bullheads from the St. Lawrence River than from Lac La Pêche and relate to observed changes in phase I and II enzyme activities. Higher activities of EROD and GST in various tissues from St. Lawrence bullheads may indicate the possibility of a preferred detoxication route through MO and conjugation with glutathione. The low TGSH concentrations in the different body tissues further imply a weakened antioxidant status in these fish. Both processes are affected in white muscle together with liver and kidney, which may impair the overall physiological status of these fish.

CHAPTER 3.

IMPAIRED INDUCIBILITY OF XENOBIOTIC AND ANTIOXIDANT RESPONSES IN RAINBOW TROUT EXPOSED TO POLYCHLORINATED BIPHENYL CONTAMINATED SEDIMENTS IN THE ST. LAWRENCE RIVER

Introduction

Polychlorinated biphenyls (PCBs) are major pollutants, ubiquitously distributed in the aquatic and terrestrial environments (Bush *et al.* 1989; Lake *et al.* 1995). Although the industrial utilization of PCBs has been largely discontinued, approximately half of the world's production of PCBs has yet to escape to the environment from older electrical equipment and landfill sites (Hargrave *et al.* 1992). PCBs are highly persistent and are among the most prevalent organochlorine hydrocarbons accumulated in the fauna of the Arctic Ocean (Hargrave *et al.* 1992).

Continuous exposure of organisms to polluted environments often leads to specific biochemical changes. The induction of phase I (monooxygenases) and II (conjugation) enzymes in combination with antioxidant defenses have been suggested as sensitive bioindicators of aquatic pollution (Roy *et al.* 1995a,b). Cytochrome P450 dependent monooxygenases such as ethoxyresorufin O-deethylase (EROD) activities are induced in fish harvested from PCB polluted waters (Branchaud *et al.* 1995; Livingstone *et al.* 1995; Chapter 2). Glutathione (L-γ-glutamyl-L-cysteinylglycine; GSH) is a major cellular antioxidant and participates in two detoxication pathways. Conjugation of a pollutant with GSH is initiated by glutathione S-transferase (GST), an

inducible phase II enzyme. Reactive oxygen species generated by some pollutants are detoxified by the concerted action of antioxidant enzymes, including glutathione peroxidase (GPX) and glutathione reductase (GR). The glutathione system together with other cellular antioxidants plays an important role in the biochemical responses of fish exposed to PCBs and related pollutants (Bucher *et al.* 1993; Thomas & Wofford 1993; Roy *et al.* 1995b; Chapters 2 and 5).

Dredging at the General Motors Superfund site on the St. Lawrence River at Massena, NY, an area of known PCB contamination (Kadlec, 1994), provided a unique opportunity to examine the uptake of PCB congeners and the biochemical responses of rainbow trout (*Oncorhynchus mykiss*) continuously exposed to the leachates associated with the removal of these contaminated sediments. Trout were caged for 21 and 42 days at the GM site during a period of ongoing dredging and at a relatively nonpolluted site seven kilometers downstream. Fish were analyzed for PCB congeners and activities of EROD, GST, GPX and GR, as well as total glutathione (TGSH; reduced + oxidized glutathione) content in various tissues. The present study was therefore aimed at determining whether exposure of trout to the dredging site would evoke tissue PCB accumulation and trigger detoxication processes in this species.

Materials and Methods

Chemicals

Ethoxyresorufin, NADPH, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), GSH, oxidized glutathione (GSSG), Bakers yeast glutathione reductase were obtained from Sigma Chemical Co. All other reagents were of analytical grade.

Animals and experimental procedure

Rainbow trout were obtained from Hitchenbrooke Hatchery, Chateauguay, NY. Fish were transported in an aerated tank (1 h) to the experimental sites. Fish were removed by dip net and distributed into previously located and secured floating cages. The cages are applicable for aquaculture of various teleost species and are not in contact with the sediments (Buttner 1992). One cage was placed near (30 m) the sealed iron wall enclosing the area dredged by GM. Controls were caged at the reference (control) site, about 7 km downstream in the Snye channel. The control site was shown from previous studies to have low PCB contamination (Kadlec 1994). The effect of the dredging operation on fish condition was monitored for a total of 42 days, while fish were harvested at 21 and 42 days during June and July of 1995. Fish were fed commercial diets 3 times/week for the first 21 days, but feeding was discontinued for the remaining period at both sites due to the inaccessibility to the cage at the GM site.

At harvest, fish were dip netted from the cage into an aerated holding tank and transferred by boat (15 min) to the shore. Trout were killed immediately by a blow to the head. Blood was collected from the caudal vessels for TGSH analysis as previously described (Chapter 2). Tissues were excised, immersed in liquid nitrogen, transported in dry ice and stored at -80°C until analyzed.

Biochemical analyses

Frozen white muscle samples were extracted and analyzed for PCB concentrations by capillary gas chromatography as described by Bush *et al.* (1989) and described in Chapter 2. Tissues were homogenized for TGSH analysis as previously described (Chapter 2) and TGSH was determined according to Sen *et al.* (1992). Tissues analyzed for enzyme activities were suspended in Tris buffer (50 mM, containing 0.25 M sucrose and 1 mM EDTA, pH 7.5) and microsomal and

cytosolic fractions were obtained as described previously (Chapter 2). Microsomes and supernatants were stored at -80°C. Microsomal and cytosolic enzymes were determined as described in Chapters 2 and 5. Glutathione peroxidase (GPX) activity was determined by the method of Tappel (1978) using 6 mM GSH as substrate. Protein concentrations were determined by the method of Lowry *et al.* (1951).

Statistical analyses

Significant differences between trout from the GM and control sites at a time period were determined with a Student's t-test for unpaired sample means using the software Statistix 4.1. Time-related differences between parameters of fish before the time of caging (0-controls) and fish caged at the control or GM sites at 21 and 42 days were tested with a one-way ANOVA. The level of significance was located using the Least Significant Difference method (LSD).

Results

Conditions of fish and uptake of PCB congeners

Alkalinity, pH, temperature and dissolved oxygen were monitored during the caging period and remained within ranges considered suitable for rainbow trout (alkalinity 83-94 mg/l $CaCO_3$; pH 7.6-8.6; temperature 17.4-23.5 °C; dissolved oxygen 8.3-10.0 mg/l). Physical parameters of the caged rainbow trout are summarized in Table 3.1. Body weight and length were reduced for trout from the GM site ($p<0.05$; 42 days) compared with fish from the control site and 0-controls. Condition factor (CF) did not differ between same day groups,

Table 3.1. Physical parameters of rainbow trout caged at the GM and the control (Snye) site in the St. Lawrence River

	day 0	day 21	day 42
weight in g			
control	158±8.6	167±11.8	161±10.9
GM site		141±8.4	134±5.14$^{*\#}$
length in cm			
control	22.9±0.6	23.6±0.5	24.3±0.4
GM site		22.7±0.4	23.0±0.3$^{\#}$
liver weight in g			
control	1.43±0.10	1.54±0.23	1.86±0.14
GM site		1.10±0.09*	1.90±0.11$^{**\blacksquare}$
CF			
control	1.32±0.11	1.25±0.05	1.12±0.11*
GM site		1.20±0.05	1.10±0.02*
LSI			
control	0.90±0.05	0.89±0.07	1.20±0.04$^{*\blacksquare}$
GM site		0.78±0.03	1.44±0.10$^{**\blacksquare\blacksquare}$

Data represent mean ± SEM; n=6-8. Condition factor (CF, 100 x body weight/length^3); Liver somatic index (LSI, 100 x liver weight/body weight). *, **, ***: significantly different from 0-controls ($p<0.05$; $p<0.01$; $p<0.001$, respectively). $^{\#}$: significantly different from same day controls ($p<0.05$).$^{\blacksquare}$,$^{\blacksquare\blacksquare}$: significantly different from 21-day same exposure group ($p<0.05$, $p<0.01$, respectively).

although CF was significantly lower at 42 days at both sites compared with the 0-controls ($p<0.05$). Liver weight and liver somatic index (LSI) were significantly higher at 42 day at both sites than in 0-controls ($p<0.05$), particularly in fish from the GM site ($p<0.01$). However, no differences were found between the 42 day groups, but differences were noted between the 21 and 42 day same exposure groups.

Total PCB concentration increased significantly in fish caged at the GM site versus same day controls ($p<0.001$) (Table 3.2), with differences of 117- and 34-fold at 21 and 42 days, respectively. The marginally lower difference between the 42 day groups resulted from an increase in PCB content in controls at 42 days compared with 21 days (2.8-fold, $p<0.01$). But 21 day controls contained 2.5-fold lower PCB concentrations than 0-controls. Table 3.2 lists the most concentrated congeners in muscle of a total of 74 PCB congeners analyzed; these represent 60-80% of the PCBs identified in fish from each site. Thus most accumulated PCB congeners in fish at the GM site are lower molecular weight PCBs. Also trout caged at the control site showed an accumulation of lower weight PCBs (di- to tetrachlorinated) with caging time, while penta- to octachlorinated biphenyls are absent in most samples analyzed. On the other hand, 0-controls contained detectable amounts of most congeners analyzed (mono- to octochlorinated). Although total PCB concentrations did not significantly differ between 0-controls and fish from the control site at 42 days, a marked shift from higher to lower chlorinated PCB congeners occurred in muscle of trout with increased caging time (Table 3.2). Controls at 42 days showed significantly higher di- to tetrachlorinated biphenyl concentrations than the 21 day controls, suggesting a bioconcentration of these PCB congeners with caging in controls, but to a marginally smaller extent than in trout from the GM site. There was no

Table 3.2. Mean content of PCB congeners in white muscle tissue of caged rainbow trout

congener	0-control	21 day control	21 day GM site	42 day control	42 day GM site
2	11.4±4.9	2.40[g]	143±27	1.23±0.23	46±7.4
2,2'[a]	0.70[g]	2.23±0.52	703±101	6.80±1.60[*]	407±64
2,4'[b]	0.4-1.5[h]	0.82±0.17	87±14	2.18±0.38[*]	69±14
2,6,2'	0.09±0.01	0.36±0.06	146±22	2.37±0.45[**]	122±22
2,5,2'	0.2±0.04	0.30±0.04	47±12	1.35±0.23[*]	42±13
2,3,6[c]	0.06[g]	0.28±0.04	100±16	1.97±0.39[**]	104±22
2,3,2'[d]	0.18±0.05	0.40±0.07	110±19	2.50±0.46[**]	116±25
2,5,3'	0.10±0.00	0.13±0.03	21±5	0.63±0.11[**]	28±6
2,4,4'	0.80±0.29	1.08±0.24	193±41	5.28±1.17[*]	210±72
2,5,2',5'	1.7±0.54	0.90±0.16	89±16	3.05±0.53[*]	103±23
2,4,2',5'	0.88±0.36	0.54±0.12	53±12	1.67±0.29[*]	55±13
2,4,2',4'[e]	0.48±0.16	0.30±0.04	42±7	1.30±0.23[*]	50±12
2,3,2',5'	0.64±0.19	0.32±0.06	34±6	1.20±0.21[*]	43±10
2,3,6,4'	0.40±0.12	0.18±0.04	22±4	0.69±0.17[*]	24±6
2,5,3,4'	0.70±0.23	0.26±0.02	17±3	0.58±0.10[*]	21±5
2,4,3',4'	2.12±0.61	0.64±0.17	40±5	1.40±0.21[*]	49±10
3,4,3',4'[f]	2.40±0.92	0.87±0.23	19±4	1.64±0.39	26±9
total PCB	49.0±9.2	19.3±4.5[•]	2261±323[#]	53.3±10.1[**]	1829±352[#]
% fat	4.1±1.21	1.9±0.93	2.7±1.25	2.14±0.59	1.31±0.14

Data represent mean ± SEM and are expressed in ng/g wet weight; n=5-6;

[a-f]: also contains congeners 2,6([a]), 2,3([b]), 2,6,3'([c]), 2,6,4'([d]), 2,4,5,2'([e]) and 2,3,6,3',4'([f]). [g]: detected in 1 sample of 5 assayed. [h]: range of 2 samples of 5 assayed. [*],[**]: significantly different from 21 day same exposure group ($p<0.05$, $p<0.01$, respectively). [#]: significantly different from same day and 0-controls ($p<0.001$). [•]: significantly different from 0-controls ($p<0.01$).

further accumulation of congeners between 21 and 42 days in fish caged at the GM site. Percentage of fat in white muscle remained unchanged for all groups (Table 3.2). Water column total PCB content increased from 0.34 to 1.34 µg/l at the GM site from day 0 to 42; levels at the control site were below detection (<0.062 µg/l).

Biochemical responses

Hepatic and renal EROD activities remained unchanged in all groups (Table 3.3). GST activities in the 4 tissues analyzed significantly decreased with caging at both sites compared with 0-controls. Significant differences between trout caged at the GM site and controls were detected only in liver with 1.18-fold lower GST activities in GM caged trout ($p<0.05$; 42 days), and in muscle (1.24-fold higher activities; $p<0.05$; 21 days).

GPX activities increased in caged trout at 21 and 42 days compared with 0-controls (Table 3.4), but significantly lower activities were monitored in GM caged trout in liver at 42 days and in muscle at 21 days than in same day controls ($p<0.05$). In contrast, hepatic and renal GR activities tended to decrease in GM caged fish and controls at both caging periods versus 0-controls, while higher muscle GR activities were noted in caged trout at both sites at 21 days compared with 0-controls. However, GR activities were unchanged between both caged groups.

Total glutathione (GSH + GSSG) concentrations also decreased in liver and plasma of caged trout compared with 0-controls (Table 3.5), but increased in muscle with caging time ($p<0.05$). This increase in muscle TGSH content occurred more rapidly in controls compared with trout at the GM site; TGSH levels were significantly lower in fish at the GM site than in same day controls at 42 days ($p\leq0.05$).

Table 3.3. Ethoxyresorufin O-deethylase (EROD) and glutathione S-transferase (GST) activities in tissues of rainbow trout caged in the St. Lawrence River

	day 0	day 21	day 42
EROD			
liver			
control	11.91±2.36	6.33±0.54	5.83±0.75
GM site		9.92±1.58	7.44±0.96
kidney			
control	5.33±0.66	8.82±1.02	7.75±0.89
GM site		9.29±1.21	7.57±0.87
GST			
liver			
control	725±59	776±151	538±15
GM site		627±61*	455±25$^{**\#}$
kidney			
control	269±14	178±29**	180±18**
GM site		187±11**	206±14**
gill			
control	315±29	174±25**	196±51*
GM site		175±16***	167±21***
muscle			
control	35.2±3.0	26.8±1.4*	21.6±2.1***
GM site		33.2±2.6$^{\#}$	21.9±1.3**

Data represent mean ± SEM (n=6-8) and are expressed in pmol/(min x mg protein) [EROD] and nmol/(min x mg protein) [GST]. *, **, ***: significantly different from 0-controls ($p<0.05$; $p<0.01$; $p<0.001$, respectively). $^{\#}$: significantly different from same day controls ($p<0.05$).

Table 3.4. Glutathione peroxidase (GPX) and glutathione reductase (GR) activities in tissues of rainbow trout caged in the St. Lawrence River

	day 0	day 21	day 42
GPX			
liver			
control	120±20	218±35	391±57*
GM site		230±34*	243±35*#
kidney			
control	408±34	565±28*	557±62*
GM site		483±53	417±82
gills			
control	159±29	261±40*	270±29*
GM site		220±16	255±34*
muscle			
control	24.0±2.0	43.1±3.7**	41.4±5.0**
GM site		32.7±3.1#	42.3±7.2*
GR			
liver			
control	39.5±6.5	24.5±4.1*	22.6±4.0*
GM site		20.3±1.2**	14.8±1.3**■
kidney			
control	51.3±3.9	39.0±2.1**	40.1±2.4*
GM site		45.7±3.2	47.8±5.4
gill			
control	13.6±2.5	13.7±2.2	16.8±1.7
GM site		10.8±0.8	16.7±2.0■
muscle			
control	2.53±0.19	4.3±0.9**	2.7±0.3
GM site		3.5±0.2**	3.2±0.3

Data represent mean ± SEM (n=6-8) and are expressed in nmol/(min x mg protein). *, **, ***: significantly different from 0-controls ($p<0.05$; $p<0.01$; $p<0.001$, respectively). #: significantly different from same day controls ($p<0.05$). ■: significantly different from 21 day same exposure group.

Table 3.5. Total glutathione (TGSH) concentrations in tissues of rainbow trout caged in the St. Lawrence River

	day 0	day 21	day 42
TGSH			
liver			
control	3.06±0.204	2.59±0.295	2.30±0.142*
GM site		2.51±0.244	2.21±0.088*
kidney			
control	2.50±0.141	2.98±0.112*	2.61±0.168
GM site		2.73±0.112	2.62±0.203
gill			
control	1.38±0.097	1.73±0.083*	1.59±0.05
GM site		1.67±0.115	1.62±0.103
muscle			
control	0.129±0.024	0.260±0.035**	0.334±0.016**
GM site		0.195±0.040	0.266±0.027*#
plasma			
control	58±6.8	40±3.9*	42±5.2
GM site		44±5.2	32±1.1*

Data represent mean ± SEM (n=6-8) and are expressed in µmol/g wet weight, except for blood plasma (µM). *, **: significantly different from 0-controls ($p<0.05$; $p<0.01$, respectively). #: significantly different from same day controls ($p \leq 0.05$).

Discussion

Caged rainbow trout at the GM Superfund site during the dredging period accumulated significant levels of PCBs (Table 3.2). Most notable are the differences between congeners. Muscle accumulated lower molecular weight PCB (di- to tetrachlorinated) congeners with higher water solubility. Several studies monitoring PCB load and congener pattern in feral fish species show highest tissue concentrations of tri- to heptachlorinated biphenyls (Bush *et al.* 1989; Lake *et al.* 1995; Chapter 2). Teleost fish accumulate aquatic pollutants largely from ingested food or the water column (Makarewicz *et al.* 1993; Kadlec 1994). Trout in the present study were fed commercially available feed for 21 days, suggesting that uptake of PCBs from the water through the gills and/or skin represents the most significant uptake route. Uptake of lipophilic pollutants such as PCBs depends largely on the chemical and physical characteristics of tissues which may influence diffusive exchange of the congeners between organism and water (Roy *et al.* 1995a). Higher molecular weight PCBs have lower water solubility and may thus be accumulated through ingestion of invertebrates or by having a benthic life habitat. A modest bioconcentration of lower molecular weight PCBs was also observed in trout at the control site, which tended to increase with caging time, consistent with previous findings (Kadlec 1994). The notable weight loss in trout caged at the GM site remains difficult to explain, as both sites were under similar feeding schedules and fish did not show any noticeable signs of stress. However, the dredging operation at the GM site may have caused multiple physical disturbances leading to altered behaviour of these fish. Whether these physical disturbances ultimately influenced the results of this study could not be determined.

The marked accumulation of PCB congeners did not concur with increased biochemical

responses. Enhanced phase I and II enzyme activities result from increased transcription of their respective genes induced by pollutant binding to the Ah receptor (Nebert 1989). Induced phase I and II enzyme activities have been repeatedly linked to increased muscle PCB and related contaminant loads (Livingstone *et al.* 1995; Chapter 2). However, winter flounder collected from highly PCB contaminated areas showed decreased EROD activity, while cytochrome P450IA (CYP1A) protein, the EROD catalyst, was significantly induced (Elskus *et al.* 1989). We observed in the present study unchanged EROD and mildly depressed GST activities (Table 3.3), enzyme activities which are both regulated by the Ah receptor (CYP1A transcription and protein expression were not analyzed). Not all PCB congeners induce transcription of genes regulated by the Ah receptor, with substituent position and structure being important parameters for the mediation of enzyme changes (Parkinson *et al.* 1983; Gooch *et al.* 1989). Present data show high accumulation in trout muscle of the 2,2' substituted congener (Table 3.2), which exhibits primarily phenobarbital type of induction in rats (Dragnev *et al.* 1995). However, there seems to be a lack of response to phenobarbital type inducers in fish (Kleinow *et al.* 1987). Even the established Ah receptor ligand 3,3',4,4'-tetrachlorobiphenyl (TCB), a congener also detected in the present study (Table 3.2), suppressed EROD activity while inducing CYP1A mRNA and CYP1A protein expression at high exposure concentrations (Gooch *et al.* 1989; Hahn *et al.* 1993). We observed previously that total glutathione content is a sensitive determinant of TCB induced CYP1A gene expression and catalytic (EROD) activity (Otto *et al.* 1996a). These studies do indicate that the concentration of a toxicant or the interference of cellular factors (i.e. glutathione status) modify the biochemical response of the organism. The decrease in hepatic TGSH content demonstrated in trout at both sites may be interfering with the sensitivity of EROD activity (Table 3.3). Our

previous studies have shown that single exposure of trout to TCB and exposure of feral bullheads to PCB contaminated waters significantly affected phase I and II enzymes and antioxidant defenses and identified white muscle along with liver and kidney as key detoxication organs (Chapters 2 and 5). Neither liver nor muscle displayed unambiguous changes in antioxidant enzymes or total glutathione, although GPX was lower in liver and muscle and TGSH levels were lower in muscle of fish caged at the GM site (Tables 3.4 and 3.5). This may indicate that the mixture of PCB congeners and/or the continuous high exposure suppresses the inducibility of these detoxication parameters.

In summary, the present study monitored accumulation of PCB congeners and the biochemical detoxication processes in rainbow trout caged at the GM Superfund site during a dredging operation. Trout muscle displayed significant uptake of PCB congeners, yet this marked accumulation resulted in either unchanged or minor suppression of phase I and II enzyme activities and antioxidant defenses in various tissues. These observations suggest either a lack of response to this type of PCB mixture and/or an impaired inducibility of biochemical detoxication processes in trout exposed continuously to high PCB concentrations. In either case the accumulation of this high toxicant load by the trout did not induce detoxication parameters examined in this study.

CHAPTER 4.

ENDOGENOUS ANTIOXIDANT SYSTEMS OF TWO TELEOST FISH, THE RAINBOW TROUT AND THE BLACK BULLHEAD, AND THE EFFECT OF AGE

Introduction

Salmonid and Ictalurid species have been extensively used in studies of oxidative stress and the effects of pollutants on antioxidant defenses (Hasspieler *et al.* 1994a,b; Otto *et al.* 1994; Chapter 5). During oxidative stress the balance between prooxidant and antioxidant forces shifts toward a more prooxidizing status (Sohal & Allen 1986). A number of conditions alter this balance, one of which is aging. One theory of aging postulates that aging occurs as a consequence of deleterious and irreversible changes initiated by partially reduced oxygen species or free radicals (Harman 1956; Darr & Fridovich 1995). Free radicals are atoms or molecules containing an unpaired electron in their outer valence shell and are thus generally highly reactive. Under physiological conditions there is a continuous production of reactive oxygen species which are controlled to a great extent by endogenous antioxidants. A primary component of this antioxidant defense is the glutathione system (Meister 1991). The cellular tripeptide glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) exerts protective antioxidant functions through a complex enzyme system including glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferase (GST) and γ-glutamyl transpeptidase (GGT). These enzymatic reactions lead to the production of byproducts including glutathione disulfide (GSSG) and mercapturate and aid to maintain GSH in its reduced state. Other enzymes involved in cellular defenses against uncontrolled oxidative

processes are superoxide dismutase (SOD) and catalase (CAT) which catalyze the dismutation of superoxide radical and H_2O_2. The change toward a pro-oxidizing status in aging animals has been argued to arise from lowered antioxidant enzyme activities and antioxidant concentrations, particularly GSH (Kitani 1994).

Piscine antioxidant defenses are being used increasingly for environmental monitoring (Hasspieler *et al.* 1994a,b; Otto *et al.* 1994; Palace *et al.* 1993), so it is important to examine the susceptibility of these systems to age. The effect of age has been studied to a limited extent in fish, although the involvement of free radicals during the terminal spawning migration of salmon has been reported (Sawada *et al.* 1993). Maturation dependent changes in carbohydrate metabolism have been demonstrated in rainbow trout (Barciela *et al.* 1993) and several other physiological and biochemical parameters have been reported in different age-groups of various fish species (Patnaik *et al.* 1994). Many fish species continue growth throughout life and are thus very distinct from mammals (Patnaik *et al.* 1994), but it remains to be demonstrated that general age-related changes in antioxidant defenses do exist in teleosts.

To test whether endogenous antioxidant defenses are altered in relatively young fish groups, we have compared mature rainbow trout (*Oncorhynchus mykiss*) and black bullhead (*Ameiurus melas*) with their immature counterparts. The responsiveness of the antioxidant machinery including glutathione status (GSH and GSSG) and antioxidant enzymes (GPX, GR, SOD and CAT) to environmental stimulants has previously been tested in the related brown bullhead and the rainbow trout (Hasspieler *et al.* 1994a; Chapter 5). These plus the 2 GSH-dependent enzymes GST and GGT will be estimated in various tissues of both species of the 2 age groups. The involvement of kidney and white muscle in extrahepatic antioxidant defenses has been suggested

in rainbow trout (Chapter 5) and the impact of these tissues in age- or maturation-dependent changes is also investigated.

Materials and Methods

Hatchery reared immature 1+ year (15 months) and sexually mature, 3+ year (41 months) rainbow trout in non-spawning conditions and of mixed sexes were obtained from Linwood Acres (Campellcroft, ON). Hatchery reared immature 1+ year (13-15 months) and sexually mature, 3+ year (37-39 months) female bullheads in non-spawning conditions were kindly provided by Dr. J. Buttner, SUNY Brockport, NY. These fish were derived from 5th and 6th-generation parental fish spawned at SUNY Brockport and were raised under general fish farming procedures. The fish were laboratory acclimated in tanks supplied with dechloraminated city of Ottawa tap water for at least 3 weeks prior to harvesting. Water temperature was 12±1°C for trout and 20±1°C for bullheads and water was saturated with oxygen, as determined once/day. Fish were fed daily with a complete standard diet. Experiments were undertaken during the months of July (trout) and September (bullhead).

Fish were killed by a blow to the head and blood was collected into heparinized syringes from the caudal vessels. After rapid centrifugation (14,000g for 15 sec; Fisher Micro-Centrifuge, Model 235B) the plasma and erythrocytes were acidified with 5% 5-sulfosalicylic acid. The precipitated protein was removed by repeated centrifugation and the cleared supernatants were stored at -20°C. Organs were quickly excised, a small piece immediately suspended in buffer and analyzed for GGT (trout and bullhead) and GR (bullhead), which are freeze-sensitive, and the remaining tissue was freeze-clamped in liquid nitrogen and stored at -80°C until analyzed (<1

week). Tissues were homogenized in ice-cold Tris buffer (50 mM, containing 0.25 M sucrose and 1 mM EDTA, pH 7.5) using a Potter-Elvehjem-type glass homogenizer (liver and kidney) or a Polytron PCU-2 homogenizer (Brinkmann Instruments, Rexdale, ON) (other tissues). Homogenates were centrifuged at 10,000g for 15 min at 4°C. Portions of not centrifuged homogenates and the post-mitochondrial supernatants were used immediately (GGT and bullhead GR) or stored at -80°C (< 2 months) until analyzed. Enzyme assays were measured as specified in Chapters 2 and 5, with substrate concentrations that were those of the original assay and optimal for both species except for GPX. GPX activity was measured by the method of Tappel (1978), and the estimation of the Michaelis-Menten value (K_m) for GPX used 10 concentrations of GSH and the data were analyzed using a double reciprocal plot (Lineweaver-Burk) and linear regression analysis. Unless otherwise indicated, enzymes were assayed at 15°C for trout and 20°C for bullhead, which are temperatures experienced by these species. γ-Glutamyl transpeptidase was estimated in kidney crude homogenates by the method of Tate and Meister (1985), at a temperature of 20°C for both species. Protein content was determined according to Lowry *et al.* (1951). Tissues for total glutathione (TGSH) concentrations were processed as described previously (Chapter 2) and stored at -20°C (< 3 months). TGSH levels were assessed as described by Sen and coworkers (1992) and GSSG levels were determined by the method of Griffith (1980). Thiobarbituric acid reactive substances (TBARS) were quantified as described by Hermes-Lima *et al.* (1995). In short, tissue samples were homogenized in ice-cold 1.1% phosphoric acid, the homogenate was mixed with 1% TBA/50 mM NaOH/0.1 mM butylhydroxytoluene solution and 0.2 ml 7% phosphoric acid (final pH was 1.6-1.7 in muscle and 2.2-2.3 in liver). Subsequently samples were heated for 15 min at 100°C and mixed with 1.5 ml of butanol. The organic layer was

removed after separation by benchtop centrifugation. TBARS were calculated by using an absorbance difference of 532-600 nm and. Significant differences were tested between the different age groups using the Student's t-test for unpaired sample means (Statistix 4.1).

Results

Physical parameters of the 2 age groups of rainbow trout and black bullheads used in this study are shown in Table 4.1. Body weights, lengths and liver weights were significantly different in the 2 age groups of both species, reflecting a different condition factor (CF), but no difference in liver somatic index (LSI).

Antioxidant enzyme activities are shown in Table 4.2 and Fig. 4.1-4.4. GPX activities were significantly lower (1.3-fold) in kidney of 3+ year than in 1+ year trout, but significantly higher activities were detected in gill and white muscle (1.5- and 1.7-fold, respectively) (Table 4.2). GPX activities were also higher in gill (2.1-fold) and red muscle (1.7-fold) of 3+ year versus 1+ year bullheads. GST activities were significantly lower (2.2-fold) in kidney of 3+ year in comparison with 1+ year trout, but were significantly increased in red and white muscles (2.9- and 1.4-fold, respectively) of the same fish. In 3+ year bullheads GST activity in gill was higher (1.5-fold) than in 1+ year fish. Similarly, liver CAT activities (Table 4.2) were decreased significantly (1.4-fold), while gill and red muscle were higher (1.4- and 1.7-fold, respectively) in 3+ year trout compared with the younger trout. In bullheads, CAT activities were significantly elevated in white muscle of 3+ year versus 1+

Table 4.1. Physical parameters of the different age groups of rainbow trout and black bullhead

	Body weight (g)	Length (cm)	Liver weight (g)	CF	LSI
Trout					
1+ yr	98±7	21.4±0.6	1.0±0.1	1.0±0.04	1.0±0.06
3+ yr	1115±89"	45.0±1.2"	11.9±0.9"	1.2±0.04#	1.1±0.10
Bullhead					
1+ yr	100±5.4	20.4±0.3	3.1±0.2	1.2±0.03	2.6±0.5
3+ yr	218±11.6"	24.9±0.4"	6.2±0.4"	1.4±0.05"	2.6±0.4

Data represent means ± SE. Number of fish (n) are 10-12 animals. CF: condition factor (100 x body weight/length^3); LSI: liver somatic index (100 x liver weight/body weight); #, ": significantly different from 1+ fish ($p<0.01$, $p<0.001$, respectively) by the Student's t-test.

Table 4.2. Glutathione peroxidase (GPX), glutathione S-transferase (GST) and catalase (CAT) activities of different age groups of rainbow trout and black bullhead.

	Liver	Kidney	Gill	Red m	White m
GPX[a]					
Trout					
1+ yr	6.6±0.5	10.9±0.6	5.9±0.4	3.2±0.5	2.5±0.2
3+ yr	6.6±0.6	8.3±0.9*	9.1±0.7■	3.9±0.5	4.3±0.4■
Bullhead					
1+ yr	127±15	59.7±6.4	19.1±2.3	7.4±0.7	2.4±0.1
3+ yr	185±30	57.1±9.4	39.4±4.6#	12.3±1.9*	3.8±0.6
GST[a]					
Trout					
1+ yr	183±15	247±41	184±15	28.7±1.5	20.6±1.1
3+ yr	184±10	114±17#	213±18	82.4±3.4■	29.1±3.2*
Bullhead					
1+ yr	664±41	243±16	193±9	183±10	160±3
3+ yr	773±104	253±11	287±24#	179±10	184±11
CAT[b]					
Trout					
1+ yr	65.5±4.2	9.5±0.6	4.0±0.2	0.7±0.07	0.04±0.01
3+ yr	47.5±4.3#	9.0±0.5	5.7±0.5*	1.2±0.12#	0.06±0.01
Bullhead					
1+ yr	31.5±1.3	7.3±0.4	4.0±0.2	2.6±0.2	0.6±0.04
3+ yr	30.9±3.2	7.0±0.5	4.6±0.4	4.3±0.9	1.7±0.4*

Data represent means ± SE (n=10-12). Activities are expressed as nmol/(min*mg protein) ([a]) and µmol/(min*mg protein) ([b]). In 3+ trout, white muscle GPX represents the mean ± SE of males (3.4±0.4, n=5) and females (5.1±0.6, n=5), while kidney GST is the mean ± SE of males (147±25) and females (81±8.7), $p<0.05$ for both enzymes; no other significant sex-related differences were noted for any other enzyme. *, #, ■: significantly different from 1+ fish ($p<0.05$, $p<0.01$, $p<0.001$ respectively).

FIGURE LEGENDS

Fig. 4.1. Glutathione reductase activities (nmol/(min*mg protein)) in liver, kidney, gill, red and white muscles of 1+ year (open bars) and 3+ year (hatched bars) rainbow trout. Data represent means ± SE, n=10-12. *, **: significantly different from 1+ year trout ($p<0.05$, $p<0.01$, respectively).

Fig. 4.2. Glutathione reductase activities (nmol/(min*mg protein)) in liver, kidney, gill, red and white muscles of 1+ year (open bars) and 3+ year (hatched bars) black bullheads. Data represent means ± SE, n=6-8. *: significantly different from 1+ year bullheads ($p<0.05$).

Fig. 4.3. Superoxide dismutase activities (U/(min*mg protein)) in liver, kidney, gill, red and white muscles of 1+ year (open bars) and 3+ year (hatched bars) rainbow trout. Data represent means ± SE, n=10-12. *, **, #: significantly different from 1+ year trout ($p<0.05$, $p<0.01$, $p<0.001$, respectively).

Fig. 4.4. Superoxide dismutase activities (U/(min*mg protein)) in liver, kidney, gill, red and white muscles of 1+ year (open bars) and 3+ year (hatched bars) black bullheads. Data represent means ± SE, n=10. *, **, #: significantly different from 1+ year bullheads ($p<0.05$, $p<0.01$, $p<0.001$, respectively).

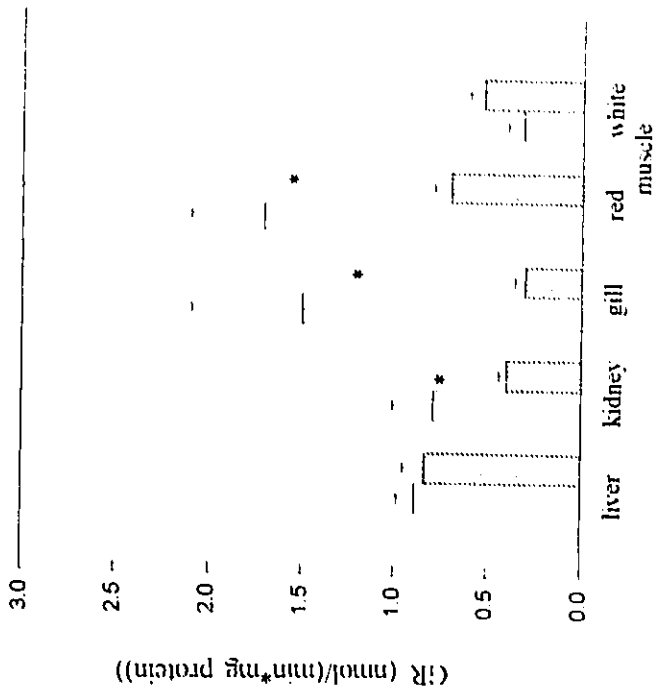

3.0
2.5
2.0
1.5
1.0
0.5
0.0
liver
kidney
gill
red muscle
white muscle
(iR (nmol/(min*mg protein))

Fig. 4.2

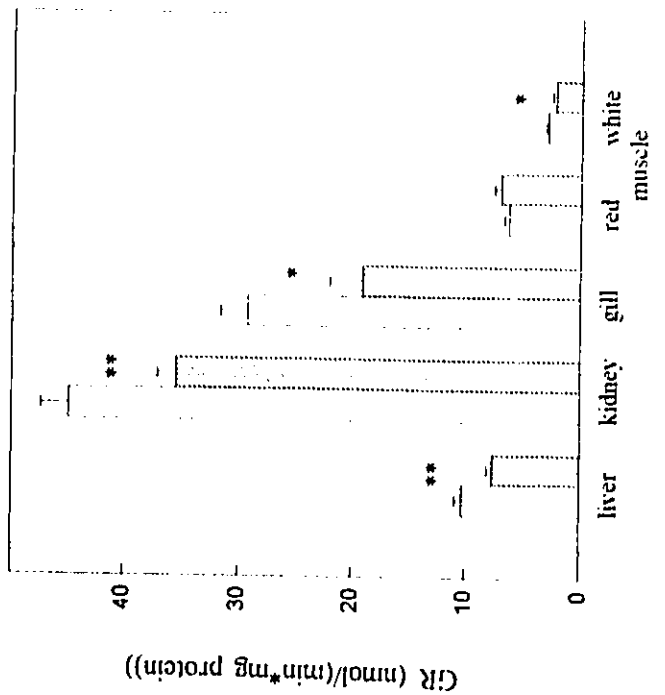

40
30
20
10
0
liver
kidney
gill
red muscle
white muscle
CiR (nmol/(min*mg protein))

Fig. 4.1

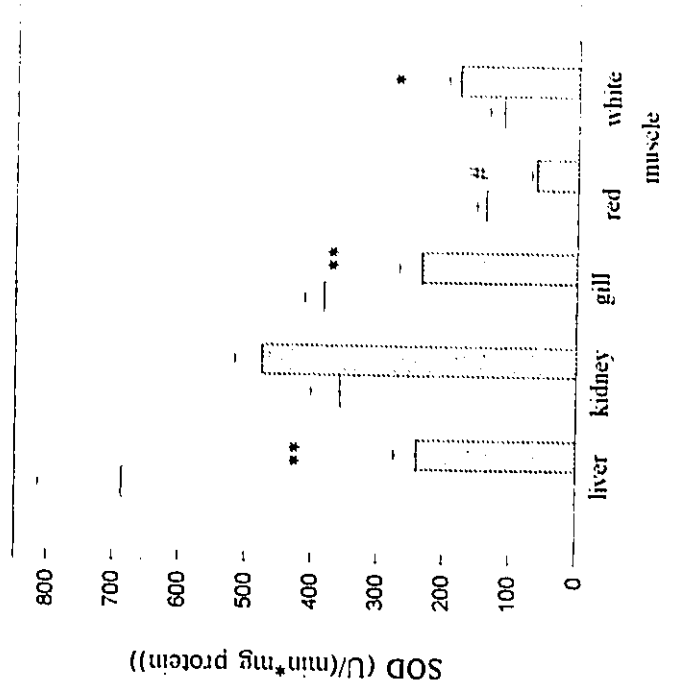

SOD (U/(min*mg protein))
800
700
600
500
400
300
200
100
0
**
**
*
#
liver
kidney
gill
red
white
muscle

Fig. 4.4

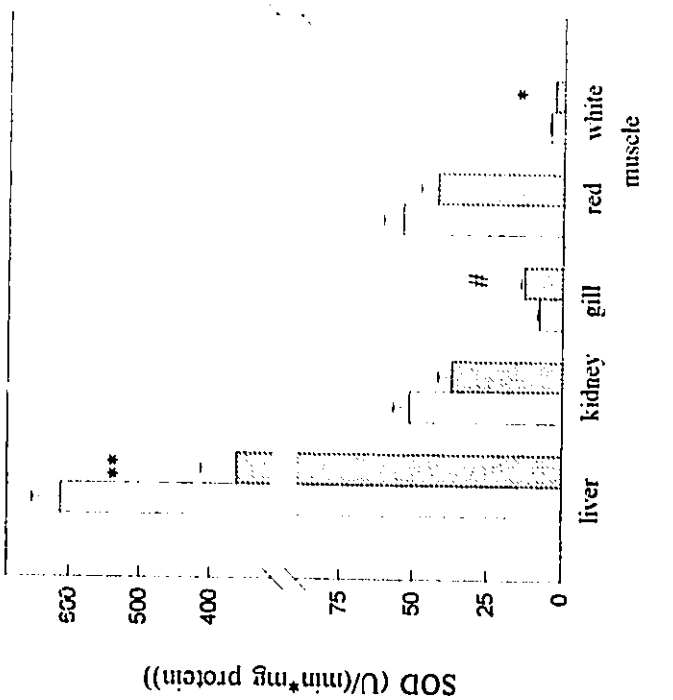

SOD (U/(min*mg protein))
550
500
400
75
50
25
0
**
*
#
liver
kidney
gill
red
white
muscle

Fig. 4.3

year fish, but remained unchanged in other tissues.

More consistent changes are shown in GR and SOD of both species (Fig. 4.1-4.4). GR activities were significantly lower in liver, kidney, gill and white muscle of 3+ year than in 1+ year trout (1.4-, 1.3-, 1.5- and 1.3-fold, respectively) (Fig. 4.1). A similar trend was observed in bullheads, as GR activities of kidney, gill and red muscle were significantly decreased in 3+ year versus 1+ year fish (2-, 5- and 2.4-fold, respectively) (Fig. 4.2). Rainbow trout SOD activities were significantly lower in liver (1.7-fold) and white muscle (1.5-fold), but significantly higher in gill (1.3-fold) of 3+ year than in 1+ year fish (Fig. 4.3). Also bullhead SOD activities were significantly decreased in liver, gill and red muscle (2.9-, 1.6- and 2.2-fold, respectively), while white muscle SOD activity was increased 1.3-fold in 3+ year compared with 1+ year bullheads (Fig. 4.4). SOD activities represent Cu,Zn-SOD activities in all tissues, except for red muscle where Mn-SOD activities contributed 5-20% in rainbow trout and 3-10% in bullheads of presented activities, as assessed by inhibition of Cu,Zn-SOD with cyanide.

Activities of GGT could be spectrophotometrically detected only in kidney and values are presented in Table 4.3 for both species. GGT activities were significantly lower (8.3-fold) in 3+ year than in 1+ year trout. Although GGT activities were lower (2.5-fold) in 3+ year bullheads, this result was not significantly different.

Rainbow trout GSH concentrations were significantly higher in liver, kidney, red muscle, heart and brain (1.7-, 1.41-, 2.2-, 1.8- and 1.3-fold, respectively) in 3+ year than in 1+ year trout (Table 4.4). But in the same fish, gill GSH levels were decreased (1.3-fold) compared with younger fish. Significantly lower GSH concentrations were noted in gill (1.3-fold) and

Table 4.3. Kidney γ-glutamyltranspeptidase (GGT) activities in trout and bullhead

Species	Kidney GGT
Rainbow trout	
1+ yr	4.09±0.98
3+ yr	0.50±0.21*
Bullhead	
1+ yr	0.65±0.18
3+ yr	0.26±0.09

Data represent means±SE expressed as nmol/(min*mg protein), n=8-10. *: significantly different from 1+ trout.

Table 4.4. Reduced and oxidized glutathione concentrations in rainbow trout and black bullhead

Tissue	Trout GSH	Trout GSSG	Bullhead GSH	Bullhead GSSG
Liver				
1+ yr	910±87	85±13	1103±52	74.1±4.4
3+ yr	1539±238*	120±10	1106±57	94.2±16
Kidney				
1+ yr	1411±70	99±9.6	811±27	61.6±7.3
3+ yr	1993±66**	103±12.4	840±52	48.2±9.3
Gill				
1+ yr	1291±72	69.4±7.3	713±34	18.3±1.3
3+ yr	982±49#	74.1±8.2	563±31#	14.6±1.1
Red Muscle				
1+ yr	214±28	27.1±2.8	346±19	9.5±4.1
3+ yr	476±36**	23.5±1.9	394±27	5.0±6.3
White Muscle				
1+ yr	174±24	3.5±0.7	284±17	1.6±0.3
3+ yr	238±13	3.5±0.5	264±23	2.0±0.3
Heart				
1+ yr	604±64	35.0±4.3	671±96	31.1±7.1
3+ yr	1086±77**	87.9±23.0	704±108	50.9±16.4
Brain				
1+ yr	384±32	54.8±3.6	1064±108	15.3±3.7
3+ yr	516±49*	47.0±2.3	999±146	5.3±0.8**
Plasma				
1+ yr	35±3.3	4.8±0.7	5.13±1.3	ND
3+ yr	30±2.7	4.0±0.5	1.38±0.33#	ND
Erythrocytes				
1+ yr	1619±73	84.5±12.9	690±117	159±20
3+ yr	1758±84	120±10.4	826±77	96±13.6

Data represent means±SE, expressed as nmol/g wet tissue (except for plasma, which is expressed in µmol/l), n=10-12. *, #, **: significantly different from 1+ fish at $p<0.05$, $p<0.01$, $p<0.001$ respectively. ND, not detectable.

plasma (3.7-fold) of 3+ year than 1+ year bullheads, but remained unchanged in other tissues. On the other hand, GSSG concentrations were significantly lower in brain (2.9-fold) of 3+ year than in 1+ year bullheads. No other differences in GSSG concentrations were noted in either species. GSH levels in both species were similar in most tissues, although kidney, plasma and red blood cell GSH concentrations appeared to be lower and brain higher in bullheads than in rainbow trout.

Thiobarbituric acid reactive substances (TBARS) were only determined in liver and white muscle of black bullheads and white muscle of rainbow trout (Table 4.5). No significant differences were found between values in older versus younger bullheads. Although mean muscle TBARS were 1.4-fold higher in 3+ year than in 1+ trout, this finding was not significantly different.

For most parameters measured interspecies and intertissue differences were noted. With the exception of white muscle, GPX activities were higher in all tissues of bullheads compared with trout, with hepatic GPX activities almost 20-fold higher than in trout (Table 4.2). As GPX activities were measured at a single assay substrate concentrations, which was found to be suboptimal for both species, the Michaelis-Menten constant (K_m) for GSH of hepatic GPX was determined. Mean K_m values did not differ significantly between trout (2.7±0.4 mM) and bullhead (4.1±1.4 mM) (n=4); these values were not influenced by the age of the fish.

Also hepatic GST activities were 3-fold higher in bullheads compared with rainbow trout, and similar differences were noted in red and white muscles of the 2 species (Table 4.2). Catalase activities were substantially higher in red and white muscles of bullheads than of trout, as were SOD activities of white muscle (Fig. 4.3 and 4.4). Most enzymes assayed had

Table 4.5. Thiobarbituric acid reactive substances (TBARS) in tissues of bullhead and rainbow trout

	Bullhead		Trout white muscle
	Liver	White muscle	
1+ yr	8.32±1.06	29.14±4.37	29.27±2.99
3+ yr	6.22±0.49	31.46±5.45	42.34±10.31

Data represent means ± SE (n=9-10 bullheads and n=4-6 trout), expressed as nmol/g tissue.

higher tissue activities in bullheads than rainbow trout, except for GR and GGT where activities in rainbow trout are much higher than those of bullhead in all tissues examined (Fig. 4.1 and 4.2; Table 4.3). Also intertissue GR activity differences were noted in rainbow trout, with markedly higher activities in kidney (4-fold) and gill (2.5-fold) than in liver. Other trout enzyme activities were in general marginally higher in liver than in other tissues (Fig. 4.3; Table 4.2).

Sex differences were not detected in trout except white muscle GPX activities were 1.5-fold higher ($p<0.05$) and kidney GST activities which were lower (1.8-fold, $p<0.05$) in 3+ year females than in the same age males (Table 4.2). In both cases 3+ females, but not males, are significantly different from 1+ trout.

Discussion

Recent reviews on aging in "cold-blooded" vertebrates (Goss 1994; Patnaik 1994; Patnaik *et al.* 1994) have stressed the importance of a comparative approach to such studies. Fish show three patterns of aging: i) rapid senescence and death at first spawning; ii) gradual senescence; and iii) indeterminate growth and very slow or negligible senescence (Patnaik *et al.* 1994). Gradual senescence occurs in most vertebrates including mammals. However, while mammals stop growth at a certain age, most teleosts continue to grow throughout life, although at a reduced rate. Thus fish show a different aging pattern compared with mammals and represent an ideal comparative model when asking the question if growth cessation is a necessity for the appearance of senescence (Goss 1994; Patnaik *et al.* 1994). Should determinate growth be a prerequisite for senescence, the proposed shifts in oxidative status (Sohal and Allen 1986; Sohal *et al.* 1994) would consequently be less pronounced in fish compared with mammals.

Sawada *et al.* (1993) have found significantly increased formation of free radicals in brain of 2 year versus 1 month old salmon. Although lipid peroxidation did not change significantly in these age-groups, it was significantly increased in 4 year old salmon. Both parameters were significantly enhanced in spawning versus prespawning salmon (Sawada *et al.* 1993). These data suggest that age-related changes in prooxidant forces already exist in relatively young fish and also in individuals that differ in spawning conditions and imply a need to examine the antioxidant defenses in fish species with different age.

Rainbow trout and black bullheads are representatives of fish species showing gradual senescence. The age groups compared in this study are stages of the young versus the maturation phase, which are both commercially available and used for environmental monitoring; all fish showed physical signs of well-being (Table 4.1). The 3+ age groups represent relatively young individuals that have reached the maturation phase. A life-span of 7-9 years is reported for both species (Carlander 1969). Indices such as condition factor and liver somatic index are conventionally used growth indices (Dutta 1994). However, the changed condition factor in 3+ year compared with 1+ year trout and bullheads could be interpreted as either a slowing of growth or as a greater weight gain for the same length. Table 4.1 shows that change is primarily due to weight gain rather than length. On the other hand, LSI did not change in either species.

The present study documents several changes indicating a weakening of endogenous antioxidant defenses in rainbow trout and black bullhead with age and/or maturation. The design of the present experiment cannot distinguish between both processes in the two different age groups. Current knowledge in fish does not show any evidence that maturation alone accounts for shifts in the prooxidant/antioxidant balance (Otto 1992; Sawada *et al.* 1993).

This study documents decreases in several antioxidant enzymes, especially in the liver and kidney. Several studies have elucidated the changes in antioxidant status in laboratory rodents (Cappozza *et al.* 1994; Liu & Mori 1993; Rikans *et. al.* 1992; Stio *et al.* 1994), showing a clear-cut pattern of decreasing antioxidant enzyme activities with age (He *et al.* 1994; Liu & Mori 1993; Véricel *et al.* 1994). In contrast, the changes in antioxidant enzyme activities differed in different tissues in aging mice (Sohal *et al.* 1994). In the present study, activities of GPX, GST and CAT show contrary effects in the different tissues (Table 4.2). Enzyme activities generally decreased with age in trout liver and kidney, but increased in gill and muscle tissues of both species. The different organs appear to have different age-related patterns with regard to these enzymes. Conjugation enzyme activities are reported to be stable with age (Kitani 1994), but isozyme-specific changes do occur with aging (Chen *et al.* 1994). Conjugation with CDNB measures most GST isozymes (Chen *et al.* 1994) and GST activities were relatively stable in both species, although activities were increased in trout muscle and bullhead gill, but depressed in trout kidney with age. An effect of age on GST activity had also been shown in different strains of mice (He *et al.* 1994). GPX activities have been reported to decrease with age in rodents (He *et al.* 1994; Stio *et al.* 1994), while CAT activities increased in liver of reptiles (Patnaik 1994). Neither GPX nor CAT activities changed in a consistent manner in all tissues of trout and bullhead.

Although GPX, CAT and GST showed rather contrary effects, the associated enzymes GR and SOD exhibited more consistent changes that could be related to age and/or maturation (Fig. 4.1-4.4). Exposure of nematodes to oxidative stress is reported to induce SOD activities in young, but not in middle aged and old worms (Darr and Fridovich 1995). A recent review by Warner (1994) on SOD and aging summarized the important role SOD is thought to play in attenuating oxidative

damage in vivo and thereby delaying the aging process. Although consistency in SOD activities did not occur in all tissues studied, the predominant decreases seen for both enzymes, SOD and GR, point to a weakened antioxidant system in the 2 species that appears to be age- or maturation-related. It is thus tempting to speculate that both enzymes underlie a similar age-dependent pattern in both species, despite the use of relatively young fish. The literature on these enzymes in mammals is inconclusive (Liu & Mori 1993; Rikans *et al.* 1992; Stio *et al.* 1994; Warner 1994) and it has been proposed that with the current disparate information on SOD activities and aging, innovative studies need to be undertaken (Warner 1994). Teleost fish appear to serve as suitable vertebrates in this respect.

Interestingly, white muscle in trout exhibited decreased SOD and GR activities with age, but similar changes did not occur in bullhead. This confirmed our previous observation that white muscle may play a key role, together with liver and kidney, in whole body antioxidant status in rainbow trout (Chapter 5). The decreased GR activities with age appeared to hold for most organs in trout (Fig. 4.1). We recently demonstrated that toxicant exposure increased GR activities several-fold in different body tissues of rainbow trout (Chapter 5). Such alterations of GR activities under different physiological conditions support an important role for this enzyme in antioxidant metabolism of teleost fish. In rats GR increased with age (Rikans *et al.* 1992; Stio *et al.* 1994). GR is a major regulatory enzyme that regenerates GSH from GSSG. Also GGT is involved for increasing cellular GSH concentrations, as it couples the γ-glutamyl moiety to a suitable amino acid acceptor for transport into the cell (Meister 1991). Interestingly, both enzymes are considerably decreased in older rainbow trout (Fig. 4.1; Table 3.3); nonetheless, tissue GSH concentrations generally increased in trout (Table 4.4). This may suggest de novo

synthesis of GSH by GSH synthesizing enzymes. In rats GSH synthesizing enzymes decreased with age in liver and heart as did GSH levels (Stio *et al.* 1994). Age-dependent decreases in GSH concentrations are often reported in laboratory rodents (Cappozza *et al.* 1994; Liu & Mori 1993; Véricel *et al.* 1994), but this is not the case in the present study.

GSH concentrations in bullheads generally remained unchanged in most tissues, although it did decrease in gill and plasma. The present study does not show a clear effect of age on GSH concentrations, providing no new information to the debate whether age affects GSH levels (Kitani 1994).

Lipid peroxidation, which has been described as indicative of progressive aging in several other studies on mammals (Véricel *et al.* 1994) and salmon (Sawada *et al.* 1993), did not change in the present study, but TBARS were only measured in 1-2 tissues of each species (Table 4.5). Also the sex differences in parameters of aging animals that have been noted in mammals (Rikans *et al.* 1992) are negligible in rainbow trout of the age-groups studied.

In conclusion, despite the use of relatively young fish, trout and bullheads exhibited age-related decreases in some antioxidant enzymes in various tissues. Although enzymes including GST, GPX and CAT, as well as GSH status lack a clear pattern, SOD and GR activities do decrease in both species and suggest possible age- and/or maturation-related effects. These data invite further studies to test if these parameters change in senescent fish, but do suggest that age and/or maturation of the fish may be a confounding variable for studies in environmental monitoring.

CHAPTER 5.

3,3',4,4' TETRACHLOROBIPHENYL EFFECTS ON ANTIOXIDANT ENZYMES AND GLUTATHIONE STATUS IN DIFFERENT TISSUES OF RAINBOW TROUT

Introduction

Polychlorinated biphenyls (PCBs) are ubiquitously distributed and are major environmental pollutants (Zile 1992). Although the production and use of these toxic chemicals are now discontinued, they are resistant to biodegradation and their persistence is enhanced with increasing chlorination of the compound (Safe 1994). PCBs are known to accumulate in fish and induce cytochrome P450-dependent monooxygenases (MO) and various studies have examined the MO system and its role in mediating the effects of anthropogenic pollutants in aquatic species (Kleinow *et al.* 1987; Gooch *et al.* 1989; Collier *et al.* 1992). It has been suggested that the determination of phase I (monooxygenases) and phase II (conjugation) enzymes after toxicant exposure may predict the fate of a toxicant in an organism (Kleinow *et al.* 1987). The impact of environmental pollutants on conjugation enzymes in fish has been described (Parker *et al.* 1993; Otto *et al.* 1994).

Various studies on avian and mammalian species find that PCB congeners have a major impact on antioxidant defenses (Kamohara *et al.* 1984; Saito 1990; Spear & Moon 1986), implicating the involvement of other detoxication routes apart from the cytochrome P450 system for such toxicants. Exposure of different fish species to PCB mixtures have demonstrated shifts in the prooxidant/antioxidant forces in various tissues (Thomas & Wofford 1993; Rudneva-Titova &

Zherko 1994).

Various xenobiotic compounds like biphenyls as well as nitroaromatics and quinones are known to undergo redox cycling following univalent reduction by NADPH electron transport leading to the formation of reactive oxygen species, i.e. the superoxide anion radical ($O_2^{\cdot}$) (Kappus 1986; Winston & Di Giulio 1991). To evade oxygen toxicity, aerobic life forms have acquired a defense mechanism represented by the physiological antioxidants. Primary components of this system in vertebrates are superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6), involved in the detoxication of $O_2^{\cdot}$ and H_2O_2, as well as the glutathione system (Harris 1992).

Glutathione (L-γ-glutamyl-L-cysteinylglycine; GSH) serves a diverse series of critical cellular defensive functions including free radical scavenging, detoxication of electrophiles and maintenance of thiol-disulfide status (Deneke & Fanburg 1989; DeLeve & Kaplowitz 1991). Glutathione peroxidase (GPX, EC 1.11.1.9) is specific for its hydrogen donor GSH, but may use a wide range of substrates extending from H_2O_2 to organic hydroperoxides. As a consequence of GPX action, GSH is oxidized to glutathione disulfide (GSSG), which in turn is rapidly reduced back to GSH by glutathione disulfide reductase (GSSG reductase, GR, EC 1.6.4.2). This reaction requires reducing equivalents derived from the NADP-dehydrogenases glucose-6-phosphate dehydrogenase (G-6-PDH), 6-phosphogluconate dehydrogenase (6-PGDH), isocitrate dehydrogenase (IDH) and malic enzyme (ME) (Reed 1986). Glutathione also functions in a second detoxication pathway initiated through glutathione S-transferase (GST, EC 2.5.1.18), catalyzing the conjugation between the sulfhydryl group of GSH and the electrophilic atom of a xenobiotic.

This study hypothesizes that PCBs will affect not only P450-dependent enzyme activities, but as importantly, the endogenous antioxidant system in tissues of fish. To test this hypothesis the effects of a single i.p. injection of 3,3',4,4'-tetrachlorobiphenyl (TCB), a PCB congener, on activities of the P450-dependent monoxygenase ethoxyresorufin O-deethylase (EROD), the conjugation enzymes UDPglucuronosyltransferase (UDPGT) and GST, the antioxidant enzymes GPX, GR, SOD and CAT, and GSH status of rainbow trout are examined. Most studies focus on toxicant metabolism in the liver, a major detoxication organ. Our previous work has suggested that environmental pollutants also affect the GSH system in muscle of rainbow trout (Otto *et al.* 1994). We have, therefore, investigated the effect of TCB on P450 species and antioxidant defenses in tissues other than liver, including kidney, red and white muscles. The effects of short-term exposures of mammals and fish to PCB congeners on antioxidant defenses have been monitored (Schramm *et al.* 1985; Saito 1990; Rudneva-Titova & Zherko 1994), but it was our specific interest to test the impact of the TCB on the metabolic machinery by examining trout tissues 6 weeks following injection. A 6 week post acute exposure to TCB allows the organism to adapt and reestablish a new equilibrium where it is now dealing with the toxicant impact.

Materials and Methods

Chemicals

TCB was purchased from Ultra Scientific (Kingstown, RI). Ethoxyresorufin, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), xanthine, xanthine oxidase (XOD), Bakers yeast GR, GSH, GSSG, 1 chloro-2,4-dinitrobenzene (CDNB), NADP, NADPH, uridine 5'diphosphoglucuronic acid

(UDPGA), p-nitrophenol were obtained from Sigma Chemical Co. (St. Louis, MO) and 2-vinylpyridine was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other reagents were of analytical grade.

Animals

Hatchery reared, immature rainbow trout (180-310g) were obtained from Linwood Acres (Campbellcroft, Ont.). The fish were laboratory acclimated in tanks supplied with dechloraminated city of Ottawa tap water before separating the fish randomly into 2 tanks supplied with identical water. Water temperature was 12±1°C and water was saturated with oxygen. Fish were pair-fed 1.5% of body weight per day and except for the day of injection, all fish ate continuously throughout the experiment. All experiments were undertaken during the fall.

Exposure of fish to TCB

TCB was dissolved in corn oil with heating to 60°C at a stock concentration of 4 mg/ml. The solution was cooled to approximately 30°C and was injected i.p. at 5 mg/kg (200-400 µl) and the trout (n=8) were placed back into their respective tanks. Controls received equivalent volumes of corn oil. Fish were killed 6 weeks after injection by a blow to the head and blood was collected into heparinized syringes from the caudal vessels. After rapid centrifugation the plasma was acidified with 5% 5-sulfosalicylic acid in a 1:2 ratio and centrifuged to remove precipitated protein. The supernatant was stored at +4°C. Organs were quickly excised, freeze-clamped in liquid nitrogen, and stored at -80°C until analysed.

Preparation of cytosolic tissue fractions

Frozen tissue samples were rapidly weighed and homogenized in ice-cold 0.25 M sucrose in a Potter-Elvehjem-type glass teflon homogenizer for liver and kidney, and with a Polytron PCU-2

homogenizer (Brinkmann Instruments, Rexdale, Ont.) for muscles. Weight to volume ratios used were 1:5 for liver and kidney and 1:2 for red and white muscles. Homogenates were centrifuged at 10,000 g for 15 min at 4°C (Sorvall RC 28S). The post mitochondrial supernatants were further centrifuged at 105,000 g at 4°C for 60 min (Beckman L8-70M Ultracentrifuge, 50Ti rotor). Microsomes were resuspended by homogenization in 0.25 M sucrose (containing 60 mM Tris, 5 mM EDTA and 20% glycerol). Supernatants and microsomes were stored at -80°C until further analysed for cytosolic and microsomal enzyme activities.

Glutathione measurements

Frozen tissue samples were homogenized in ice-cold 5% 5-sulfosalicylic acid and centrifuged at 4°C and 4,000 g for 10 min. Dilutions were 1:5 for liver and kidney and 1:2 for muscle tissues. Supernatants were recovered by centrifugation and stored at 0-4°C. Total glutathione (TGSH) was determined according to Sen *et al.* (1992) in which the rate of reduction of DTNB is correlated to the sum of GSH and GSSG using a standard curve. GSSG was quantified by the method of Griffith (1980).

Cytosolic enzyme assays

All enzymes were assayed at 15°C, a temperature which is within the range experienced by this species. Substrate concentrations were those of the original assay, all were found to be optimum for this species, except for GPX which was not optimized. Protein content was chosen to give linear change in enzyme activity and it was estimated according to Lowry *et al.* (1951).

GPX activity was measured by the method of Tappel (1978) using cumene hydroperoxide (0.05 mg/ml) and GSH (0.25 mM) as substrates, in 0.05 M Tris buffer, pH 7.6. The data are expressed as nmol NADPH oxidized per min per mg of protein. GR activity was determined as

described by Carlberg & Mannervik (1985) and expressed as nmol NADPH oxidized per min per mg of protein. Final substrate concentrations are 1 mM GSSG and 0.1 mM NADPH, pH 7.0. GST activity was monitored according to Habig *et al.* (1974) by measuring conjugate derivative formation as a function of time, with final concentrations of 1 mM GSH and 1 mM CDNB, pH 6.5. Specific activity was expressed as nmol GSH conjugated per min per mg of protein using an extinction coefficient at 340 nm of 9.6 x 10^3/mol per cm^2. Superoxide dismutase activity was assessed by the Beauchamp & Fridovich (1971) procedure, in which xanthine-xanthine oxidase is the superoxide anion generating system and nitro blue tetrazolium is the indicator of superoxide production. By inhibition of Cu,Zn-SOD with cyanide, the activity of Mn-SOD was determined. Catalase activity was measured as reported by Cohen *et al.* (1970) by estimation of residual H_2O_2 concentrations using linear regression of a standard curve. NADPH generating enzymes were assayed using a final concentration of 1 mM of each glucose-6-phosphate (for the combined activities of G-6-PDH and 6-PGDH), malate (ME) and isocitrate (IDH), as well as 0.4 mM NADP, 8 mM $MgCl_2$ in 0.05 M imidazole, pH 7.8 as described by Foster & Moon (1991).

Microsomal enzyme activities

Deethylation of ethoxyresorufin to resorufin (EROD activity) was assessed as described by Burke & Mayer (1974), with final concentrations of 1 µM ethoxyresorufin and 50 µM NADPH, pH 7.8. Glucuronidation of p-nitrophenol (UDPGT activity) was measured according to Hänninen (1968), with 0.23 mM p-nitrophenol and 4 mM UDPGA, pH 7.0. Reaction times were 10 min for liver and 40 min for kidney. Microsomal enzyme activities were determined at 18°C, a preferred reaction temperature for these assays in fish (Koivusaari 1983).

Statistical analysis

Significant differences between control and treatment groups were tested using the student t-test for unpaired sample means. With nonhomogeneous variance in many variables, data were transformed into their common logarithm and analysed with the Student's t-test. A level of significance of $p < 0.05$ was accepted as significant.

Results

Injection of TCB in doses of 5 mg/kg resulted in no mortalities to the fish during the 6 week experimental period. The behavior of the fish was similar in both groups throughout the experiment, although the TCB injected fish appeared to feed more actively than controls. Table 5.1 shows measured physical parameters in control and TCB-injected fish. Body weight did not differ between groups, but liver weight and liver to body weight ratio (LSI) were significantly higher in TCB-injected fish than in controls ($p \leq 0.01$). These findings are in concordance with similar exposure studies in mammals in which PCB exposure resulted in significant increases in liver weight and LSI (Anders *et al.* 1983; Kamohara *et al.* 1984).

The effect of TCB on EROD activities in liver and kidney of rainbow trout are shown in Fig. 5.1. EROD activity increased 11-fold in liver of injected trout, while EROD induction

Table 5.1. Measured physical parameters in TCB-injected and control rainbow trout

	BW	LW	LSI
Control (8)	234±17.5	3.1±0.24	1.3±0.08
TCB (8)	227±22.1	4.2±0.27*	1.9±0.12*

Data represent means ± SE. Number of fish (n) is shown in parenthesis. Abbreviations: BW: body weight; LW: liver weight; LSI: liver somatic index (100 x LW/BW). *: significantly different from controls ($p \leq 0.01$).

Table 5.2. Glutathione-dependent and NADPH-producing enzymes in various rainbow trout tissues after TCB exposure

	LIVER	KIDNEY	RED M	WHITE M
GST				
Control	426±83	360±87	43.2±4.6	16.2±0.96
TCB	971±252#	333±85	31.7±3.4	14.6±1.02
GPX				
Control	8.8±0.98	14.7±1.2	1.7±0.21	0.56±0.09
TCB	11.7±0.82*	15.6±0.4	1.9±0.18	0.86±0.10*
HMP				
Control	225±28.4	158±12.5		
TCB	373±23.1*	143±17.9		
ME				
Control	63.3±7.7	11.9±1.2		
TCB	80.1±6.4	15.2±2.3		
IDH				
Control	119±14.4	84±8.4		
TCB	130±7.7	101±11.9		

Data represent means ± SE (n=8), expressed in nmol/(min*mg protein). Red M and White M indicate red and white muscles, respectively; HMP represents the combined activities of G-6PDH and 6-PGDH. *, *, #: significantly different from controls ($p<0.05$, $p<0.01$, $p<0.001$, respectively).

FIGURE LEGENDS

Fig. 5.1. EROD (pmol/(min*mg protein)) and UDPGT (nmol/(min*mg protein)) activities in liver and kidney of control (open bars) and TCB-injected (hatched bars) rainbow trout. Data represent means ± SE, n=8. ***: significantly different from controls ($p<0.001$).

Fig. 5.2. Glutathione reductase activities (nmol/(min*mg protein)) in liver, kidney, red and white muscles in control (open bars) and TCB-injected (hatched bars) rainbow trout. Data represent means ± SE, n=8. *, **, ***: significantly different from control values ($p<0.05$, $p<0.01$, $p<0.001$, respectively).

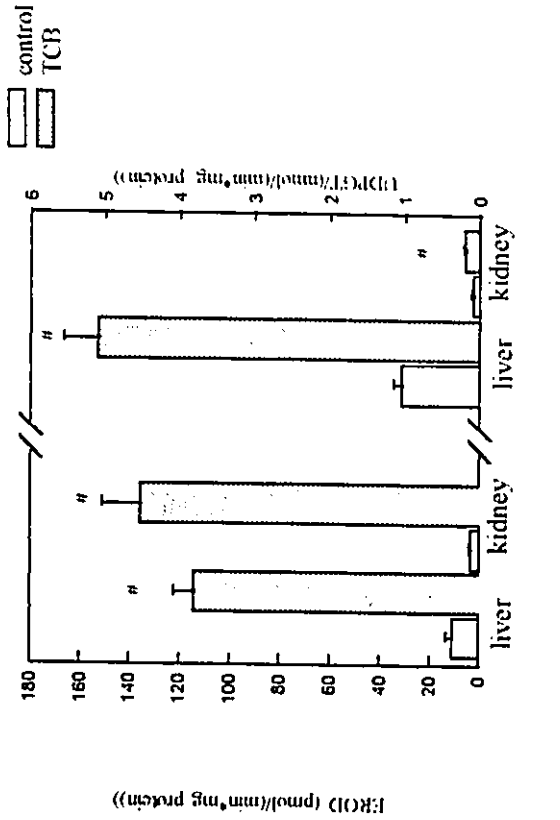

control
TCB
EROD (pmol/(mm*mg protein))
liver
kidney
liver
kidney
#

Fig. 5.1

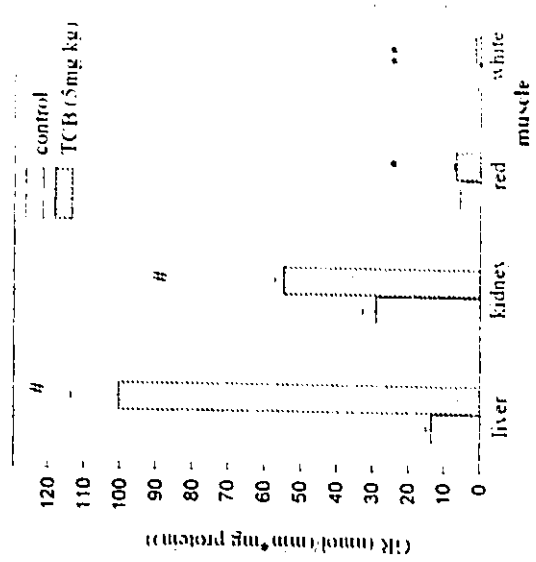

control
liver
kidney
red muscle
white muscle
#

Fig. 5.2

was more strongly increased in kidney with a 40-fold higher activity in TCB-exposed fish compared with controls. Concomitantly with enhanced phase I enzyme activities, increased activities of phase II enzymes were observed. UDPGT activity was elevated 5-fold in liver of TCB-injected fish (Fig. 5.1). In comparison to the high EROD induction in kidney, UDPGT activity in this organ increased only 2-fold after biphenyl exposure. In addition to UDPGT, other phase II pathways involve the conjugation with glutathione, catalyzed by GST (Kleinow *et al.* 1987). TCB-injected trout showed a 2.3-fold induction of GST activities in liver compared with controls (Table 5.2). However, GST activities remained unchanged in kidney, red and white muscles.

PCB congeners have been previously shown to affect the antioxidant status in rats through changes in GSH-dependent enzymes (Kamohara *et al.* 1984; Saito 1990; Schramm *et al.* 1985). GPX activities (Table 5.2) were significantly increased in liver and white muscle (32 and 54%, respectively), but not in kidney or red muscle. Such data implicate toxicant-induced production of H_2O_2 and/or organic peroxides which are detoxified by GPX. In order to maintain the redox status of the cell, the GSSG so formed must be reduced by GR. GR activities increased in all tissues assayed in the TCB-injected trout (Fig. 5.2). Liver GR activity was induced 7-fold, while increases in kidney, red and white muscle were lower, but significantly elevated (88, 21 and 67%, respectively).

Regeneration of GSH from GSSG by GR occurs at the expense of NADPH. NADPH can be generated by a number of dehydrogenases, but the contribution of the two dehydrogenases of the hexose monophosphate pathway (HMP; glucose-6-phosphate and 6-phosphogluconate dehydrogenases) are thought to be the most important (Reed 1986). As changes in GR activities

were most pronounced in liver and also kidney of TCB-injected fish, NADPH generating enzymes were therefore determined only in these tissues. HMP enzyme activities increased significantly by 65% in liver of TCB-injected fish (Table 5.2). Most of this increase was due to enhanced activity of G-6-PDH (64% versus controls), while 6-PGDH was increased slightly (12%; data not shown). HMP enzyme activities remained unchanged in kidney, as were ME and IDH activities in liver and kidney (Table 5.2).

The activities of SOD and CAT are shown on Table 5.3. SOD remained unchanged in liver, kidney and red muscle, but was significantly increased (43%) in white muscle. Cytosolic SOD activity represents mainly Cu,Zn-SOD, as Mn-SOD is thought to be predominantly in the mitochondria, but may also be found in the cytosol (Ichikawa *et al.* 1994). Mn-SOD activities were not detected in the cytosolic fraction in any tissue examined except for red muscle where cytosolic Mn-SOD activity was 7-22% of total SOD activities (data not shown). Mn-SOD activity in this tissue may result from a breakage of mitochondria during homogenization, as red muscle is highly enriched with mitochondria. Cytosolic CAT activities were significantly reduced in livers of TCB-injected trout (by 17%), but were unaltered in kidney and red muscle. White muscle displayed a 4.7-fold increase in CAT activity, although activities in this tissue were at least 10-fold lower than those of any other tissue assayed.

GSH is described as the predominant defense against toxic products of oxygen metabolism and cellular glutathione status (GSSG/GSH, GSH and GSSG levels) are most indicative of cellular oxidative stress (DeLeve & Kaplowitz 1991). Table 5.4 shows a significant 50% increase in hepatic GSH levels in TCB-injected trout compared with controls. Kidney GSH content was significantly increased (16%), but GSH levels in blood plasma, red and white

Table 5.3. Superoxide dismutase (SOD) and catalase (CAT) activities in different tissues of TCB-injected rainbow trout

	SOD	CAT
LIVER		
Control	996±80	54.5±3.0
TCB	1066±58	45.5±1.8*
KIDNEY		
Control	200±23.0	10.9±0.86
TCB	188±13.2	9.9±0.37
RED MUSCLE		
Control	138±18.6	1.4±0.13
TCB	117±7.8	1.7±0.10
WHITE MUSCLE		
Control	15.7±0.89	0.03±0.01
TCB	22.3±2.08*	0.14±0.04#

Data represent means ± SE (n=8). SOD is expressed in U/(min*mg protein); CAT in µmol/(min*mg protein). *, #: significantly different from controls ($p<0.05$, $p<0.001$, respectively).

Table 5.4. Reduced (GSH) and oxidized (GSSG) glutathione concentrations in tissues and blood plasma of TCB-injected rainbow trout

	GSH	GSSG	GSSG/GSH
LIVER			
Control	3.3±0.25	0.14±0.01	0.04±0.004
TCB	5.0±0.26#	0.24±0.03■	0.05±0.008
KIDNEY			
Control	3.0±0.14	0.12±0.01	0.04±0.004
TCB	3.5±0.17•	0.13±0.01	0.04±0.004
PLASMA			
Control	50.4±7.0	4.2±0.41	0.09±0.002
TCB	65.8±7.6	6.4±0.48■	0.11±0.002
RED MUSCLE			
Control	0.63±0.04	0.067±0.005	0.11±0.010
TCB	0.66±0.04	0.068±0.004	0.11±0.013
WHITE MUSCLE			
Control	0.28±0.03	0.008±0.001	0.03±0.004
TCB	0.24±0.02	0.007±0.001	0.03±0.004

Data represent means ± SE (n=8). Concentrations are µmol/g wet weight for all tissues and µmol/l blood plasma. •, ■, #: significantly different from controls ($p<0.05$, $p<0.01$, $p<0.001$, respectively).

muscles were not significantly different between the TCB-injected and control fish. On the other hand, GSSG concentrations were significantly increased in liver (73%) and blood plasma (51%) in TCB-injected fish compared with controls. Despite these changes, the calculated GSSG/GSH ratio for the studied tissues did not differ significantly between groups.

Discussion

This study supports our hypothesis that the PCB congener, TCB, not only affects cytochrome P450 activities, but that it modifies enzymes associated with antioxidant defenses in a number of tissues of rainbow trout. In addition, these changes suggest that TCB may generate reactive oxygen species in these tissues which still impact on antioxidant defenses even at 6 weeks after the injection.

Induction of monooxygenases by various PCB congeners has been repeatedly shown in mammals (Safe 1994; Anders *et al.* 1983). Fish MOs are known to be induced by aromatic hydrocarbon-type inducers including PCB congeners and changes in EROD activity are a useful indicator in such exposure studies (Collier *et al.* 1992; Gooch *et al.* 1989). Also in our study EROD activities are indicative of TCB induction (Fig. 5.1), with EROD activities in the kidney exceeding that of the liver in TCB-injected trout. This result supports a role for the MO pathways in the detoxication of TCB in both hepatic and extrahepatic organs. Concentrations of TCB greater than 1 mg/kg have been reported to inhibit EROD activity in scup 3 days after exposure (Gooch *et al.* 1989), but we see strong induction even at 5 mg/kg in trout after a 6 week period.

The 2- and 5-fold induction of UDPGT activity in kidney and liver (Fig. 5.1) implies the poosibility that glucuronidation may be a preferred conjugation route for TCB and metabolites.

Increased UDPGT activity has been reported in aflatoxin-induced hepatic neoplasms in rainbow trout (Parker *et al.* 1993) and glucuronidation is thought to be quantitatively the most important phase II pathway for many xenobiotics in fish (Clarke *et al.* 1991). Significant changes in GST activities were also observed in liver, while other organs displayed unchanged activities (Table 5.2); rat liver cytosol also demonstrated increased GST activities after exposure to various PCB congeners (Kamohara *et al.* 1984; Schramm *et al.* 1985). Changes in GST activity generally affect the GSH status of a cell, as with increasing conjugation, more GSH will be withdrawn from the cell. However, such an effect on GSH concentrations was not observed in our study (Table 5.4).

Elevations of antioxidant enzyme activities indicate the production of reactive oxygen species by PCB congeners as previously reported (Kamohara *et al.* 1984; Kappus 1986). The generation of oxyradicals may also arise from cytochrome P450-dependent mechanisms, by uncoupling at the level of NADPH-cytochrome P450 reductase (Terelius & Ingelman-Sundberg 1988). Radical production from this source can modify enzyme activities and can lead to suicide inactivation of P450 species (Karuzina & Archakov 1994). Activation of MO detoxication processes, which could be observed in the present study, may thus increase the likelihood of radical production from this source as well.

Administration of TCB resulted in increased GPX activity in liver and white muscle of rainbow trout (Table 5.2). These results are in contrast to studies using rats fed PCB containing diets for short and long durations which reported significantly decreased hepatic GPX activities (Kamohara *et al.* 1984; Saito 1990). In other studies, however, rat GPX activity remained unchanged after injection with different PCB congeners, while administration of various other compounds including isosafrole, phenobarbital or 2-acetylaminofluorene resulted in significantly increased

GPX activities (Schramm *et al.* 1985; Shamaan *et al.* 1993). Short- and long-term exposures of different fish species to PCB mixtures resulted in increased lipid peroxidation within liver, muscles and ovarian tissues (Thomas & Wofford 1993; Rudneva-Titova & Zherko 1994). Atlantic croaker fed with a PCB mixture displayed increased GPX activities in liver and ovary, while other antioxidant defenses were not affected (Thomas & Wofford 1993). Elevated GPX activity would enhance the potential of the cell to detoxify H_2O_2. As a result, GSSG is produced which is rapidly converted to GSH by GR at the expense of NADPH. Significant GR activation was detected in all tissues of TCB-injected trout in this study (Fig. 5.2), confirming similar results in rodents after PCB or other toxicant exposures (Kamohara *et al.* 1984; Shamaan *et al.* 1993). GR is known to consume NADPH at rates comparatively higher than other NADPH-dependent enzymes, such as those of fatty acid synthesis or MO activity, implying that the glutathione redox cycle when functioning at maximum capacity could impose a significant impact upon cellular NADPH levels (Reed 1986). The increased activities of the hexose monophosphate shunt enzymes in liver (Table 2) support a higher demand for NADPH in TCB-injected trout, as would be expected with increased GR activities.

Increased cellular $O_2^{\cdot-}$ should also elevate SOD, and the resulting H_2O_2 would impose a further pull on GPX and CAT activities (Harris 1992). This cascade of reactions resulting in increased antioxidant enzyme levels could only be observed in white muscle, where significant increases in activities of SOD, CAT, GPX and also GR occurred in TCB-injected trout (Tables 5.2 and 5.3; Fig. 5.2). Liver CAT was decreased (Table 5.3), in contrast to rats where hepatic CAT increased after PCB exposure (Kamohara *et al.* 1984). A 24h PCB-exposure of 2 fish species resulted in decreased muscle CAT activities, but increased SOD activities (Rudneva-Titova & Zherko 1994).

Catalase was previously thought to be a peroxisomal marker, but its presence in the cytosol under normal physiological conditions has been discussed (Eriksson *et al.* 1992).

Although some studies have been made in fish to examine the effects of PCB on antioxidant defenses, the literature remains inconclusive (Thomas & Wofford 1993; Rudneva-Titova & Zherko, 1994). The long-term effects of toxicants on biochemical responses are often environmentally more relevant. Administration of a PCB mixture to Atlantic croaker through their food for 17 days led to an increased mortality of greater than 40%, indicating an enormous stress on the fish (Thomas & Wofford 1993). A single injection of a toxicant and sampling 6 weeks post injection may enable the fish to achieve a new physiological equilibrium and thus more effectively deal with the toxicant.

Although not conclusive, the changes in all measured antioxidant enzymes in the white muscle may suggest an involvement of white muscle in the detoxication of TCB in rainbow trout. Changes in antioxidant status have been studied in mammalian muscle after exercise (Sen *et al.* 1992) and in snakes after freezing or anoxia (Hermes-Lima & Storey 1993). Sen *et al.* (1992; 1993) have suggested that rat skeletal muscle may be a significant component of interorgan GSH homeostasis supported by the characterization of GSH dependent enzymes *in vivo* and *in vitro*. Although liver is the organ with primary detoxication functions, but white muscle in trout may also be included in the detoxication process. In comparison to mammals, fish have a much higher percentage of white muscle over total body weight (70 - 80%). Thus, if our hypothesis is correct, white muscle would greatly increase the detoxication potential in a fish injected with a toxicant like TCB. An effect of environmental pollutants on GSH status in muscle of rainbow trout had been previously discussed (Otto *et al.* 1994).

Despite the significant changes in antioxidant enzymes in the different tissues, the concentrations of GSH increased only in liver and kidney (Table 5.4). The observed increase in GST should decrease GSH content in liver, but this did not occur at 6 weeks after a single exposure to TCB. The observed increase in GR (Fig. 5.2) does support the maintenance of the cellular glutathione pool in the reduced state. Liver is thought to be a major participant in interorgan GSH homeostasis, as it may release large quantities of GSH into the blood, which can then be taken up by other organs (Deneke & Fanburg 1989). The observed elevated hepatic GSH concentrations may not only enhance liver detoxication and protection, but may also contribute to constant glutathione levels in other tissues (i.e. muscle) to enable proper detoxication processes there. Although GSSG concentrations increased in liver and plasma of TCB-injected trout, the unchanged GSSG/GSH ratios indicate that antioxidant forces remain high, which may result from increased GSH concentrations particularly in the liver.

In conclusion, the observed changes in antioxidant enzymes and GSH status in concert with the cytochrome P450-dependent and conjugation enzymes, support the involvement of a complex enzyme system in the liver and other tissues in TCB exposed rainbow trout. Certainly white muscle, with the highest relative body mass proportion, may play an important role in the detoxication process, as all antioxidant enzymes displayed enhanced activities. TCB is, therefore, likely to increase oxidative stress in fish and stimulates GSH-dependent detoxication.

CHAPTER 6.

ROLE OF EXOGENOUS GLUTATHIONE IN TELEOST FISH AND ITS EFFECTS ON ANTIOXIDANT DEFENSE RESPONSES IN RAINBOW TROUT EXPOSED TO 3,3',4,4'-TETRACHLOROBIPHENYL

Introduction

Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is a low molecular weight thiol with major cellular antioxidant functions. GSH serves in a multitude of critical cellular defensive functions including free radical scavenging, detoxication of electrophiles and maintenance of thiol-disulfide status and signal transduction (Meister & Anderson 1983; DeLeve and Kaplowitz, 1991; Dröge *et al.* 1994; Sen 1995; Sen & Packer 1996).

GSH metabolism in lower vertebrates like fish is thought to resemble that of mammalian species (Gallagher *et al.* 1992; Chapter 5). Sex differences as well as age- and maturation-dependent changes in the GSH system of teleosts appear to show notable differences to mammalian species (Chapter 4). Such observations increase the likelihood that mammalian GSH biochemistry cannot be directly extended to teleost species. Further elucidation of GSH metabolism in fish may therefore enhance knowledge of the general biochemistry of this ubiquitously present thiol. The glutathione system and the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), which detoxicate superoxide anion ($O_2^{\cdot-}$) and H_2O_2, act as a primary defense as endogenous physiological antioxidants. A second line of defense is established by antioxidants, which can only be provided by nutritional supplements (Sen 1995).

These exogenous antioxidants such as vitamins C and E have been described as vital components of fish health (Frischknecht *et al.* 1994; Thorarinsson *et al.* 1994). Both vitamins are thus added to commercial fish feeds, and vitamin E plays an important role on fish flesh quality including maintenance of colour and rendering oxidative stability to salmon muscle (Sigurgisladottir *et al.* 1994a,b). Vitamin E is a generic term that includes the tocopherols (α-, β-, γ- and δ-tocopherols) and tocotrienols (Sies and Stahl 1995). All four tocopherols have been identified in fish species. Fish deposit some tocopherols more readily than others, whereby γ-tocopherol appears to be preferentially incorporated into salmon muscle (Sigurgisladottir *et al.* 1994b). However, the beneficial antioxidant actions of tocopherols and vitamin C produce radical byproducts that are efficiently scavenged by GSH or α-lipoic acid (Packer *et al.* 1995; Sen 1995).

GSH serves thus as a significant regenerator for other antioxidants and the enhancement of tissue GSH content has been sought in mammalian species (Sen *et al.* 1994). The majority of tissues in mammals do not respond to exogenous GSH, so GSH pro-agents such as N-acetylcysteine and lipoate have been employed as dietary supplements (Sen *et al.* 1994; Packer *et al.* 1995). In mammals, intracellular GSH content is kept constant by consistent supply of GSH by glutathione synthesizing enzymes, γ-glutamylcysteine synthetase and GSH synthetase. Substrates for such synthesis are provided by direct amino acid transport or γ-glutamyl-transpeptidase (GGT). GSH is also regenerated from oxidized glutathione (GSSG) by glutathione reductase (GR). All these enzymes are important contributors to intracellular GSH homeostasis in teleost species (Gallagher *et al.* 1992; Chapters 4 and 5). However, in contrast to mammals, there are presently no reports available on GSH delivery to fish tissues.

The first part of this study aims to establish whether GSH administration serves as an effective

delivery agent of GSH to tissues of two teleost species, the rainbow trout (*Oncorhynchus mykiss*) and the American eel (*Anguilla rostrata*). We have previously shown that manipulation of the trout tissue GSH content by either GSH, L-buthionine-[S,R]-sulfoximine (BSO; a specific GSH depletor) and lipoate, significantly alters 3,3',4,4'-tetrachlorobiphenyl (TCB) induced cytochrome P4501A metabolism (Chapter 7). However, uptake of GSH into tissues may have been influenced by TCB exposure. We now show that exogenous GSH effectively increases tissue GSH concentrations in teleost fish. Given that vitamins E, C and carotenoids are effectively used in fish feed (Sigurgisladottir *et al.* 1994a,b), GSH may also serve as a suitable candidate for dietary supplementation, which is yet unexplored. GSH tissue enhancement may improve the overall physiological conditions of the fish and could find commercial applications within the aquacultural industry. GSH plays major roles in immunological and immunopathological responses (Dröge *et al.* 1994). Maintenance of a favourable immunological status in fish is a major objective for the fish farming industry.

Polychlorinated biphenyls (PCBs) are environmental pollutants which influence physiological antioxidant defenses in various teleosts (Thomas & Wofford 1993; Chapters 2 and 5). The PCB congener TCB strongly impacts on the GSH system and associated antioxidant enzymes in rainbow trout (Chapter 5). PCBs are known to increase lipid peroxidation in fish tissues (Thomas & Wofford 1993). Tissue tocopherols in fish have been shown to play a vital role in diminishing tissue lipid peroxidation when exposed to oxidizing conditions (Williams *et al.* 1992; Sigurgisladottir *et al.* 1994a). Tocopherols, GSH and lipoate act synergistically against prooxidant forces (Sen 1995). In the second part of the study, we intend to show whether thiol manipulation modifies lipid peroxidation, vitamin E and antioxidant enzymes in TCB exposed

trout.

Materials and Methods

For GSH injection, 100-180 g hatchery reared rainbow trout were obtained from Linwood Acres (Campellcroft, ON, Canada). Feral eels (80-300 g) were obtained from the St. Lawrence River by a local fishermen at Cornwall, ON. Both species were held for 3-5 months in tanks supplied with dechloraminated City of Ottawa tap water at 12±1°C and saturated with oxygen. Experiments were performed in the spring of the year. Trout were fed 1.5% of body weight per day, whereas eels were not feed in the facility. Tank water levels were lowered (to approximately 40 l) and trout were mildly anaesthesized by adding 0.05 g/l 3-aminobenzoic acid ethyl ester (MS 222) and 0.1 g/l sodium bicarbonate to the tank. GSH (0.25 g/kg; in physiological saline and pH adjusted to 6.5-6.8) was injected intraperitoneally (i.p.) once per day. Control fish received equivalent volumes of saline. Eels received the same treatment, but were placed on ice 10 min before injections instead of using chemical anaesthesia. Fish were harvested the morning following 3 or 6 days of daily GSH injections.

In the second part of the study, trout (50-80 g) were injected intraperitoneally (i.p.) with TCB dissolved in corn oil at 1 mg/kg; control fish received equivalent volumes of corn oil. For injections, trout were mildly anaesthesized as described above. The day following the TCB injection, fish were given daily (every evening) i.p. injections of either saline, GSH (0.25 g/kg), BSO (6 mmol/kg) or lipoate (16 mg/kg). All agents were dissolved in physiological saline (pH 6.8), except lipoate which was dissolved in 1 part 6 N NaOH and 19 parts 0.2 M sodium phosphate buffer (pH 7.0) (see Chapter 7, for detailed experimental design). Fish were harvested

following 3 or 6 days of daily injections.

For both experiments, trout were killed by a blow to the head and eels were decapitated. Tissues were collected and treated as previously described (Chapter 4). For biochemical analyses, tissues were homogenized and analyzed using the original assays as previously described (Chapter 4). Tocopherols (α and γ) were assayed in liver and white muscle by high pressure liquid chromatography according to the method of Thompson and Hatina (1979).

Significant differences between saline and GSH injected trout and eels were tested using the Student's t-test for unpaired sample means (Statistix 4.1). Significant changes between corn oil controls and the four TCB thiol treatment groups were tested with one-way analysis of variance (ANOVA). The location of significance was determined using the Least Significant Difference method (LSD). Significant differences between 3 and 6 day treatments were tested using the Student's t test for unpaired sample means.

Results

Tissue delivery of exogenous GSH in teleosts

Daily supplementation with exogenous GSH increased tissue total glutathione (TGSH; GSH + GSSG) levels in both trout and eel. TGSH content significantly increased in trout liver 1.6-fold, kidney 1.7- to 2-fold and plasma 21- to 25-fold (3 and 6 days) (Table 6.1). TGSH levels were only significantly increased in trout gill following 3 days and muscle following 6 days of GSH supplementation. GSH supplementation significantly activated kidney γ-glutamyltranspeptidase (GGT) activities (3.9-fold) following 3 days, but not following 6 days. GGT in trout can be detected spectrophotometrically only in kidney (Chapter 4).

Table 6.1. Tissue total glutathione (TGSH) content and kidney γ-glutamyl-transpeptidase activity in rainbow trout receiving daily glutathione injections (0.25 g/kg body weight)

	3 days	6 days
TGSH		
liver		
control	2.44±0.10	2.14±0.18
GSHinj.	3.85±0.21*	3.51±0.54*
kidney		
control	2.41±0.17	2.39±0.23
GSHinj.	4.81±0.67**	4.14±0.33*
gill		
control	1.66±0.02	1.71±0.02
GSHinj.	2.25±0.14*	1.93±0.13
white M		
control	0.29±0.05	0.22±0.01
GSHinj.	0.30±0.03	0.29±0.01*
plasma		
control	33.0±3.10	19.5±1.93
GSHinj.	838±259#	397±105#
GGT kidney		
control	1.10±0.35	1.85±0.74
GSHinj.	4.32±1.39*	2.93±0.25

Data represent means ± SEM (n=3-4) and are expressed in μmol/g tissue except for plasma (μM) and GGT (nmol/(min x mg protein). *,**,#: significantly different from saline injected controls ($p<0.05$; $p<0.01$; $p<0.001$, respectively).

Table 6.2. Tissue total glutathione (TGSH) content in American eels receiving daily glutathione injections (0.25g/kg body weight)

	3 days	6 days
TGSH		
liver		
control	1.33±0.12	1.09±0.51
GSHinj.	5.76±1.00**	8.05±1.34**
kidney		
control	0.62±0.06	0.57±0.03
GSHinj.	1.21±0.06**	1.15±0.20*
gill		
control	0.67±0.08	0.29±0.09[1]
GSHinj.	0.60±0.02	0.58±0.06*
white M		
control	0.075±0.016	0.047±0.004
GSHinj.	0.080±0.012	0.077±0.012
plasma		
control	1.78±0.49	1.03±0.28
GSHinj.	90.9±29.2**	126.5±31.0#
GGT		
liver		
control	0.92±0.33	1.5±0.47
GSH inj.	1.35±0.37	2.02±0.27
kidney		
control	16.5±1.13	11.3±3.06
GSH inj.	16.5±1.99	15.7±1.45
gill		
control	5.2±0.15	6.9±0.46
GSHinj.	4.4±0.53	6.8±0.84

Data represent means ± SEM (n=3-5) and are expressed in µmol/g tissue (TGSH) except for plasma (µM) and nmol/(min x mg protein) (GGT). *,**,#: significantly different from saline injected controls ($p<0.05$; $p<0.01$; $p<0.001$, respectively). [1]: significantly different from 3 day controls ($p<0.05$).

TGSH content significantly increased in eel liver (4.3- to 7.4-fold), kidney (2-fold) and plasma (51- to 123-fold) following 3 and 6 days of GSH supplementation (Table 6.2). However, neither gill nor muscle TGSH levels changed; gill TGSH was significantly higher in GSH injected eels than in controls following 6 days, but this resulted from a decline of gill TGSH content from 3 to 6 days in the saline treated control eels. GGT could be determined in liver, kidney and gill of eels, but did not significantly change between control and GSH supplemented eels.

Thiol treatment effects on associated antioxidant defenses in TCB exposed trout

TCB exposure and daily thiol manipulation changed only few physical characteristics of the rainbow trout (Table 6.3). In spite of random selection of fish, the GSH supplemented TCB treated trout had significantly lower body weights than controls following 6 days of thiol treatment. Significant changes in liver somatic index (LSI) resulted from the TCB exposure even in the absence of significant changes in liver weight, and these changes appeared to be influenced by the various daily thiol treatment. LSI increased more rapidly in TCB-saline and GSH depleted (BSO) trout to values significantly higher than control or GSH and lipoate supplemented trout following 3 days of thiol treatment. Following 6 days of thiol treatment the LSI was not significantly different between the various TCB groups, but was significantly higher in all TCB exposed groups except the GSH depleted trout compared with controls (Table 6.3).

Thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation, significantly increased in the liver of TCB-saline, GSH and lipoate supplemented trout compared with control and GSH deficient groups following 3 days of thiol treatment ($p<0.05$) (Table 6.4). Following 6 days of thiol treatment, TBARS were significantly higher in livers of lipoate supplemented trout than in controls or TCB-saline treated groups ($p<0.05$). TBARS in muscle were significantly

Table 6.3. Physical characteristics of TCB exposed rainbow trout

	control	TCB-saline	TCB-GSH	TCB-BSO	TCB-lipoate
WEIGHT					
3 days	70±4.4	68±2.9	68±3.4	66±3.0	71±2.5
6 days	71±1.8[a]	65±2.3	62±2.7[a]	64±4.0	64±2.6
LENGTH					
3 days	19.6±0.4	19.1±0.3	19.1±0.3	18.9±0.3	19.3±0.3
6 days	19.4±0.3	19.3±0.2	18.8±0.3	19.1±0.3	19.2±0.2
LW					
3 days	0.69±0.04	0.82±0.05	0.74±0.05	0.83±0.04	0.72±0.05
6 days	0.68±0.01	0.81±0.07	0.71±0.03	0.69±0.04	0.74±0.03
CF					
3 days	0.92±0.02	0.95±0.02	0.97±0.02	0.97±0.03	0.99±0.03
6 days	0.97±0.02	0.90±0.02	0.92±0.03	0.92±0.04	0.89±0.02
LSI					
3 days	1.00±0.04[■▲]	1.19±0.05[▲]	1.09±0.07[■]	1.27±0.04[•]	1.02±0.08[■]
6 days	0.96±0.03[■]	1.24±0.09[•]	1.16±0.03[•]	1.09±0.06	1.16±0.03[•]

Data represent means ± SEM and are expressed in g (body weight) and cm (length), n=5-8. LW, liver weight. CF, condition factor (body weight/length^3). LSI, liver somatic index (100 x liver weight/body weight). [•]: significantly different from [■]-labeled groups of the same day (p,0.05).[▲]: significantly different from same-labeled groups of the same day ($p<0.05$).

Table 6.4. Thiobarbituric acid reactive substances (TBARS) and α- and γ-tocopherol (α- andγ-TOC) levels in TCB exposed rainbow trout

	control	TCB-saline	TCB-GSH	TCB-BSO	TCB-lipoate
TBARS					
liver					
3 days	6.2±0.65$^{\blacksquare}$	10.7±1.9$^{\bullet}$	11.6±2.0$^{\bullet}$	8.9±1.4	10.7±0.8$^{\bullet}$
6 days	8.5±1.26$^{\blacksquare}$	9.3±1.4$^{\blacksquare}$	12.8±1.1	12.6±2.9	15.4±1.5$^{\bullet}$
muscle					
3 days	53±8.8	54±7.0	56±7.3	61±6.2$^{\bullet}$	39±6.0$^{\blacksquare}$
6 days	44±1.3	56±7.6	49±8.0	57±5.6	42±4.6
α-TOC					
liver					
3 days	83±18	60±12	83.8±16	67±14	85±11
6 days	72±13	72±12	67.3±12	88±15	65±10
muscle					
3 days	4.7±0.6	4.4±0.4	3.4±0.4	4.9±0.3	4.5±0.8
6 days	3.7±0.5	4.9±0.4	3.3±0.4	3.9±0.9	4.6±0.6
γ-TOC					
muscle					
3 days	0.60±0.10$^{\bullet}$	0.63±0.08$^{\bullet}$	0.45±0.05	0.31±0.08$^{\blacksquare}$	0.31±0.08$^{\blacksquare}$
6 days	0.56±0.04$^{\bullet a}$	0.52±0.05$^{\bullet}$	0.38±0.07	0.23±0.03^{a}	0.23±0.03$^{\blacksquare}$

Data represent mean ± SE, n=5 and are expressed in nmol/g wet tissue (TBARS) and μg/g (tocopherols). $^{\bullet}$: significantly different from $^{\blacksquare}$-labeled groups of the same day ($p<0.05$); ^{a}: significantly different from same labeled group of the same day ($p<0.05$).

higher in GSH deficient than in lipoate supplemented trout following 3 days of thiol treatment. However, these differences disappeared following 6 days of thiol treatment.

The relative insensitivity of oxidative stress parameters (like TBARS) to the various thiol treatments was also observed for α-tocopherol content in liver and muscle of TCB exposed fish (Table 6.4). All groups analyzed showed a similar α-tocopherol content. However, muscle γ-tocopherol content of controls and TCB-saline injected fish was significantly higher than in GSH deficient and lipoate supplemented trout following 3 days and 6 days of treatment ($p<0.05$). γ-Tocopherol was only detected in few livers.

Thiol treatment showed an effect on superoxide dismutase (SOD) activities in the TCB exposed fish in the four tissues analyzed (Table 6.5). Hepatic, renal and muscle SOD activities were significantly lower in GSH deficient and lipoate supplemented trout than in control, TCB-saline and GSH supplemented groups following 3 days of treatment ($p<0.05$). Following 6 days of thiol treatment, hepatic SOD activity remained significantly lower in GSH deficient compared with GSH supplemented fish ($p<0.05$). SOD activities in kidney and muscle of controls declined following 6 days versus the 3 days of treatment. Renal SOD activity was significantly elevated in TCB-saline treated fish compared with all other groups following 6 days of thiol treatment ($p<0.01$). Muscle SOD activity was unchanged in all groups following 6 days of thiol treatment. Gill SOD activity remained unchanged in all groups following 3 days of thiol treatment. Following 6 days of thiol treatment, gill SOD activity was significantly higher in all TCB exposed groups than in controls ($p<0.05$). Again, this effect reflects a decrease of gill SOD activity in controls from 3 to 6 days ($p<0.05$) rather than changes provoked by TCB exposure and thiol treatment.

Liver, kidney and muscle catalase (CAT) activities remained unaffected by TCB exposure and

Table 6.5. Superoxide dismutase (SOD) and catalase (CAT) activities in various tissues of TCB exposed rainbow trout

	control	TCB-saline	TCB-GSH	TCB-BSO	TCB-lipoate
SOD					
liver					
3 days	650±59*	748±73^{*c1}	530±76^{abc}	326±30$^{\blacksquare a}$	457±27$^{\blacksquare b}$
6 days	526±133	347±69	554±87^{a}	270±40^{a}	516±69
kidney					
3 days	172±14^{*3}	163±24^{*a}	159±15^{*1}	79±7.8$^{\blacksquare}$	92±8.2$^{\blacksquare}$
6 days	55±4.2	157±20$^{\#\#}$	95±18	65±14	78±11.7
gills					
3 days	61±10.0^{1}	56±8.8	85±18	77±23	75±5.0
6 days	29±4.3$^{\blacksquare}$	63±2.7*	55±5.8*	67±5.9*	59±11*
muscle					
3 days	82±10.3^{*2}	86±9.9*	96±20^{*a}	47±7.8$^{\blacksquare}$	60±3.8^{a}
6 days	39±7.4	70±2.6	55±14	55±8.3	43±14
CAT					
liver					
3 days	55±2.5	53±2.2	54±3.5	48±2.2	52±5.7
6 days	50±1.5	44±4.4^{a}	54±1.7^{a}	52±4.5	47±2.7
kidney					
3 days	7.7±0.08	6.7±0.36	6.8±0.20	7.2±0.52	7.8±0.64
6 days	7.1±0.34*	5.8±0.22$^{\blacksquare}$	6.5±0.19	7.5±0.20*	6.6±0.56
gills					
3 days	4.8±0.23^{ab1}	5.1±0.24$^{\blacksquare 2}$	7.8±1.36^{a}	7.2±0.75^{b1}	8.5±0.26*
6 days	8.2±0.90$^{\blacksquare}$	12.4±0.8^{**a}	10.3±0.60*	9.8±0.60^{a}	8.2±0.63$^{\blacksquare}$
muscle					
3 days	0.41±0.04^{2}	0.34±0.05	0.31±0.02	0.30±0.02	0.30±0.06
6 days	0.24±0.02$^{\blacksquare}$	0.30±0.02^{a}	0.36±0.04	0.45±0.06^{**a}	0.41±0.04**

Data represent means ± SEM, n=5 (except TCB-BSO at 6 days n=4) and are expressed in U/(min x mg protein) (SOD) or µmol/(min x mg protein) (CAT). $^{\#,\#\#}$: significantly different from all other groups of the same day ($p<0.05$ and $p<0.01$, respectively). *,**:significantly different from $^{\blacksquare}$-labeled groups of the same day ($p<0.05$ and $p<0.01$, respectively).^{a,b}: significantly different from same-labeled groups of the same day ($p<0.05$). ^{1,2,3}: significantly different from 6 day same treatment ($p<0.05$; $p<0.01$; $p<0.001$, respectively).

following 3 days of thiol treatment (Table 6.5). However, following 6 days of thiol treatment, hepatic CAT was significantly higher in GSH supplemented than in TCB-saline treated trout. Renal CAT activity was significantly decreased in TCB-saline treated fish compared with controls and GSH deficient groups. Muscle CAT activity decreased in the control group following 6 days, which led to significantly higher activities in GSH deficient and lipoate supplemented trout than in controls or TCB-saline treated fish ($p<0.05$). Gill CAT activities were significantly elevated in GSH supplemented, GSH deficient and lipoate supplemented trout compared with controls or TCB-saline treated fish following 3 days of thiol treatment ($p<0.05$). Following 6 days of thiol treatment, CAT activities were significantly higher in TCB-saline and GSH supplemented than in controls and lipoate supplemented trout ($p<0.05$).

Discussion

Exogenous GSH, administrated i.p., is an effective delivery agent of GSH to tissues not only in rainbow trout but also in the American eel (Tables 6.1 and 6.2). This increase was particularly apparent in liver, kidney and plasma with varied results in gill and white muscle. We previously observed that administration of GSH increased tissue GSH content in TCB exposed trout (Otto *et al.* 1996a), but present results show this is a direct effect of GSH rather than indirectly mediated by toxicant exposure. The efficiency of GSH as delivery agent in teleost fish is further supported by preliminary observations of black bullhead hepatocytes incorporating exogenous GSH (D. Shangavi, D. Otto, C. Sen, T. Moon, unpublished results). Although the GSH system of teleost fish resembles that of mammals in many aspects (Gallagher *et al.* 1992; Chapter 4), exogenous GSH serves as a markedly more effective delivery agent for GSH in teleosts than in mammals.

Sen *et al.* (1994) observed TGSH increases in kidney and blood of rats receiving i.p. administered GSH, while liver, heart, lung and muscle tissue remained noticeably unaffected. In addition, the piscine GSH system appears to show other differences compared with mammalian species. Mammalian liver TGSH concentrations are 2-4 times higher than in any other body tissue, including kidney, lung, heart and muscle tissues which show similar TGSH contents (Sen *et al.* 1994). At least for trout, liver, kidney and gill have similar TGSH concentrations. Similar TGSH tissue distributions are also observed in black bullhead (Chapter 4). In contrast, eel hepatic TGSH levels are double those of kidney and gill. However, eels had been starved for several months and tissue TGSH distribution patterns may have been affected, as liver is a net synthesizer of GSH, while other tissues like kidney and muscle mostly import GSH from the circulating blood (DeLeve & Kaplowitz 1991). Starvation could also affect the distinct TGSH values observed in tissues of eel compared with trout. The present results in combination with our previous study (Chapter 4) imply that tissue TGSH distributions are different in teleosts than in mammals and may be a factor for efficient GSH uptake. GSH uptake into cells is known to be catalyzed by γ-glutamyltranspeptidase (GGT), a membrane bound enzyme (DeLeve & Kaplowitz 1991). Administration of GSH activated renal GGT activity in trout only initially (following 3 days of GSH supplementation) and not at all in eels. Whether GSH can be incorporated into cells by mechanisms other than by GGT has not been resolved. At present little is known about the implication of efficient GSH uptake in fish.

GSH also serves as a storage for cysteine (Meister & Anderson 1983), fish growth and overall health could be positively affected by exogenous GSH. Given the numerous functions of GSH as an antioxidant, coenzyme, involvement in electrophilic conjugation, DNA and protein synthesis

and many more, these observations invite further research, particularly in the aquacultural and fish physiology field. Several tissues are known to be attacked by parasites in farmed fish. *I.e.* bacterial kidney disease is caused by manifestation of kidney by *Renibacterium salmoninarum* and impaired immunological responses in salmonids (Thorarinsson *et al.* 1994). Nutrient supplementation including vitamin C, iodine, vitamin E and selenium have been tested to alter the resistance of salmonids to this disease (Thorarinsson *et al.* 1994). Improved tissue GSH status, particularly in liver and kidney, may ameliorate the deleterious effects of such diseases.

Previous long-term studies have shown that PCB affect prooxidant/antioxidant status in fish tissues (Thomas & Wofford 1993; Chapters 2 and 5). A direct effect of TCB on antioxidant status would thus be expected shortly after exposure to the toxicant. We have previously shown that under the same treatment, TCB acted alone on cytochrome P450 metabolism, which was markedly affected by thiol treatment; however, conjugation and glutathione redox enzymes were only little affected (Chapter 7). The efficiency of thiol treatment against TCB toxicity may thus depend on biochemical changes evoked by TCB. Such changes did not occur within the present short time period, although activities of conjugation enzymes and antioxidant defenses are markedly altered by TCB in long-term toxicity studies in the same species (Chapter 5). The lack of TCB induced biochemical changes may have made the present parameters rather insensitive to thiol treatment.

L-buthionine-[S,R]-sulfoximine (BSO) is a specific inhibitor of GSH synthesis and is effective on GSH metabolism in teleost fish (Gallagher *et al.* 1992; Chapter 7). Physical parameters were not affected by TCB or thiol treatment over the 6 day treatment (Table 6.3), except LSI increased in all TCB exposed groups following 6 days of thiol treatment, an observation previously reported

in TCB exposed trout (Chapter 5). Also TCB treatment barely induced lipid peroxidation and was negligibly modified by thiol treatment (Table 6.4). However, γ-tocopherol content was influenced by thiol treatment, although not by TCB exposure. GSH and lipoate are known to serve as reductants for the regeneration of tocopherols from their radical forms (Packer *et al.* 1995; Sen 1995). Tocopherols are known to diminish lipid peroxidation in fish (Williams *et al.* 1992; Sigurgisladottir *et al.* 1994b). The present results are therefore insufficient to predict efficiencies of thiol treatment in trout exposed to toxicants like TCB. In contrast, these results are again showing further evidence that time and type of exposure may affect sensitivity of biochemical parameters to PCB congeners, which has been previously discussed (Chapter 3).

SOD and CAT activities have been shown to be induced in muscle of TCB exposed trout (Chapter 5). Again, the present results show little sensitivity of SOD and CAT activities to TCB exposure and thiol manipulation (Table 6.5). Interestingly, hepatic, renal and muscle SOD activities often were lowest in GSH depleted and lipoate supplemented trout compared with other groups. This is also correlated with a low γ-tocopherol content in muscle of these groups. TGSH contents were generally reduced in GSH deficient and unchanged in lipoate supplemented trout, but both appear to suppress TCB induced cytochrome P4501A metabolism to similar extent (Chapter 7). This type of metabolic similarity between a GSH depletor and a pro-GSH agent remains unresolved. It also shows that pro-GSH agents like lipoate certainly exert different metabolic effects than GSH itself. This again makes fish an interesting model for studies on thiol metabolism.

In conclusion, exogenous GSH efficiently enhances tissue TGSH content in tissues of rainbow trout and American eel. Significant GSH uptake into tissues of vertebrates has previously not

been reported. The physiological and biochemical effects of such GSH enhancement in tissues are therefore largely unknown. A short-term study using TCB exposed trout revealed only little sensitivity to thiol treatment. However, γ-tocopherol content and superoxide dismutase activities are decreased in GSH deficient and lipoate supplemented TCB exposed trout. GSH is a major cellular antioxidant and has important immunological function that may positively influence the immune system of an organism. The beneficial role of GSH enhancement in teleosts should be further examined with possible prospects in the aquacultural industry i.e. by altered feed composition.

CHAPTER 7.

REGULATION OF 3,3',4,4'-TETRACHLOROBIPHENYL INDUCED CYTOCHROME P450 METABOLISM BY THIOLS IN TISSUES OF RAINBOW TROUT

Introduction

Polychlorinated biphenyls (PCBs) are major industrial pollutants which have been heavily produced in several industrial settings. Their production has now discontinued, but PCBs remain a worldwide pollution problem (Asplund *et al.* 1994). The toxicity of various PCB congeners to terrestrial and aquatic organisms are wideranged causing physical and biochemical changes including activation of xenobiotic metabolism in mammals and fish (Safe 1994; Dragnev *et al.* 1995; Chapter 5). PCBs share many properties with polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans, including some of the most toxic chemicals known such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Asplund *et al.* 1994; Hahn *et al.* 1993).

The xenobiotic responses predominantly induced by PCB congeners in mammals and fish are phase I and II enzyme activities (Dragnev *et al.* 1995, Gooch *et al.* 1989; Chapter 5). Phase I enzymes are almost exclusively cytochromes P450, while phase II enzymes usually function by conjugating phase I metabolites to *e.g.* glutathione or glucuronide to facilitate the excretion of a xenobiotic (Nebert *et al.* 1990). Pollutant induced cytochrome P450 enzymes have been characterized in various fish species (Berndtson & Chen 1994; Husøy *et al.* 1994; Hahn & Stegeman 1994; Morrison. *et al.* 1995). Induction of these enzymes in fish as in mammals is known to occur through ligand (e.g. TCDD) binding to the Ah receptor (AhR), translocation and

transformation of this complex in the nucleus involving ARNT (Ah receptor nuclear translocator) protein, and ultimately DNA binding of the complex to the aromatic hydrocarbon responsive element (AhRE) (Hahn *et al.* 1994; Denison & Whitlock 1995). Such DNA binding induces the *Ah* gene battery, which consists in mammals of at least six genes, two phase I (CYP1A1 and CYP1A2) and four phase II genes which include UDP (uridine diphosphate)-glucuronosyltransferase (UDPGT) and one glutathione S-transferase form (Nebert *et al.* 1990). In fish the main focus has been on phase I induction mechanisms, and two distinct CYP1A genes have been described in rainbow trout (Berndtson & Chen 1994).

A phylogenetic analysis of CYP1A genes in vertebrates has been presented and suggests an early appearance in vertebrate evolution (Morrison *et al.* 1995). In spite of a better knowledge of the function of the Ah receptor, little is known of the physiological conditions that regulate the *Ah* gene battery. Nebert *et al.* (1990) proposed that the *Ah* gene battery responds to oxidative stress. It was recently shown that the *in vitro* DNA binding of the Ah receptor is redox regulated (Ireland *et al.* 1995).

PCBs are known to perturb the balance between cellular pro-oxidant and antioxidant forces in vertebrates and thus induce oxidative stress (Yamamoto *et al.* 1994; Rudneva-Titova & Zherko 1994). Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is a major cellular antioxidant with numerous key functions. Glutathione S-transferase (GST) catalyzes the conjugation of the sulfhydryl group of GSH and an electrophilic atom of a xenobiotic compound. Glutathione dependent antioxidant defenses are catalyzed by glutathione peroxidase (GPX) which decomposes hydrogen peroxide and organic peroxides generating oxidized glutathione (GSSG) as endproduct. The tissue GSH pool is maintained in its reduced state by the action of glutathione reductase (GR)

at the expense of NADPH. Other antioxidants including vitamins E and C and α-lipoic acid, act synergistically with glutathione and have often been used in antioxidant supplementation studies (Meister 1994; Packer *et al.* 1995). Although vitamins E and C are powerful antioxidants and their tissue availability can be enhanced by oral supplementation, their beneficial antioxidant actions involve radical byproducts which are reduced by GSH and lipoate (Meister 1994; Packer *et al.* 1995). Increasing the cellular thiol pool (primarily established by -SH either as GSH or protein bound -SH) has been a challenging task in mammalian studies. Administration of GSH to rats was shown to increase GSH levels in the intestinal mucosa (Aw 1994), but i.p. injections of GSH failed to increase tissue GSH concentrations (Sen *et al.* 1994). Supplementation of lipoate as an effective pro-GSH agent has become a more successful approach (Busse *et al.* 1992; Packer *et al.* 1995).

3,3',4,4'-Tetrachlorobiphenyl (TCB) influences xenobiotic metabolism and GSH dependent antioxidant defenses in rainbow trout (Chapter 5). Yet there is no evidence that alteration of the cellular thiol pool concomitantly interferes with the expression and activity of phase I enzymes. The arrest of tissue glutathione synthesis by L-buthionine-[S,R]-sulfoximine (BSO) is well established to study the biochemical adaptation in mammals in a glutathione deficient and thus oxidative stress susceptible state (Leeuwenburgh & Ji 1995). GSH deficiency induces the expression of phase II genes in a non-Ah receptor mediated fashion (Shertzer *et al.* 1995), and Ireland *et al.* (1995) proposed that redox conditions regulate the DNA binding of the Ah receptor. These data suggest a possible influence of GSH on *Ah* battery genes, but no effect on CYP1A expression has yet been reported. The importance of CYP1A subfamilies in xenobiotic metabolism warrants a better understanding of the physiological conditions that modulate the expression of

these genes and their related enzymatic activities. By manipulating tissue glutathione status we examined the inducibility of the *Ah* gene battery in TCB exposed trout. We evaluated the role of two different pro-GSH agents (lipoate and GSH) versus GSH depletion in TCB induced P450 metabolism and present novel evidence that tissue thiol status regulates cytochrome P4501A function.

Materials and Methods

Chemicals

3,3',4,4'-Tetrachlorobiphenyl was purchased from Ultra Scientific (Kingstown, RI). Ethoxyresorufin, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), Bakers yeast GR, GSH, GSSG, BSO, α-lipoic acid, 1 chloro-2,4-dinitrobenzene, NADPH, uridine 5'diphosphoglucuronic acid (UDPGA), p-nitrophenol, 3-aminobenzoic acid ethyl ester (MS 222) were obtained from Sigma (St. Louis, MO) and 2-vinylpyridine was obtained from Aldrich (Milwaukee, WI). BSO was obtained from ICN (Montreal, Canada). All other chemicals were of analytical grade.

Animals

Hatchery reared, immature rainbow trout (50-80g) were obtained from Linwood Acres (Campellcroft, ON, Canada). The fish were placed in tanks supplied with dechloraminated City of Ottawa tap water and laboratory acclimated for 1 month before separating the fish randomly into 5 tanks, 15 fish per tank, supplied with the same water. Water was saturated with oxygen and maintained at 12±1°C. Fish were fed 1.5% of body weight per day, but after TCB injection feeding rates declined. Experiments were undertaken during the spring.

Exposure of fish to TCB and thiol manipulation

TCB was dissolved in corn oil heated to 60°C to obtain a stock concentration of 0.5 mg/ml. The solution was cooled to approximately 30°C and injected intraperitoneally (i.p.) at 1 mg/kg (100-160 µl); controls received equivalent volumes of corn oil. The day following the TCB injection, fish were given daily i.p. injections of saline, GSH, BSO or lipoate in the evening. Tank water levels were lowered (to approximately 40 l) and fish were mildly anaesthesized by adding 0.05 g/l MS 222 and 0.1 g/l sodium bicarbonate to the tank. All agents were made up in physiological saline (pH 6.8), except that lipoate was dissolved in 1 part 6 N NaOH and 19 parts 0.2 M sodium phosphate buffer (pH 7.0). Controls and TCB-saline trout received saline injections (125-200 µl) and TCB-GSH (GSH supplemented) trout received GSH (0.25 g/kg, 125-200 µl; pH was adjusted to 6.5-6.8 by cautious addition of NaOH just before use). BSO stock was dissolved in saline with careful heating to 30°C and injection volume was 300-480 µl (6 mmol/kg). TCB-lipoate (lipoate supplemented) fish received volumes of 100-160 µl of lipoate solution (16 mg/kg).

Trout were harvested the morning following either the 3 days or 6 days of treatment. Fish were killed by a blow to the head and blood was collected into heparinized syringes from the caudal vessels. Whole blood and plasma were acidified with 5% 5-sulfosalicylic acid and centrifuged to deproteinize. Supernatants were stored at -20°C. Organs were quickly excised, freed from blood and snap frozen in liquid nitrogen, and stored at -80°C until analyzed.

Preparation and biochemical analysis of tissue samples

Tissues were homogenized in ice-cold Tris buffer (50 mM, containing 0.25 M sucrose and 1 mM EDTA, pH 7.5) using a Potter-Elvehjem-type glass homogenizer (liver and kidney) or a Polytron PCU-2 homogenizer (Brinkmann Instruments, Rexdale, ON) (gills and white muscle).

Homogenates were centrifuged at 10,000 g for 15 min at 4°C. Supernatants were stored at -80°C (< 2 months). For glutathione measurements, frozen tissue samples were homogenized in ice-cold 5% 5-sulfosalicylic acid, centrifuged and supernatants were stored at -20°C. Biochemical analysis were conducted as described in Chapters 2 and 5. GPX was assayed as described previously (Chapter 2), but using 6 mM GSH as substrates.

RNA slot blot analysis

Total RNA was isolated from 50 mg of frozen liver tissue using a guanidinium isothiocyanate lysis, membrane binding system (RNeasy, Qiagen, Chatsworth, CA). RNA (5 µg/sample) was denatured in 50% formamide, 7% formaldehyde at 65°C for 15 minutes, then transferred to a nylon membrane using a vacuum slot manifold (Gibco/BRL, Burlington, Canada). In addition, an equivalent amount of total RNA from mouse liver was blotted on the same membrane as a negative hybridization control. Blots were probed with a ^{32}P-labelled rainbow trout CYP1A cDNA probe, synthesized from a template ,fP1-450-3', obtained from ATCC (Rockville, MD). Probes were hybridized for 1 hour at 68°C in the presence of 100 mg/ml sonicated calf thymus DNA using a rapid hybridization system (Quickhyb, Stratagene, La Jolla, CA), and washed with 0.1 X SSC, 0.25% SDS at 62°C. Blots were exposed to X-ray film for 18 hours, stripped for 5 minutes with boiling 0.1 X SSC, 0.1% SDS, and then re-exposed for
24 hours to confirm stripping. Stripped blots were re-hybridized with a ^{32}P labelled oligonucleotide probe (5'-CCGACATCGCCGCTGACCCCTGGCGCCAGTTTACGTGAGCCGATC-3') directed against the D2 region of the trout 28S RNA using the same hybridization and washing conditions as described above. Relative optical densities of the CYP1A and 28S blots were determined for each sample by densitometry scanning of the X-ray films using a Molecular Dynamics scanning

laser densitometer with Imagequant software.

Western blot analysis

Aliquots of equivalent protein content from liver homogenates of each animal were pooled for each treatment group and separated by 12% SDS-PAGE according to Laemmli (1970), then transferred to nitrocellulose membranes. Blots were incubated for 2 hours with a rabbit polyclonal anti-rat CYP1A1/2 antibody (Human Biologics, Phoenix, AZ). Antibody binding was detected by chemiluminescence using a biotinylated anti-rabbit IgG followed by a streptavidin-horseradish peroxidase conjugate (ECL, Amersham, Oakville, Canada). After development, blots were exposed to Hyperfilm ECL (Amersham).

Statistical analysis

Significance of changes in means of same day groups was tested with one-way analysis of variance (ANOVA), using the software Statistix 4.1. The location of significance was determined using the Least Significant Difference method (LSD). Significant differences between 3 days and 6 days of each treatment (control, saline, GSH, BSO and lipoate) were tested using the Student's t test for unpaired sample means.

Results

Injection of TCB and treatments resulted in only 1 mortality in the lipoate supplemented group (6 d). Otherwise no fish showed any sign of distress as a result of the daily injections.

GSH status

GSH supplementation. Following 3 days of GSH supplementation, GSH levels in trout significantly increased in liver (3.6-fold), kidney (2.1-fold) and plasma (6.5-fold) compared with the TCB-saline treated group (Table 7.1). Consistent effects were observed in TGSH results; blood TGSH levels were 1.3-fold higher in GSH supplemented compared with TCB-saline treated fish. Tissue GSH and TGSH contents did not differ between controls, TCB-saline and lipoate treated fish. However, plasma GSH content was significantly higher in TCB-saline and lipoate supplemented fish than in controls.

Following 6 days of GSH supplementation, GSH levels were significantly elevated in liver (2.5-fold), kidney (2.1-fold), gill (1.5-fold), muscle (2-fold) and plasma (8.9-fold) compared with TCB-saline injected fish. GSH and TGSH levels remained unaffected in controls, TCB-saline and TCB-lipoate treated groups for all tissues. But GSH levels in liver, gill or muscle were significantly different in TCB-saline and lipoate supplemented trout compared with controls.

GSH deficiency. BSO treatment for 3 days resulted in multifold decreases of tissue GSH levels. Compared with TCB-saline trout, GSH concentration in GSH deficient fish were decreased in liver 4.1-fold, kidney 2.8-fold, gill 1.7-fold, muscle 5.8-fold and blood 1.8-fold. Following the 6 day BSO treatment, tissue GSH levels further decreased. Tissue GSH content was 1.2- to 2.1-fold lower following 6 days than 3 days of BSO treatment. White muscle was the only tissue not to show continued decreases in GSH and TGSH content following 6 days of BSO treatment.

Lipoate supplementation. Lipoate supplementation did not significantly influence tissue

Table 7.1. Glutathione status in different tissues of TCB exposed rainbow trout

a) concentrations in liver, kidney and gills

	control	TCB-saline	TCB-GSH	TCB-BSO	TCB-lipoate
liver					
3 days					
GSH	1423±176	1098±120[2]	3957±811[##]	270±60[###]	1091±203
GSSG	65±21	84±15	148±53[a]	5±1[###]	47±11[a]
TGSH	1554±201	1266±127[2]	4252±859[##]	280±60[###]	1186±218
ratio	44±12	79±17[#1]	37±11	23±7.9	43±8
6 days					
GSH	1136±140[a]	1715±88[a]	4214±637[###]	126±36[###]	1483±105
GSSG	50±10	67±8	212±42[###]	3±0.3[###]	47±4
TGSH	1236±153[a]	1850±88[a]	4638±716[###]	132±36[###]	1576±100
ratio	44±7	40±6	50±4[*]	31±6[■]	33±4[■]
kidney					
3 days					
GSH	1674±147	1826±169	3762±344[###]	651±36[###2]	1728±242
GSSG	85±13[2]	72±10[a1]	111±9[a]	6±0.4[###]	32±5[##]
TGSH	1844±156	1970±182	3986±357[###]	663±36[###2]	1792±243
ratio	52±9[*1]	39±5	30±2[■]	10±5[##]	20±4[#]
6 days					
GSH	2051±27	1897±229	3942±445[###]	426±9.8[###]	1630±176
GSSG	36±2	44±4[a]	122±8[###]	6±0.4[###]	30±5[a]
TGSH	2122±26	1986±228	4185±445[###]	438±10[###]	1690±182
ratio	17±9[■]	26±5[a]	32±4[*]	14±3[■a]	18±2[■]
gill					
3 days					
GSH	1267±45[a]	1387±79	1677±153[a]	808±57[##2]	1360±164
GSSG	36±4[*]	38±4[**1]	80±9[###]	16±3[■1]	29±5[*]
TGSH	1339±48	1462±83	1836±161[#]	840±60[#2]	1418±171
ratio	28±3	27±3	48±5[##]	20±3	22±2
6 days					
GSH	1003±128	1188±62	1783±49[#]	501±48[###]	1445±53[#]
GSSG	29±5	24±3	66±15[#]	5±2[###]	29±0.4
TGSH	1061±139	1236±67	1915±50[##]	512±50[###]	1502±52[#]
ratio	28±3	20±2[■]	37±8[*]	10±4[##]	20±10[■]

b) concentrations in white muscle, blood and plasma

	control	TCB-saline	TCB-GSH	TCB-BSO	TCB-lipoate
muscle					
3 days					
GSH	74±13^{2}	81±33	97±20^{a}	14±4$^{\#\#2}$	40±7^{a1}
GSSG	2±0.2*	1±0.2$^{\blacksquare}$	7±0.5$^{\#\#}$	0.6±0.03$^{\blacksquare}$	1±0.3$^{\blacksquare}$
TGSH	78±13^{2}	83±33	111±20^{a}	15±4$^{\#\#2}$	42±7^{a1}
ratio	31±9$^{\blacksquare}$	25±7$^{\blacksquare a}$	78±12*	60±13^{ab2}	29±8$^{\blacksquare b2}$
6 days					
GSH	132±7*	81±21$^{\blacksquare}$	162±22^{**a}	39±6$^{\blacksquare b}$	99±18^{ab}
GSSG	2±0.5*	2±0.3*	8±1.9$^{\#\#\#}$	0.4±0.1$^{\blacksquare}$	1±0.2$^{\blacksquare}$
TGSH	135±6.8^{ab}	84±20$^{\blacksquare a}$	177±24**	40±6$^{\blacksquare bc}$	101±18$^{\blacksquare c}$
ratio	16±4$^{\blacksquare}$	29±9^{a}	48±10*	13±3$^{\blacksquare}$	8±1$^{\blacksquare a}$
blood					
3 days					
GSH	756±96$^{\blacksquare}$	820±97	1054±98*	455±54$^{\#}$	765±90$^{\blacksquare}$
GSSG	279±35**	207±23**	278±35**	118±19$^{\blacksquare}$	109±9$^{\blacksquare}$
TGSH	1313±73*	1234±132^{ab}	1610±154^{*a}	690±83$^{\blacksquare b}$	982±84$^{\blacksquare}$
ratio	407±89	258±24	266±23	262±38	152±23$^{\#}$
6 days					
GSH	803±120$^{\blacksquare}$	871±170	1225±126*	371±25$^{\#\#}$	681±87$^{\blacksquare}$
GSSG	242±16**	169±14^{*a}	373±66^{**a}	109±14$^{\blacksquare}$	96±14$^{\blacksquare}$
TGSH	1287±123*	1209±179^{a}	1970±163$^{\#\#}$	588±34$^{\blacksquare a}$	873±96$^{\blacksquare}$
ratio	331±54*	223±44	321±71*	300±45*	146±24$^{\blacksquare}$
plasma					
3 days					
GSH	26±2.7$^{\blacksquare}$	42±4.8*	275±38$^{\#\#\#}$	22±2.5$^{\blacksquare 2}$	54±9.1*
GSSG	7±1.0	6±1.3^{1}	229±30$^{\#\#\#}$	1±0.2$^{\#}$	3±1.8$^{\#}$
TGSH	40±4.3	54±6.2	733±76$^{\#\#\#}$	24±2.5$^{\#2}$	61±12
ratio	276±28$^{\#2}$	148±35$^{\#1}$	874±147$^{\#\#}$	44±12	49±19
6 days					
GSH	36±4.6	38±1.9	339±69$^{\#\#\#}$	11±1.0$^{\#\#\#}$	41±5.7
GSSG	5±0.7$^{\#}$	2±0.5	309±76$^{\#\#\#}$	1±0.2$^{\#}$	3±0.9
TGSH	47±5.3	42±2.1	957±218$^{\#\#\#}$	12±0.9$^{\#\#\#}$	47±6.6
ratio	152±22$^{\#}$	58±12	895±80$^{\#\#\#}$	76±23	62±17

Values are means ± SEM, n=5 (except TCB/BSO at 6 days: n=4), and are expressed in nmol/g wet weight for all tissues, except blood and plasma are expressed in µmol/l. GSH: reduced glutathione, GSSG: oxidized glutathione, TGSH: total glutathione (GSH + 2GSSG), ratio: 1000*GSSG/GSH. $^{\#\#\#}$P<0.001, $^{\#\#}$P<0.01, $^{\#}$P<0.05, significantly different from all other groups of the same day. **P<0.01, *P<0.05, significantly different from $^{\blacksquare}$-labeled groups of the same day; ^{a,b,c}P<0.05: significantly different from same labeled group of the same day; ^{1}P<0.05, ^{2}P<0.01: significantly different from 6 day treatment.

GSH content. TCB-lipoate supplemented fish did, however, maintain a favourable GSH redox status with lower tissue GSSG/GSH ratios than in other TCB groups and controls. GSSG/GSH ratios were particularly low in liver, kidney, muscle, blood and plasma following 3 days and 6 days of lipoate supplementation.

Tissue GSSG/GSH ratios in response to TCB treatment. GSSG/GSH ratios in TCB-saline fish were significantly higher in liver and plasma than in all other TCB treatment groups following the 3 days ($p<0.05$), but not the 6 days treatment with saline. GSSG/GSH ratios in liver, kidney and gill were the lowest in GSH deficient trout compared with all other groups for the 3 days and 6 days of thiol manipulations. In muscle and blood, GSSG/GSH ratios were significantly higher in GSH deficient than in lipoate supplemented fish. In general, BSO and lipoate treated fish had the lowest GSSG concentrations compared with controls, TCB-saline and TCB-GSH treatment groups. GSSG content in GSH supplemented trout were significantly higher in most tissues than in any of the other TCB groups or controls. Plasma GSSG levels in GSH supplemented fish were markedly increased, resulting in a (1000*) GSSG/GSH ratio of 874-895, 6- and 15-fold higher than in TCB-saline treated trout.

Glutathione redox cycle

GPX activities in tissues of GSH supplemented fish were elevated following 3 days of GSH treatment (Table 7.2). GPX activities in all tissues were significantly higher in GSH supplemented than in GSH deficient trout ($p<0.05$) following 3 days and 6 days of GSH manipulations. Gill GPX activity significantly increased in TCB-saline treated fish from 3 days to 6 days of saline treatment ($p<0.01$) and was highest compared with other groups following 6 days of treatment ($p<0.05$). However, gill GPX activity in lipoate supplemented trout

Table 7.2. Redox regulatory enzymes in various tissues of TCB exposed rainbow trout

	control	TCB-saline	TCB-GSH	TCB-BSO	TCB-lipoate
GPX					
liver					
3 days	104±7.3[2]	117±6	120±4[*]	95±6.0[■]	112±11
6 days	70±6.4[■]	104±4[*]	130±14[**]	71±6.9[■]	105±13[*]
kidney					
3 days	345±14	289±19	294±17	199±17[#]	285±34
6 days	305±12[*]	263±18	279±9	241±20[■]	247±17[■]
gill					
3 days	231±12[■ac1]	275±10[■b2]	382±39[ab1]	302±27[■c]	447±40[*1]
6 days	313±22[■]	575±45[*]	499±21[*]	361±24[■]	332±22[■]
muscle					
3 days	33.6±3.7[*2]	33.9±2.3[*2]	32.2±1.0[*]	24.3±0.8[■]	25.9±1.5[■]
6 days	19.2±2.0[■a]	22.1±1.4[■]	33.4±3.3[*]	27.5±2.5[a]	26.5±0.8[■]
GR					
liver					
3 days	11.0±0.1	12.5±0.7	12.8±1.7	11.3±0.4	10.8±0.5
6 days	10.5±0.7[■]	11.2±0.7	11.5±1.0	10.8±0.6	13.6±1.1[*]
kidney					
3 days	35.1±2.0[a]	29.2±1.5[■]	39.2±2.0[*]	27.4±1.7[■a]	29.9±4.0[■]
6 days	34.8±2.4	31.3±2.3	32.5±2.6	30.0±1.7	30.9±1.1
gill					
3 days	13.7±0.9[■a]	14.5±1.2[■2]	22.0±4.2[a]	17.4±1.3[■2]	25.3±2.4[*]
6 days	13.5±0.8[■]	38.4±0.5[#]	21.4±2.3[*]	28.7±2.0[#]	22.2±1.8[*]
muscle					
3 days	2.7±0.25[ab2]	2.2±0.23[■c]	3.1±0.17[*1]	2.0±0.17[■a]	1.6±0.17[■bc1]
6 days	1.6±0.09[■a]	2.2±0.19	2.3±0.26[a]	1.7±0.18[■]	2.5±0.26[*]

Values are means ± SEM (nmol*min^{-1}*mg protein^{-1}), n=5 (except TCB-BSO at 6d n=4).

GPX, glutathione peroxidase, GR, glutathione reductase. [#]P<0.05, significantly different from all other groups of the same day.[*]P<0.05, significantly different from [■]-labeled groups of the same day.[a,b,c]P<0.05, significantly different from same-labeled groups of the same day.[1]P<0.05, [2]P<0.01, significantly different from 6 day treatment.

decreased from 3 days to 6 days of lipoate supplementation ($p<0.05$).

Gill GR activities in TCB-saline and GSH deficient fish were significantly increased from 3 days to 6 days of saline and BSO treatments ($p<0.01$). As observed for GPX activities, GR activities were also highest in GSH rich tissues following the 3 days of GSH supplementation. GR activities in other TCB groups and controls remained for most tissues unchanged. In general, GPX and GR activities increased more rapidly in tissues of GSH supplemented fish compared with the other TCB groups, showing higher activities by 3 days of GSH supplementation. In contrast, tissue GPX and GR activities in GSH deficient fish were low compared with other TCB groups following 3 days and 6 days of BSO treatment.

Ah gene battery

Phase I enzymes. Hepatic EROD activity was 17- and 12.5-fold higher in TCB-saline trout (3 d and 6 d, respectively, $p<0.01$) than in controls (Table 7.3). This induction in EROD activity was markedly potentiated in the GSH rich livers by 2.7-fold (3 d and 6 d, $p<0.01$ and $p<0.05$, respectively) compared with TCB-saline fish. EROD activity in GSH rich livers was thus 46- and 34-fold higher (3 d and 6 d) than in controls. This effect was fully reversed in the GSH deficient livers as activities were 19- and 13-fold lower than in GSH supplemented trout, but still 2.4- and 2.5-fold higher than in controls (3 d and 6 d, $p<0.05$). TCB induced EROD activity was also lower in lipoate supplemented fish than in TCB-saline treated trout. EROD activities were 4.1- and 7.3-fold (3 d and 6 d, $p<0.01$ and $p<0.05$, respectively) higher than in controls. A similar trend was observed in the kidney. Renal EROD activities were significantly higher [2.4- (3 d) and 4.8- (6 d) fold] in TCB-saline ($p<0.01$ and $p<0.05$, respectively), 7.6- (3 d) and 5.6- (6 d) fold in GSH ($p<0.001$ and $p<0.01$), 2.2- (3 d) and

Table 7.3. Phase I and II enzyme activities in TCB exposed rainbow trout

	control	TCB-saline	TCB-GSH	TCB-BSO	TCB-lipoate
GST					
liver					
3 days	184±9	201±4*	165±10$^{\blacksquare}$	167±9$^{\blacksquare}$	159±9$^{\blacksquare}$
6 days	203±12*	162±19$^{\blacksquare}$	167±7	156±13$^{\blacksquare}$	176±3
kidney					
3 days	201±21	150±13^{1}	197±25	183±9	186±18
6 days	182±15	205±11	202±12	196±51	224±10
gill					
3 days	227±21	181±12$^{\blacksquare 3}$	309±61*	278±31*	213±16^{2}
6 days	284±63$^{\blacksquare}$	410±23*	313±42	320±46	364±32
muscle					
3 days	27.8±2.4^{*2}	25.2±2.3^{1}	23.9±1.2	22.9±1.8	22.1±1.0$^{\blacksquare}$
6 days	17.0±1.1$^{\blacksquare}$	19.0±1.1	20.8±1.8	20.8±1.8*	20.2±0.7
EROD					
liver					
3 days	2.9±0.3$^{\blacksquare}$	50±19.6$^{\#\#}$	133±41$^{\#\#}$	7.1±1.5*	12.0±2.6**
6 days	4.0±0.9$^{\#\blacksquare}$	50±13.3**	134±44$^{\#**}$	10.1±2.3$^{\blacksquare}$	29.3±14.2$^{\blacksquare}$
kidney					
3 days	5.5±0.8$^{\blacksquare}$	13.4±2.2^{**1}	41.7±5.0$^{\#\#\#}$	4.5±0.8$^{\blacksquare}$	12.1±3.2*
6 days	5.3±0.6$^{\blacksquare b}$	25.6±4.1*	29.9±5.5**	6.5±0.8$^{\blacksquare a}$	14.2±1.8$^{\blacksquare ab}$
UDPGT					
liver					
3 days	468±58^{*1}	328±37	403±83*	219±17$^{\blacksquare 2}$	251±34$^{\blacksquare 1}$
6 days	238±54$^{\blacksquare}$	256±36$^{\blacksquare}$	395±31*	369±32	418±56*
kidney					
3 days	79±3.9^{3}	93±6.8^{*2}	81±7.4	56±6.6$^{\blacksquare}$	80±15
6 days	42±3.0	50±7.8	61±6.5	49±20	51±7

Values are means ± SEM, n=5 (except TCB/BSO at 6 days: n=4), GST, glutathione S-transferase (nmol*min^{-1}*mg protein^{-1}), EROD, ethoxyresorufin O-deethylase (pmol*min^{-1}*mg protein^{-1}), UDPGT, UDPglucuronosyltransferase (pmol*min^{-1}*mg protein^{-1}). $^{\#\#\#}P<0.001$, $^{\#\#}P<0.01$, $^{\#}P<0.05$, significantly different from all other groups of the same day; $^{**}P<0.01$, $^{*}P<0.05$, significantly different from $^{\blacksquare}$-labeled groups of the same day. $^{a,b,c}P<0.05$, significantly different from same labeled group of the same day. $^{1}P<0.05$, $^{2}P<0.01$, $^{3}P<0.001$, significantly different from 6 day treatment.

2.7- (6 d) fold in lipoate supplemented trout ($p<0.05$) compared with controls. EROD induction did not differ between lipoate supplemented and TCB-saline treated fish after 3 days, but was significantly lower after 6 days of lipoate supplementation ($p<0.05$) than in TCB-saline treated trout. However, kidney EROD activity in GSH deficient tissues was not elevated compared with controls following 3 days or 6 days of BSO treatment.

Phase II enzymes. Tissue GST activities were not influenced by TCB, and also not by the different thiol supplementations (Table 7.3). GST activities significantly increased in the kidney, gills and muscle of TCB-saline, and in gills only of lipoate supplemented fish from the 3 days to 6 days of saline and lipoate supplementation. GST activity in gill of TCB-saline treated fish was significantly higher than controls following the 6 day saline treatment ($p<0.05$).

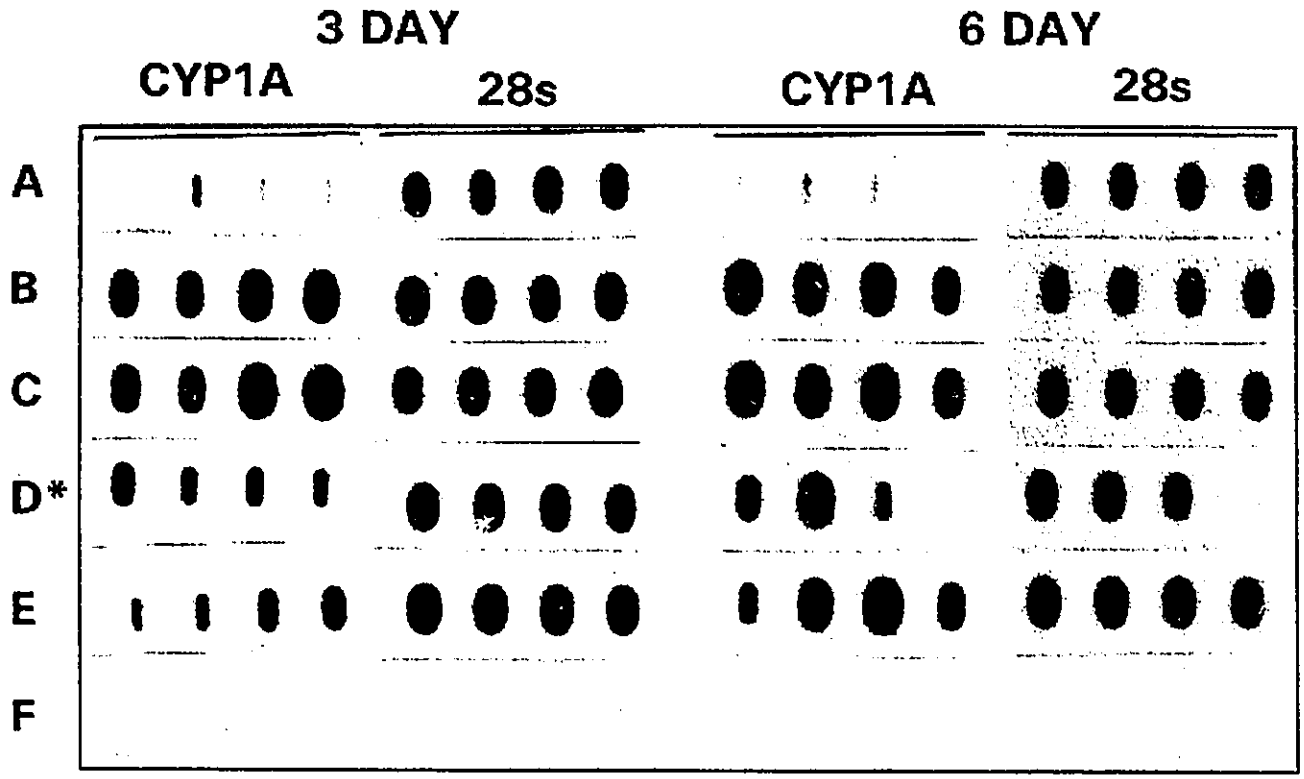
UDPGT activities in liver and kidney were unaffected by TCB exposure (Table 7.3). However, following 3 days of thiol manipulation, hepatic UDPGT activities were significantly higher in GSH supplemented trout compared with GSH deficient and lipoate supplemented groups ($p<0.05$). Following 6 days of thiol supplementation, hepatic UDPGT activities were significantly higher in GSH and lipoate supplemented groups than in controls and TCB-saline treated fish ($p<0.05$).

CYP1A expression. TCB induced CYP1A mRNA expression severalfold in TCB-saline and GSH supplemented groups following 3 days and 6 days of saline and GSH treatment as indicated by an increased signal intensity on slot blots in these treatments (Fig. 7.1). In contrast, GSH deficient and lipoate supplemented fish show suppressed TCB induced CYP1A mRNA expression following 3 days of thiol manipulation. 28S RNA signal intensities were

FIG. 7.1. CYP1A versus 28S RNA levels.

a) 5 µg samples of total RNA extracted from livers of 3 or 6 day treated animals were immobilized on nylon membrane using a vacuum slot blot apparatus. A) controls, B) TCB-saline, C) TCB-GSH, D) TCB-BSO, E) TCB-lipoate and F) negative control RNA (5 µg from mouse liver). *: indicates n=3 for TCB/BSO at 6 days. Blots were probed with a ^{32}P-labelled CYP1A cDNA probe, and exposed to x-ray film for 18 hours. Blots were then stripped, re-exposed to confirm stripping, probed with a ^{32}P-labeled oligonucleotide directed against the D2 region of the trout 28S RNA, and exposed for 6 hours.

b) RNA levels were quantified by laser densitometry scanning of x-ray films to obtain CYP1A/28S ratios. Error bars indicate standard error of means for each group. #P<0.01, ##P<0.001, significantly different from all TCB-treatments. *P<0.05, **P<0.01, significantly different from ~-labeled groups.


3 DAY
6 DAY
CYP1A
28s
CYP1A
28s
A
B
C
D*
E
F

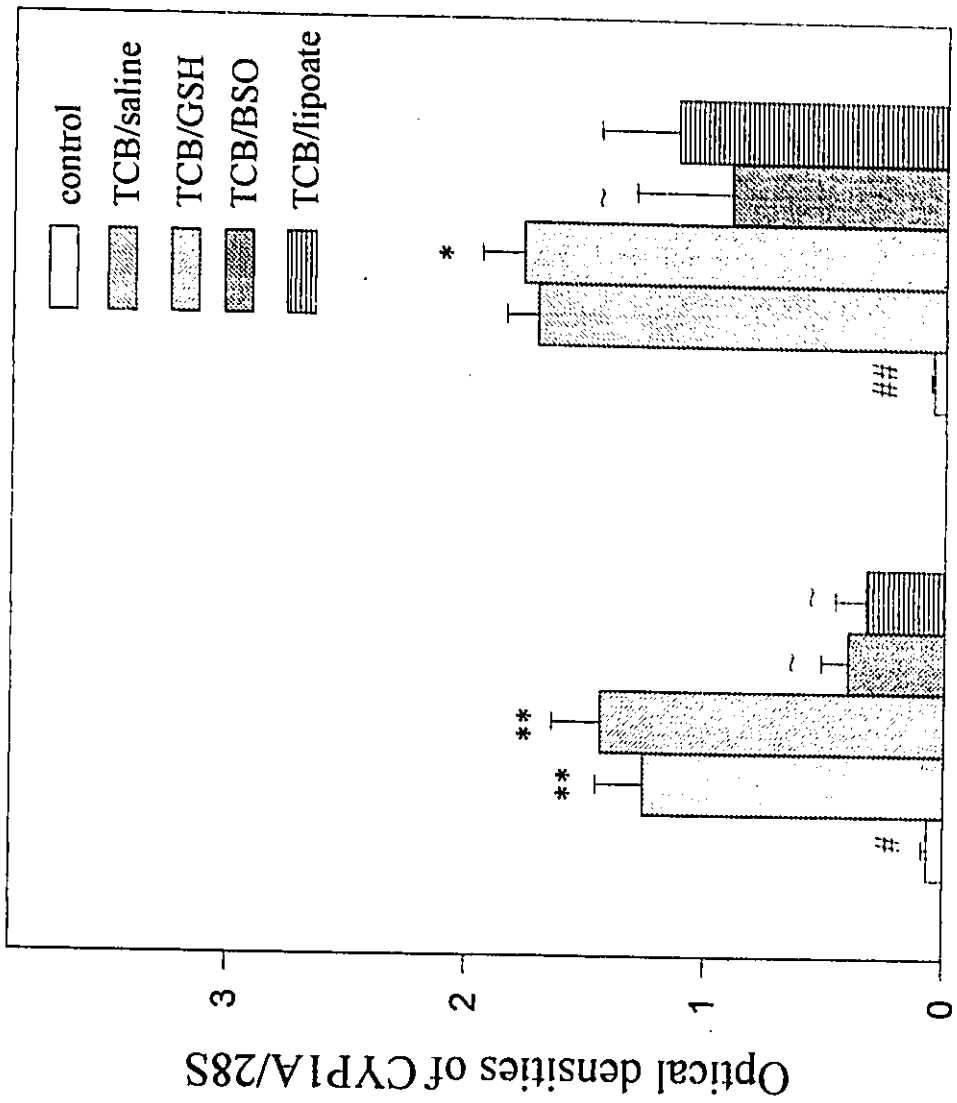

control
TCB/saline
TCB/GSH
TCB/BSO
TCB/lipoate
Optical densities of CYP1A/28S
0
1
2
3
3d
6d
#
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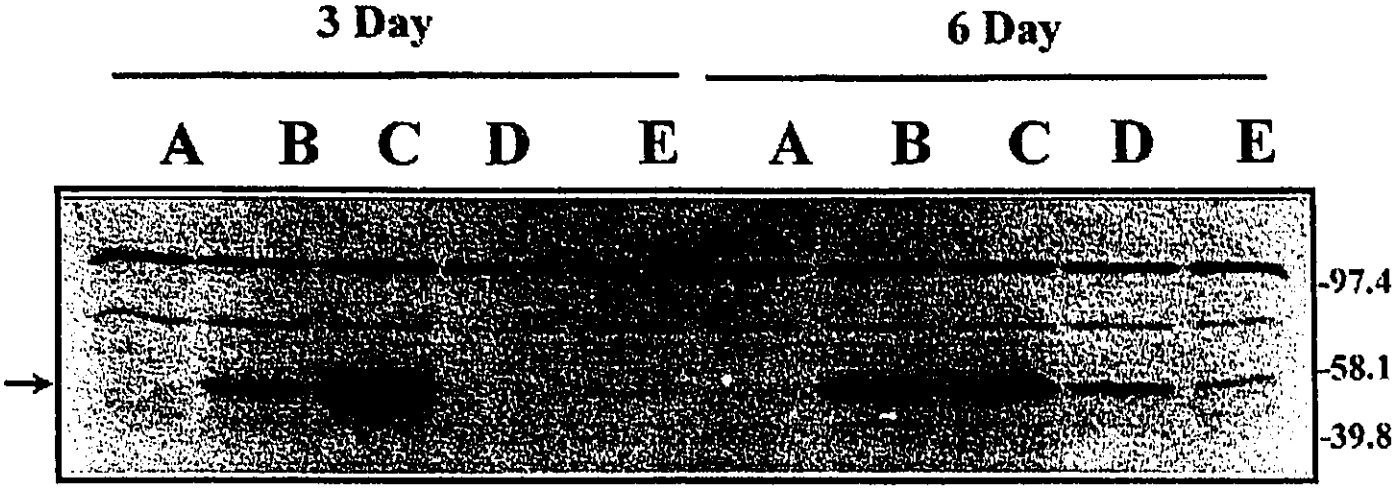
Fig. 7.1b

FIG. 7.2. Western blot of anti-rat CYP1A polyclonal antibody immunoreactive protein from trout liver.

Liver homogenates of 4 animals per treatment group were pooled and 20 μg of protein per group was separated by SDS-PAGE and electroblotted to nitrocellulose membranes. Blots were probed with polyclonal anti-rat CYP1A from rabbit and detected by chemiluminescent exposure of x-ray film. A) controls, B) TCB-saline, C) TCB-GSH, D) TCB-BSO and E) TCB-lipoate. The arrow indicates the band which co-migrates with rat CYP1A. Numbers at right indicate molecular weight standards in kDa.


3 Day
6 Day
A B C D E A B C D E
-97.4
-58.1
-39.8

significantly weaker in controls and significantly stronger in lipoate supplemented fish ($p<0.05$) following 3 days and 6 days than in the other TCB treatment groups (data not shown). The optical density ratio of CYP1A/28S revealed a 18- and 21-fold induction in TCB-saline and GSH rich livers and a 5.9- and 4.7-fold induction in GSH deficient and lipoate supplemented livers versus controls ($p<0.01$) following 3 days of thiol manipulation. This CYP1A/28S RNA density ratio was significantly higher in TCB-saline and GSH supplemented fish than in GSH deficient and lipoate supplemented groups ($p<0.01$). Following 6 days of thiol manipulation, the CYP1A/28S density ratio further increased to 34- and 35-fold in TCB-saline and GSH rich livers and 18- and 22.6-fold in GSH deficient and lipoate supplemented groups compared with controls ($p<0.001$). Only CYP1A/28S density ratios were significantly higher in GSH rich than GSH deficient livers (6 d, $p<0.05$).

Significant expression of CYP1A protein was revealed by Western blot analysis in TCB-saline treatment group which was potentiated in the GSH supplemented trout following 3 days of saline or GSH supplementation (Fig. 7.2). CYP1A protein was only weakly expressed in GSH deficient and lipoate supplemented fish. Following 6 days of thiol manipulations, CYP1A protein expression was enhanced to a similar extent in TCB-saline and GSH supplemented groups, and were more strongly expressed in GSH deficient and lipoate supplemented trout at 6 days than at 3 days of BSO and lipoate supplementation.

Discussion

GSH status

Intraperitoneally administered GSH served as an effective delivery agent of GSH to

tissues of rainbow trout. Uptake of GSH is known to be catalyzed by γ-glutamyl-transpeptidase (GGT, EC 2.3.2.2), a membrane bound enzyme with cysteine as preferred amino acid acceptor. Mammalian and piscine kidney GGT activities are known to be severalfold higher than in other tissues (Leeuwenburgh & Ji 1995; Sen *et al.* 1992; Chapter 4), yet efforts to increase intracellular GSH concentrations by administration of exogenous GSH have met with little or no success in mammals. Repeated daily i.p. injections with GSH resulted only in significant uptake of GSH in kidney and blood of rats, while there was no effect in liver, muscle, heart and lung (Sen *et al.* 1994).

To overcome the lack of response to exogenous GSH in mammals, supplementations with N-acetylcysteine or lipoate have been used to bolster tissue GSH pools. Lipoate has been reported to increase tissue GSH levels in mice with liver concentrations enhanced by 2- to 3-fold (Busse *et al.* 1992). Packer *et al.* (1995) has emphasized the role of lipoate as an antioxidant and its ability to protect other antioxidants, leading to increases in cellular glutathione concentrations. However, lipoate itself did not increase tissue GSH levels in TCB treated trout in the present study, but GSSG concentrations and GSSG/GSH ratios were lower than in other groups, perhaps suggesting a sparing of GSH oxidation (Table 7.1). In tissues, lipoate is rapidly reduced to dihydrolipoate (Packer *et al.* 1995). The dihydrolipoate-lipoate couple has a lower reduction potential (-0.32 v) than the GSH-GSSG couple (-0.24 v). Thus dihydrolipoate may directly reduce GSSG to GSH and in this way decrease the tissue GSSG/GSH ratio. BSO decreases tissue GSH content by inhibiting γ-glutamylcysteine synthetase (GCS, EC 6.3.2.2), the rate limiting enzyme of glutathione synthesis (Meister 1991).

Effect of TCB on antioxidant enzymes

Exposure of rats and fish to PCB mixtures is known to increase hepatic and muscle lipid peroxidation (Yamamoto *et al.* 1994; Rudneva-Titova & Zherko 1994). A significant correlation of PCB accumulation in fish tissues with lipid peroxidation has been shown (Rudneva-Titova & Zherko 1994). We observed a direct effect of TCB on glutathione redox enzymes which was further influenced by the various treatments. Increased substrate availability correlated positively with GPX activities in liver, gill and muscle of GSH rich tissues, while GSH deficiency decreased activities of GPX in most tissues (Table 7.2). GSH deficiency in rats has been shown to activate hepatic and renal GR activities, but decreased GPX activity in liver by over 25% (Leeuwenburgh & Ji 1995). The most prominent response of antioxidant enzymes in the various TCB-treatments were noted in the gills and may be linked to the specific function of this organ in fish. Mommsen (1984) reported that the metabolic activity of gills, as manifested by the high oxygen uptake, surpasses those of other tissues. Because gill metabolism contributes to excretion of hormones and possibly also toxicants (Mommsen 1984), the activation of redox enzymes in all TCB injected fish implies a prime involvement of the gill in the detoxication of TCB.

We have reported a significant activation of antioxidant enzymes in rainbow trout injected with 5 mg/kg TCB and studied 6 weeks post injection (Chapter 5). Our present results show marginally lower activation of GPX and GR in response to TCB exposure. As observed with increased mRNA expression of CYP1A in GSH deficient and lipoate supplemented groups following 3 days to 6 days of thiol manipulations, several metabolic processes may be suppressed after TCB injection, but appear activated with time, possibly depending on other factors e.g. cellular thiol status (Chapter 5; present data). The half-life of TCB is 7 days at a concentration of 0.8 mg/kg in this species (Stalling *et al.* 1979; Gilbert *et al.* 1995) and may play a role in the

activation of detoxication processes. Also, GSSG/GSH ratios were similar in most tissues of TCB-saline and control groups. GSSG/GSH ratios and GPX activities were lower in most GSH deficient tissues than in other TCB groups and controls. It thus appears that following drastic GSH depletion the stability of the cellular redox status is important and GPX is not activated despite exposure to TCB. GSH deficiency resulted in low GSSG content and downregulated GPX activity. This reflects a type of homeostatic adaptation that has been previously discussed by Leeuwenburgh & Ji (1995).

TCB effect on Ah receptor mediated processes

Exposure of rats and rat hepatocytes to TCDD and PCB congeners resulted in increased enzyme activities and expression of *Ah* battery genes (Dragnev *et al.* 1995; Xiao *et al.* 1995). Hahn and coworkers (Hahn *et al.* 1994; Hahn & Karchner 1995) suggested that the AhR signal transduction pathway appeared early in vertebrate evolution by demonstrating sequence homologies between Ah receptors in mammals and fish that show highly conserved regions. Fish, like mammals, are very sensitive to TCDD and PCB congeners by induction of Ah battery enzymes (Gooch *et al.* 1989; Chapter 5). TCB exposure induced phase I (EROD activity), but not phase II enzymes. Xiao *et al.* (1995) reported that glucocorticoids have both a positive and a negative regulatory effect on the subsets of the *Ah* gene battery, indicating different regulation of the various battery genes. Such studies further emphasize the need to clarify the physiological conditions that influence the expression of these genes.

The finding that tissue thiol status modulates the genetic expression of TCB induced CYP1A is novel in xenobiotic metabolism. Thiols appear to influence CYP1A expression at different steps of the cascade. While GSH deficiency and lipoate supplementation acted by transcriptional

depression of the TCB induced CYP1A gene, tissue GSH enrichment does not appear to affect transcription (Fig. 7.1). CYP1A protein expression in the GSH rich livers suggest posttranscriptional or translational modifications according to the 3 day results, although the Western blot data following 6 days do not exclude the possibility of a posttranslational potentiation by GSH (Fig. 7.2). These data again suggest a time-dependent difference in the TCB response.

In contrast to the effects of exogenous GSH is the suppression of TCB induced CYP1A transcription and CYP1A protein expression in GSH deficient tissues. Drew and Miners (1984) demonstrated that the administration of BSO in mice did not alter CYP1A catalytic activity and pointed out its usefulness in studies focusing on the effect of GSH depletion on xenobiotic-induced toxicities. According to the results reported by Nebert and coworkers (Shertzer *et al.* 1995), GSH depletion by BSO activates *Ah* battery phase II enzymes at the pre-translational level. This activation, however, does not appear to be Ah receptor mediated, but most likely acts through the electrophilic response element (EpRE) of the *Ah* gene battery and therefore does not affect CYP1A (Shertzer *et al.* 1995). This group has further suggested that AhR or related proteins participate in both the TCDD/TCB inducible AhRE mediated response and the oxidative stress inducible EpRE mediated response (Vasiliou *et al.* 1995). These reports in combination with the present data provide compelling evidence that tissue GSH status is a sensitive determinant of *Ah* battery gene expression.

We had previously hypothesized that GSH alone modifies CYP1A metabolism (Otto *et al.* 1996a). In view of the present results, it is likely that the GSH depletion *per se* does not lead to the suppression of TCB induced CYP1A expression. Lipoate injected fish had similar GSH

concentrations as did TCB-saline treated individuals, yet CYP1A induction in lipoate supplemented trout showed a similar pattern as in GSH depleted trout. Thus the CYP1A inhibition in lipoate supplemented trout was not dependent on low GSH content. GSSG concentrations and the GSSG/GSH ratio in most tissues of lipoate supplemented and GSH deficient trout were lower than those in TCB-saline and GSH supplemented groups. GSSG in the presence of GSH has been reported to activate protein kinase C activity (Kass *et al.* 1989). Other studies have postulated that GSSG modulates the activation of transcription factors such as NFκB and AP-1 (Galter *et al.* 1994; Sen & Packer 1996). The *in vitro* binding of the Ah receptor has been shown to be regulated by redox conditions by testing Ah receptor binding under oxidant conditions and the restoration by reducing agents including dithiothreitol (Ireland *et al.* 1995). Such observations raise the possibility of a regulatory role of oxidized thiols in cellular signal transduction pathways. Whether GSSG modulates TCB induced CYP1A transcription is yet unresolved.

Stegeman and coworkers (Gooch *et al.* 1989; Hahn *et al.* 1993) have described TCB as a potent CYP1A inducer at low concentrations in fish *in vivo* and *in vitro*, but TCB concentrations above 0.1 mg/kg or 0.1 μM inhibited EROD induction. This suppression of EROD activity occurred in the presence of significantly elevated CYP1A mRNA (Gooch *et al.* 1989) and/or CYP1A protein (Hahn *et al.* 1993). A similar inhibitory effect on CYP1A catalytic activities by PCB congeners has been reported for mammalian systems (Hahn *et al.* 1993). Our previous study found that compounds in unbleached pulp mill effluents can lead to a similar suppression of EROD activity, although tissue content in CYP1A protein increased (Otto *et al.* 1994), making this inhibition of EROD activity by such environmental toxicants a relevant toxicological issue.

Some tetrachlorinated dibenzofurans (TCDFs) such as 2,3,7,8-TCDF, induce scup CYP1A with good correlation of RNA, protein and catalytic activity, whereas 2,3,6,8-TCDF lack such induction potencies at the transcriptional level (Hahn & Stegeman 1994). The structure-activity relationship of toxicants certainly determine the inducibility of CYP1A, but the present results indicate that alteration of the tissue redox status modulates the inducibility and catalytic activity of this cytochrome P450.

In conclusion, exogenous glutathione is an efficient delivery agent of GSH to tissues in rainbow trout. Active manipulations of tissue glutathione status in TCB induced fish with either GSH, BSO or lipoate resulted in specific biochemical responses. Activities of glutathione redox cycle enzymes were significantly higher in GSH supplemented than in GSH deficient tissues of TCB exposed trout. TCB induced CYP1A gene expression and catalytic activity were significantly altered in the different thiol manipulated systems and correlated with tissue glutathione status. This paper presents novel evidence suggesting that tissue glutathione status is a regulator of TCB induced cytochrome P4501A function.

CHAPTER 8.

REGULATION OF CYTOCHROME P4501A METABOLISM BY GLUTATHIONE

Introduction

Polychlorinated biphenyls (PCBs) are environmental pollutants with potential carcinogenic properties. The biochemical effects of various PCB congeners, including 3,3',4,4'-tetrachlorobiphenyl (TCB), are primarily initiated by ligand binding of the biphenyl to the cytosolic Ah receptor (AhR) and subsequent gene expression of cytochromes P450 and conjugation (phase II) enzymes (Safe 1994). Ligand binding of cytosolic AhR is followed by the translocation of the complex to the nucleus where it heterodimerizes with the Ah receptor nuclear translocator (ARNT) protein (Okey *et al.* 1994b). In the form of a heterodimeric complex AhR and ARNT are known to bind to enhancer sequences termed aromatic hydrocarbon responsive elements (AhREs) or xenobiotic responsive elements that are located in the 5'-flanking region of cytochromes P4501A1 and P4501A2 (CYP1A1 and CYP1A2) and several phase II genes (Nebert 1994). The DNA binding of AhR/ARNT and subsequent CYP1A1 induction has been well characterized (Bacsi & Hankinson 1996; Dong *et al.* 1996).

CYP1A1 is induced primarily at the transcriptional level. The induction system has been described as a model for analyzing mammalian gene transcription (Whitlock *et al.* 1996). Similar to mammals, in fish ligand binding of AhR induces CYP1A. Several forms of piscine CYP1A cloned bear closer resemblance to mammalian CYP1A1 than CYP1A2 (Morrison *et al.* 1995; Hahn & Chandran 1996). Induction of CYP1A enhances lipophilic xenobiotic metabolism,

however, often the produced metabolites are more toxic and/or mutagenic than their precursors (Nebert 1994). Recently it has been shown that toxicant-induced CYP1A1 metabolic activity is associated with oxidative DNA damage. Such deleterious effects have been suggested to be triggered by excess reactive oxygen species (ROS) that are produced during CYP1A1 mediated metabolism (Park *et al.* 1996).

Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is a central player in a series of critical cell functions including antioxidant defense, detoxication of electrophiles by conjugation, maintenance of thiol-disulfide status and modulation of redox-sensitive signal transduction (Meister 1991; Dröge *et al.* 1994; Sen & Packer 1996). Most of these roles are implicated through the GSH redox cycle. ROS are detoxified either spontaneously and/or by glutathione peroxidase (GPX) catalyzed mechanisms. Oxidized glutathione or glutathione disulfide (GSSG) is formed as the endproduct. In the presence of NADPH, glutathione reductase (GR) catalyzes the reduction of GSSG to GSH thus ensuring the maintenance of a favorable oxido-reductive (redox) state in the cell (Meister 1991). Tissue GSH metabolism can be manipulated by the use of L-buthionine-[S,R]-sulfoximine (BSO) and the chemotherapeutic agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). BSO specifically inhibits glutathione biosynthesis (Meister 1991) depleting both intracellular GSH and GSSG contents. BCNU is a cytostatic drug that is commonly used as an inhibitor of GR activity (Becker & Schirmer 1995). In this way, BCNU can arrest the glutathione redox cycle resulting in the elevation of GSSG within the cell.

Another therapeutic agent that markedly influences GSH metabolism is α-lipoic acid or 6,8-thioctic acid (Packer *et al.* 1995). Following exogenous supplementation lipoate is promptly taken up by cells and reduced to the corresponding thiol, dihydrolipoate (DHLA) (Handelman *et al.*

1994). Intracellular dihydrolipoate thus produced is rapidly released by the cells to the extracellular medium (Handelman *et al.* 1994). DHLA is a strong reducing agent that reduces extracellular cystine to cysteine. By doing so, lipoate improves cysteine bioavailability within the cell and boosts GSH synthesis (Han *et al.* 1996). In addition, both lipoate and DHLA have proven radical scavenging properties. DHLA is also known to contribute to the regeneration of other crucial antioxidants (Packer *et al.* 1995). Transcription factors such as nuclear factor κB (NFκB) and activator protein-1 (AP-1) are sensitive to ROS and the cellular thiol-disulfide redox state. Exogenously supplemented lipoate and the redox state of endogenous glutathione are effective regulators of such redox sensitive gene expression (Dröge *et al.* 1994; Sen & Packer 1996; Sen *et al.* 1996).

Toxicant-induced AhR-mediated gene transcription has been suggested to be redox regulated. In support of this, decreased *in vitro* DNA binding of the AhR/ARNT heterodimer has been observed under oxidizing conditions (Ireland *et al.* 1995). Previously we have communicated that alteration in tissue GSH status sensitively regulates hepatic CYP1A gene expression and metabolic activity (Otto *et al.* 1996a). In this work we have sought to evaluate the possible role of tissue GSH level and redox state on basal and toxicant-induced cytochrome P450 metabolism. To address this the BSO-treated GSH deficient and the BCNU-treated GSSG-elevated models were used. To elevate tissue GSH levels, GSH- and lipoate- supplemented models were used. We present evidence that manipulations of tissue GSH metabolism, as mentioned above, sensitively modulated the constitutive cytochrome P4501A system as well as the responses of CYP1A metabolism to TCB.

Materials and methods

Chemicals

3,3',4,4'-Tetrachlorobiphenyl was purchased from Ultra Scientific (Kingstown, RI). 1,3-Bis(2-chloroethyl)-1-nitrosourea was a kind gift from Bristol Laboratories (Montreal, Qué). Ethoxyresorufin, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), Bakers yeast glutathione reductase, reduced and oxidized glutathione, L-buthionine-[S,R]-sulfoximine, α-lipoic acid, NADPH, 3-aminobenzoic acid ethyl ester (MS 222) were obtained from Sigma (St. Louis, MO) and 2-vinylpyridine was obtained from Aldrich (Milwaukee, WI). All other chemicals were of analytical grade.

Animals

Hatchery reared, immature rainbow trout (70-160 g) were obtained from Linwood Acres (Campellcroft, ON, Canada). The fish were placed in tanks supplied with dechloraminated City of Ottawa tap water and laboratory acclimated for 1 month before separating the fish randomly into 15 tanks, 7-10 fish per tank, supplied with identical water. Water was saturated with oxygen and maintained at 12±1°C. Fish were fed 1.5% of body weight per day, but after daily injections feeding declined. Experiments were undertaken during the spring.

Thiol manipulations and exposure of fish to TCB

Fish were daily intraperitoneally (i.p.) injected with either saline, GSH, BSO, lipoate or BCNU in the evening. Tank water levels were lowered (to approximately 40 l) and fish were mildly anaesthesized by adding 0.05 g/l 3-aminobenzoic acid ethyl ester and 0.1 g/l sodium bicarbonate to the tank. Solutions of all the abovementioned treatment agents were prepared in fish physiological saline (pH 6.8) with the exceptions that lipoate was dissolved in 1 part 6 N NaOH

and 19 parts saline (pH 7.0) and BCNU was dissolved in 1,2 propanediol, cremophore and saline (v/w/v in parts of 1:1:3). Saline-treated trout received saline injections (175-400 µl) and GSH-supplemented trout received GSH (0.25 g/kg, 175-400 µl; pH was adjusted to 6.5-6.8 by cautious addition of NaOH just before use). BSO stock was dissolved in saline with careful heating to 30°C and injection volume was 420-960 µl (6 mmol/kg). Lipoate-supplemented fish received volumes of 140-320 µl of lipoate solution (16 mg/kg). BCNU-treated trout received 140-320 µl of BCNU solution (0.15 mmol/kg). Saline, GSH, BSO and lipoate injections commenced 36 hours and BCNU 12 hours prior to TCB exposure. TCB was dissolved in corn oil heated to 60°C to obtain stock concentrations of 0.08 or 3.9 mg/ml. The solution was cooled to approximately 30°C and injected intraperitoneally (i.p.) at 0.1 mg/kg and 5 mg/kg (90-210 µl); controls received equivalent volumes of corn oil. After 12 hours of TCB injection thiol manipulation treatments (saline, GSH, BSO, lipoate and BCNU) continued for 3 consecutive days (1 injection/d).

Trout were harvested the morning following the total of 5 days of thiol treatment. Fish were killed by a blow to the head and blood was collected into heparinized syringes from the caudal vessels. Plasma was acidified with 5% 5-sulfosalicylic acid and centrifuged to remove precipitated protein. Supernatants were stored at -20°C. Organs were quickly excised, freed from blood and snap frozen in liquid nitrogen, and stored at -80°C until analyzed.

Preparation of tissue samples

Tissues were homogenized in ice-cold Tris buffer (50 mM, containing 0.25 M sucrose and 1 mM EDTA, pH 7.5) using a Potter-Elvehjem-type glass homogenizer. Homogenates were centrifuged at 10,000 g for 15 min at 4°C. The post-mitochondrial supernatants were further centrifuged at 105,000 g at 4°C for 60 min. Microsomes were resuspended by homogenization in

60 mM Tris (containing 0.25 M sucrose, 5 mM EDTA and 20% glycerol, pH 7.4). Microsomes and supernatants were stored at -80°C (< 2 months) until analyzed for enzyme activities. For glutathione measurements, frozen tissue samples were homogenized in ice-cold 5% 5-sulfosalicylic acid and centrifuged at 4,000 g and 4°C for 10 min. Supernatants were collected and stored at -20°C.

Biochemical analyses

Total glutathione (TGSH) was determined according to Sen *et al.* (1992) and GSSG according to Griffith (1980). EROD was assayed at 18°C, a temperature which is within the range experienced by this species. EROD activity was monitored according to Burke & Mayer (1974). Protein content was estimated according to Lowry *et al.*(1951).

RNA slot blot analysis

Total RNA was isolated from 50 mg of frozen liver tissue using a guanidinium isothiocyanate lysis, membrane binding system (RNeasy, Qiagen, Chatsworth, CA). RNA (5 µg/sample) was denatured in 50% formamide, 7% formaldehyde at 65°C for 15 minutes, then transferred to a nylon membrane using a vacuum slot manifold (Gibco/BRL, Burlington, Canada). In addition, an equivalent amount of total RNA from mouse liver was blotted on the same membrane as a negative hybridization control. Blots were probed with a ^{32}P-labelled rainbow trout CYP1A cDNA probe, „fP1-450-3' (Heilmann *et al.* 1988), ATCC #37657 obtained from ATCC (Rockville, MD). Probes were hybridized for 1 hour at 68°C in the presence of 100 mg/ml sonicated calf thymus DNA using a rapid hybridization system (Quickhyb, Stratagene, La Jolla, CA), and washed twice with 2X saline sodium citrate (SSC), 0.25% sodium dodecylsulfate (SDS) at room temperature for 15 min and once with 0.1 X SSC, 0.25% SDS at 62°C for 30 min. Blots

were exposed to X-ray film for 8 hours, stripped for 10 min with boiling 0.1 X SSC, 0.1% SDS, and then re-exposed for 24 hours to confirm stripping. Stripped blots were re-hybridized with a ^{32}P labelled oligonucleotide probe (5'-CCGACATCGCCGCTGACCCCTGGCGCCAGTTTACGTGAGCCGATC-3') directed against the D2 region of the trout 28S RNA using the same hybridization conditions as described above. Blots were washed as described above with two additional washes in 0.1 X SSC, 0.25% SDS at 65°C and at 70°C (30 min), and exposed to X-ray film for 45 min. Relative optical densities of the CYP1A and 28S blots were determined for each sample by densitometry scanning of the X-ray films using a Molecular Dynamics scanning laser densitometer with Imagequant software. Three different blots were made to enable statistical comparison between signal intensities of five different thiol treatments at each TCB concentration and different TCB concentrations within one thiol treatment.

Western blot analysis

Aliquots of equivalent protein content from liver microsomes of each animal were pooled for each treatment group and separated by 12% SDS-PAGE according to Laemmli (1970), then transferred to nitrocellulose membranes. Blots were incubated for 2 hours with a rabbit polyclonal anti-rat CYP1A1/2 antibody (Human Biologics, Phoenix, AZ). Antibody binding was detected by chemiluminescence using a biotinylated anti-rabbit IgG followed by a streptavidin-horseradish peroxidase conjugate (ECL, Amersham, Oakville, Canada). After development, blots were exposed to Hyperfilm ECL (Amersham). Two different Western blots were made to allow qualitative comparison between individual thiol treatments and different TCB exposures within one thiol treatment.

Statistical analyses

Significant differences among the 5 different treatment groups were tested with a one-way analysis of variance, using Statistix 4.1. Significant differences among the various TCB exposures in each treatment group were determined also with a one-way ANOVA. The location of significance was determined using the Least Significant Difference method (LSD). A p value of $p<0.05$ or lower was accepted as significant.

Results

GSH status

Daily saline injections. TCB exposure increased hepatic GSH and total glutathione (TGSH, *i.e.*, GSH+GSSG) contents in saline-treated fish (Table 8.1). TGSH levels in 5 mg/kg TCB-exposed saline-treated trout were 1.4-fold higher than in non-TCB controls ($p<0.05$). Hepatic GSSG content increased in both 0.1 mg/kg (2.8-fold) and 5 mg/kg (2.6-fold) TCB-exposed saline-treated trout compared with non-TCB controls ($p<0.01$). The oxidative stress marker, GSSG/GSH ratio of these TCB-exposed saline-treated groups approximately doubled compared to that in non-TCB-controls ($p<0.05$). Kidney GSH, TGSH and GSSG levels were not influenced by TCB exposure in placebo saline-treated groups (Table 8.1a).

GSH supplementation. Tissue GSH and TGSH contents in non-TCB and TCB-exposed GSH-supplemented trout significantly increased compared with corresponding treatment groups. In non-TCB controls, the magnitude of tissue GSH and TGSH level increase in response to GSH supplementation was as follows: liver, 3-fold; kidney, 2-fold; and plasma 14- and 26-fold (for

Table 8.1. Glutathione status in liver, kidney and plasma of TCB exposed rainbow trout receiving different thiol treatment. Values are means ± SEM, n=5. GSH, reduced glutathione; GSSG, oxidized glutathione; TGSH, total glutathione (GSH + 2GSSG); ratio, 1000*GSSG/GSH.

a) concentrations (nmol/g wet weight) in liver and kidney

	saline	GSH	BSO	lipoate	BCNU
liver					
CON					
GSH	1314±181$^{\blacksquare}$	3893±222$^{\#\#\#}$	269±71$^{\#\#\#}$	2263±90^{***d}	1469±191^{1d}
GSSG	49±12$^{2\blacksquare}$	159±37*	9±3$^{1\#}$	40±7$^{3\blacksquare}$	117±21*
TGSH	1412±180$^{1\blacksquare}$	4210±214$^{1\#\#\#}$	287±77$^{1\#\#\#}$	2343±88^{***d}	1703±222^{1d}
ratio	40±10^{1}	42±11	32±5	18±3^{3}	81±11$^{\#\#}$
0.1 mg					
GSH	1627±124	5090±523$^{\#\#\#}$	177±35$^{4\#\#}$	2077±181	1640±135
GSSG	138±15$^{\sim}$	205±19*	5±2$^{\#\#\#}$	98±24$^{2\blacksquare}$	131±35
TGSH	1903±114	5499±497$^{\sim\#\#\#}$	187±37$^{\#\#\#}$	2273±218	1903±182
ratio	88±14$^{\sim**ab}$	43±8^{a}	26±6$^{\blacksquare}$	46±8.7^{3tb}	78±19**
5 mg					
GSH	1720±176	4126±441$^{\#\#\#}$	114±6$^{\#\#\#}$	2163±139	2113±195$^{\sim}$
GSSG	128±20$^{\sim}$	118±34^{a}	3±0.4$^{\sim\#\#\#}$	214±24$^{\sim**a}$	103±23$^{\blacksquare}$
TGSH	1975±195$^{\sim\blacksquare}$	4362±478$^{\#\#\#}$	119±6$^{\sim\#\#\#}$	2590±162*	2320±170$^{\sim}$
ratio	76±10$^{\sim**}$	29±8$^{\blacksquare a}$	23±3$^{\blacksquare b}$	100±10$^{\sim**c}$	54±16^{abc}
kidney					
CON					
GSH	2021±110	3878±259$^{\#\#\#}$	572±34$^{\#\#\#}$	2284±58	2088±124
GSSG	39±4	120±15$^{\#\#\#}$	10±2$^{\#\#\#}$	30±5	28±9
TGSH	2099±109	4118±259$^{\#\#\#}$	591±33$^{\#\#\#}$	2343±58	2145±123
ratio	19±2	32±4**	18±3$^{\blacksquare}$	13±2$^{\blacksquare}$	14±1$^{\blacksquare}$
0.1 mg					
GSH	1774±145	4414±277$^{1\#\#\#}$	502±27$^{\#\#\#}$	2046±100	1882±130
GSSG	41±6	144±13$^{\#\#\#}$	9±1$^{\#\#\#}$	31±6	35±4
TGSH	1855±152	4701±293$^{1\#\#\#}$	521±30$^{\#\#\#}$	2108±95	1951±134
ratio	23±3^{a}	33±3^{***a}	19±1$^{\blacksquare}$	16±3$^{\blacksquare}$	19±2$^{\blacksquare}$
5 mg					
GSH	1783±104	3440±299$^{\sim\#\#}$	566±68$^{\#\#\#}$	2163±103	2048±228$^{\#}$
GSSG	46±5^{a}	163±31$^{\#\#\#}$	9±0.6$^{\#\#\#}$	33±3	29±5^{a}
TGSH	1874±100	3766±332$^{\sim\#\#}$	583±68$^{\#\#}$	2228±102	2107±23$^{\#}$
ratio	26±4$^{\#}$	47±8***	16±3$^{\blacksquare}$	15±2$^{\blacksquare}$	15±2$^{\blacksquare}$

b) concentrations (µmol/l) in blood plasma

	saline	GSH	BSO	lipoate	BCNU
CON					
GSH	13±3	184±35$^{\#\#\#}$	4±1$^{\sim\#\#}$	16±2	31±2
GSSG	3±0.4$^{\blacksquare}$	157±184$^{\#\#\#}$	1±0.1$^{\#\#\#}$	5±0.3$^{\sim\bullet}$	5±0.6
TGSH	19±3	497±104$^{\#\#\#}$	6±2$^{\sim\#\#\#}$	26±2	41±6
ratio	433±190$^{\blacksquare}$	893±181$^{\#}$	492±240$^{\bullet}$	329±42$^{\bullet}$	145±22$^{\blacksquare}$
0.1 mg					
GSH	12±2	226±53$^{\#\#\#}$	9±2$^{1\blacksquare}$	18±6$^{\bullet}$	30±2$^{\#}$
GSSG	4±2	128±12$^{\#\#\#}$	1±0.2$^{\#\#\#}$	4±0.8	3±0.6
TGSH	20±2	483±75$^{\#\#\#}$	11±2$^{1\#\#}$	26±6	37±2$^{\#}$
ratio	354±91$^{\bullet}$	704±129$^{\bullet\bullet\bullet a}$	176±57$^{\blacksquare}$	299±86^{a}	119±26$^{\blacksquare}$
5 mg					
GSH	16±2	188±20$^{\#\#\#}$	6±1$^{\#\#\#}$	15±2	29±1$^{\#\#}$
GSSG	4±0.8	163±28$^{\#\#\#}$	0.8±0.2$^{\#\#\#}$	3±0.4$^{\#}$	4±0.3
TGSH	25±2	513±62$^{\#\#\#}$	8±0.2$^{\#\#\#}$	20±0.4	38±2$^{\#\#}$
ratio	286±58	901±188$^{\#\#}$	197±98	170±21	141±13

$^{\#\#\#}$P<0.001, $^{\#\#}$P<0.01, $^{\#}$P<0.05, significantly different from all thiol treatment groups exposed to the same TCB concentrations . $^{\bullet\bullet\bullet}$P<0.001, $^{\bullet\bullet}$P<0.01, $^{\bullet}$P<0.05, significantly different from $^{\blacksquare}$-labeled treatment groups exposed to the same TCB concentration; ^{a,b,c}P<0.05, ^{d}P<0.01, significantly different from same labeled treatment group exposed to the same TCB concentration; ^{1}P<0.05, ^{2}P<0.01, ^{3}P<0.001, significantly different from ~-labeled TCB exposed groups receiving same treatment. $^{\sim\blacksquare}$P<0.001, $^{\sim}$P<0.01, $^{\sim}$P<0.05, significantly different from other TCB exposed groups receiving the same treatment.

GSH and TGSH, respectively) ($p<0.001$) compared with non-TCB saline-treated controls. Similar increases in tissue GSH and TGSH were also observed in TCB-exposed fish that were GSH-supplemented. Interestingly, GSH supplementation dependent increase in hepatic GSH levels was significantly more in 0.1 mg/kg TCB-exposed fish than in the corresponding non-TCB controls. That TCB exposure may enhance tissue GSH levels was also evident in the kidney. Renal GSH content in 0.1 mg/kg TCB group was also significantly higher than that in 5 mg/kg TCB-exposed GSH-supplemented fish. Lower concentration (0.1 mg/kg) TCB exposure was more effective in elevating tissue GSH levels compared to higher (5 mg/kg) concentration exposure. The elevation of tissue GSH content in 0.1 mg/kg TCB-exposed GSH-supplemented trout were thus 3.1- (liver) and 2.5-fold (kidney) higher than that in the respective saline-treated group. In comparison, hepatic and renal GSH contents were only 2.4- and 1.9-fold higher in 5 mg/kg TCB-exposed GSH-supplemented trout compared with the respective saline-treated trout. TCB exposure did not influence tissue GSSG levels or GSSG/GSH ratio of GSH supplemented fish (Table 8.1a,b).

GSH deficiency. A multifold decrease in tissue GSH content was observed in response to BSO-treatment. In non-TCB controls, BSO treatment induced global tissue glutathione deficiency. Tissue GSH and TGSH contents of BSO-treated fish were markedly lower in liver (4.9-fold), kidney (3.5-fold) and plasma (3.3-fold) compared to those in saline-treated controls ($p<0.001$). Exposure to higher TCB concentrations potentiated BSO-dependent GSH depletion in the liver. Hepatic GSH and TGSH contents in 0.1 mg/kg TCB-exposed BSO-treated fish were 9- to 10-fold lower than in the respective TCB-exposed saline-treated group. In the 5 mg/kg TCB-exposed BSO-treated trout, hepatic GSH and TGSH levels were decreased by 15- to 17-fold compared with the corresponding TCB saline-treated group ($p<0.001$). Depletion of renal and plasma GSH

and TGSH levels, however, were much less pronounced in reponse to TCB exposure and BSO treatment. Kidney and plasma GSH and TGSH contents of 0.1 and 5 mg/kg TCB-exposed BSO-treated trout were 1.3- to 3.6-fold lower than that in the respective TCB-exposed saline-treated groups.

Hepatic GSH and TGSH contents decreased in 0.1 mg/kg (1.5-fold) and 5 mg/kg (2.4-fold) TCB-exposed BSO-treated groups versus the corresponding non-TCB BSO-treated controls (Table 8.1a). In contrast, plasma GSH content was 2.3-fold higher in 0.1 mg/kg TCB-exposed BSO-treated fish than in non-TCB controls (Table 8.1b). Liver GSSG content significantly decreased in 5 mg/kg TCB-exposed BSO-treated trout compared with non-TCB controls, but tissue GSSG/GSH ratio did not differ significantly within the different BSO treated groups (Table 8.1a,b).

Lipoate supplementation. Supplementation of lipoate clearly enhanced hepatic GSH and TGSH contents in non-TCB lipoate-supplemented controls compared with the respective saline-(1.7-fold) or BCNU-(1.5- and 1.4-fold) treated controls. GSH or TGSH level of other tissues were not influenced by lipoate supplementation. Hepatic TGSH content was 1.3-fold higher in 5 mg/kg TCB-exposed lipoate-supplemented trout than in the respective saline-treated group (Table 8.1a).

Within the different lipoate supplementation groups, hepatic GSSG levels were 2.5-fold increased in 0.1 mg/kg TCB-exposed trout compared with non-TCB controls. Also hepatic GSSG content further increased in 5 mg/kg (2.2-fold) compared with 0.1 mg/kg TCB-exposed group. However, renal and plasma GSSG and GSSG/GSH ratio did not differ significantly between the different lipoate supplemented groups (Table 8.1a,b).

BCNU administration. BCNU administration significantly increased hepatic GSSG content and

GSSG/GSH ratio in non-TCB controls compared with all other treatments. This increase in GSSG levels was accompanied by significantly decreased GR activities (1.6- to 2.5-fold) compared with respective non-TCB controls (not shown). No further change in the glutathione redox state was observed in BCNU-treated fish in reponse to TCB exposure. Hepatic, renal and plasma GSSG content and GSSG/GSH ratios in TCB-exposed groups resembled values of non-TCB BCNU-treated controls.

TCB exposure also diminished BCNU inhibition of GR activities (data not shown). Consistent with results from different saline-treated groups, hepatic GSH and TGSH contents increased in 5 mg/kg TCB-exposed BCNU-supplemented trout (1.4-fold) versus non-TCB BCNU controls (Table 8.1a,b).

Cytochrome P450 metabolism and gene expression

EROD activities. BCNU treatment strongly induced hepatic and renal EROD activities ($p<0.05$) (Table 8.2). Basal EROD activities in liver of BSO-treated, and in liver and kidney of lipoate-supplemented controls were also significantly higher than in GSH supplemented controls. EROD activities did not differ between GSH-supplemented and saline-treated controls.

Exposure to 0.1 mg/kg TCB significantly increased hepatic EROD activities in saline-, GSH- and BCNU-treated groups. Induction of hepatic EROD activity was markedly suppressed in BSO-treated (6- to 10-fold) and lipoate-supplemented (2.4- to 3.9-fold) trout compared with other treatments. Renal EROD activity was only significantly increased in 0.1

Table 8.2. Hepatic and renal EROD activities (pmol*min^{-1}*mg^{-1} protein). Values are means ± SEM, n=5.

	saline	GSH	BSO	lipoate	BCNU
LIVER					
control	5.6±0.2^{2}	4.8±0.5$^{3\blacksquare}$	10±2.5$^{\sim\bullet}$	9.5±2.4$^{\sim\bullet}$	84±43$^{\sim\#}$
0.1 mg	120±53$^{\tau\bullet\bullet}$	133±66$^{\pi\bullet\bullet}$	20±11$^{\sim\blacksquare}$	51±24$^{\sim\blacksquare}$	197±64$^{\sim\bullet\bullet\blacksquare}$
5 mg	315±83$^{\sim\blacksquare}$	540±32$^{\sim\bullet}$	51±9$^{1\#\#}$	118±4$^{2\#\#}$	517±76^{2}
KIDNEY					
control	9.3±1.0$^{\sim}$	6.6±1.2$^{\tau\tau\blacksquare}$	8±0.9$^{\sim}$	12±1.1$^{\sim\bullet}$	24±2.6$^{\sim\#}$
0.1 mg	15±2.8$^{\sim}$	16±1.8$^{\tau\tau}$	12±1.6$^{\sim}$	22±4.5$^{\sim\tau}$	40±5.6$^{\tau\#\#}$
5 mg	135±23^{3}	145±10	34±4$^{3\#\#\#}$	69±3$^{3\#\#}$	176±17^{3}

$^{\#\#\#}$P<0.001, $^{\#\#}$P<0.01, $^{\#}$P<0.05, significantly different from all other treatment groups exposed to the same TCB concentrations . $^{\bullet\bullet\bullet}$P<0.001, $^{\bullet\bullet}$P<0.01, $^{\bullet}$P<0.05, significantly different from $^{\blacksquare}$-labeled treatment groups exposed to the same TCB concentration. ^{a,b,c}P<0.05, ^{d}P<0.01, significantly different from same labeled treatment group exposed to the same TCB concentration. ^{1}P<0.05, ^{2}P<0.01, ^{3}P<0.001, significantly different from ~-labeled TCB-exposed groups receiving same treatment. $^{\tau\tau}$P<0.001, $^{\tau}$P<0.01, $^{\tau}$P<0.05, significantly different from all other TCB-exposed groups receiving the same treatment.

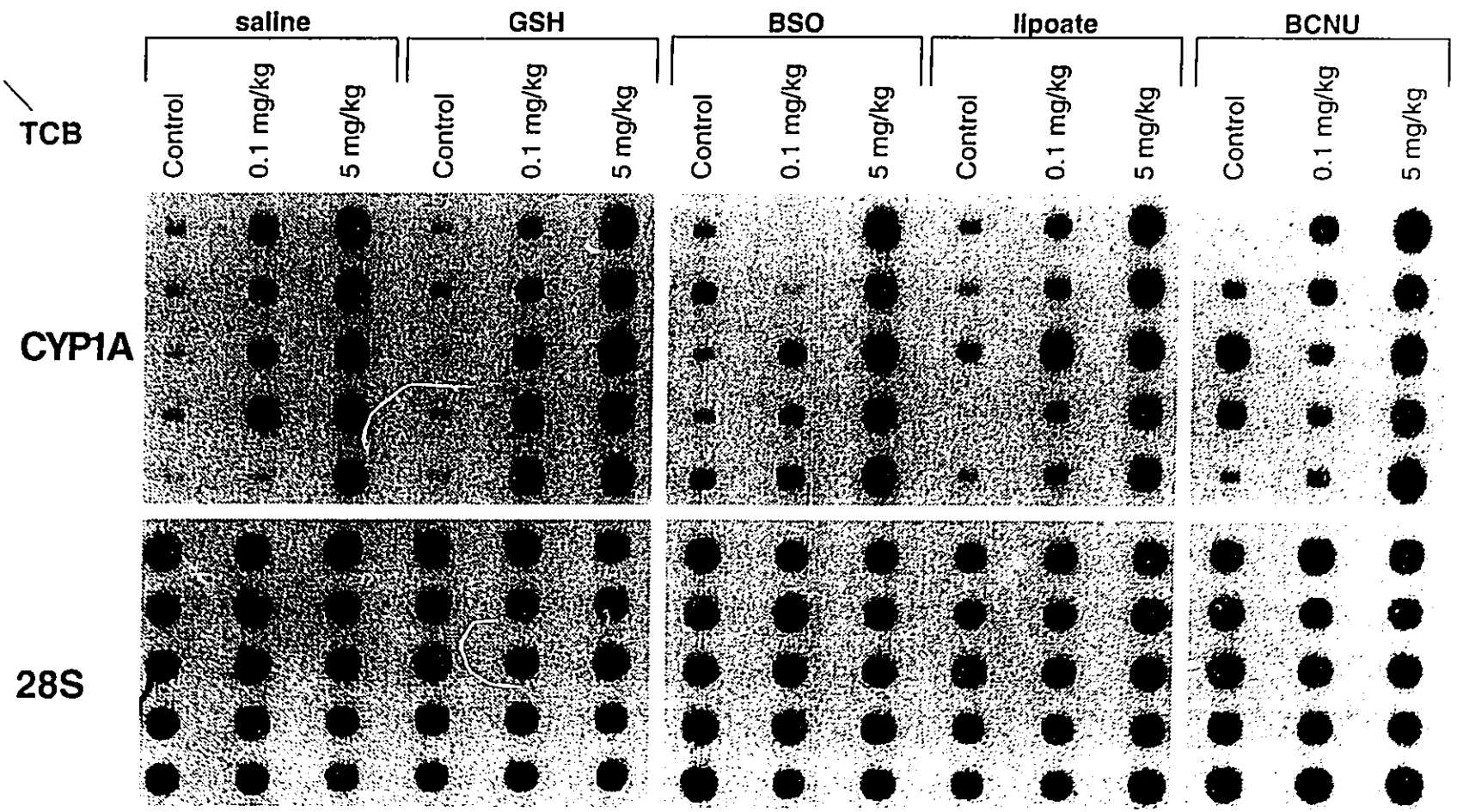
mg/kg TCB-exposed BCNU-supplemented trout compared with other respective treatment groups.

Hepatic EROD activity in 5 mg/kg TCB-exposed GSH-supplemented trout was significantly higher (1.7-fold) than in respective saline-treated controls. Higher concentration of TCB exposure was required to obtain tissue EROD responses in BSO- and lipoate-treated fish. Exposure to 5 mg/kg TCB significantly elevated hepatic and renal EROD activities in BSO-treated ($p<0.05$) and lipoate-supplemented ($p<0.01$) groups compared with the corresponding 0.1 mg/kg TCB-exposed groups and non-TCB controls. Hepatic and renal EROD activities, however, were still markedly higher (2- to 11-fold) in 5 mg/kg TCB-exposed saline-treated, GSH- and BCNU-supplemented groups than in respective BSO-treated and lipoate-supplemented groups ($p<0.01$). Thus consistent with the observation in the 0.1 mg/kg TCB group, both BSO and lipoate appeared to suppress tissue EROD response. This suppressive effect was more prominent for BSO than lipoate because hepatic and renal EROD activities were significantly higher in 5 mg/kg TCB-exposed lipoate-supplemented trout than in respective BSO-treated group ($p<0.01$).

Hepatic CYP1A gene expression. BCNU treatment markedly induced hepatic CYP1A mRNA expression (Figure 8.1). Also CYP1A and CYP1A/28S mRNA expressions were significantly induced by BSO treatment alone (Figure 8.1a,c). In all treatment groups induction of CYP1A mRNA expression was directly dependent on the concentration of TCB exposure. CYP1A and CYP1A/28S mRNA expression in response to 0.1 mg/kg TCB exposure was marginally lower in GSH deficient livers than in the respective GSH-adequate tissues. Exposure to higher concentration (5 mg/kg) of TCB strongly induced CYP1A mRNA

Fig. 8.1. a) CYP1A versus 28S RNA from livers of TCB-exposed (non-TCB, 0.1 mg/kg and 5 mg/kg TCB; single i.p. injection) trout receiving repeated treatment with saline, GSH, BSO, lipoate and BCNU. 5 µg samples of total RNA were extracted from livers and immobilized on a nylon membrane using a vacuum slot blot apparatus. 3 different blots were prepared to compare between the various TCB-exposures of each of the 5 treatments (shown above), as well as within one TCB-exposure between the 5 treatment groups (not shown). Blots were probed with a ^{32}P-labelled CYP1A cDNA probe and exposed to x-ray film for 8 hours. Blots were then stripped, re-exposed to confirm stripping, probed with a ^{32}P-labeled oligonucleotide directed against the D2 region of the trout 28S RNA and exposed for 45 min. A negative control RNA (5 µg from mouse liver) was included to confirm specificity of the probe (data not shown).

b and c) RNA levels were quantified by laser densitometry scanning of x-ray films to obtain CYP1A/28S ratio. Error bars indicate error of means for each group. Comparison were made between the 3 different TCB exposures of each treatment (b) and within one TCB-exposure between the 5 treatment groups (c). b: *,**,*** significantly different from other TCB-exposures ($p<0.05$, $p<0.01$, $p<0.001$ respectively). c: * significantly different from ~-labeled group; #significantly different from '-labeled group ($p<0.05$).


TCB
saline
GSH
BSO
lipoate
BCNU
Control
0.1 mg/kg
5 mg/kg
CYP1A
28S

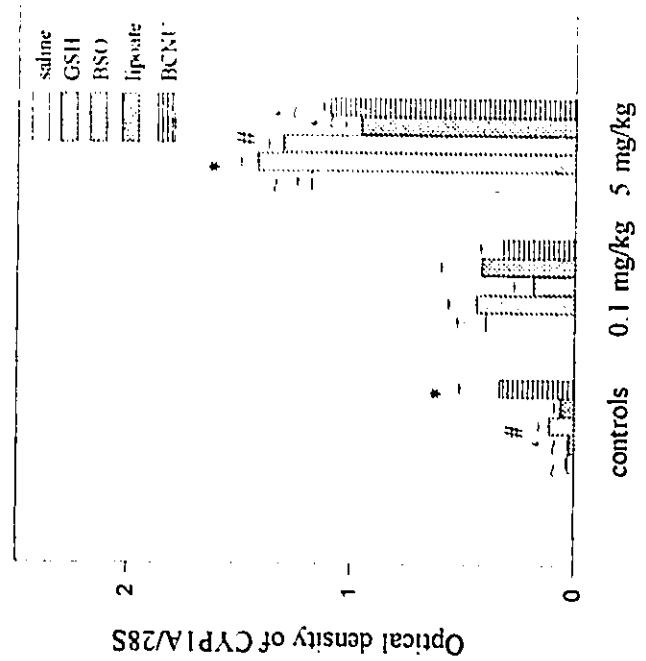


Fig. 8.1c

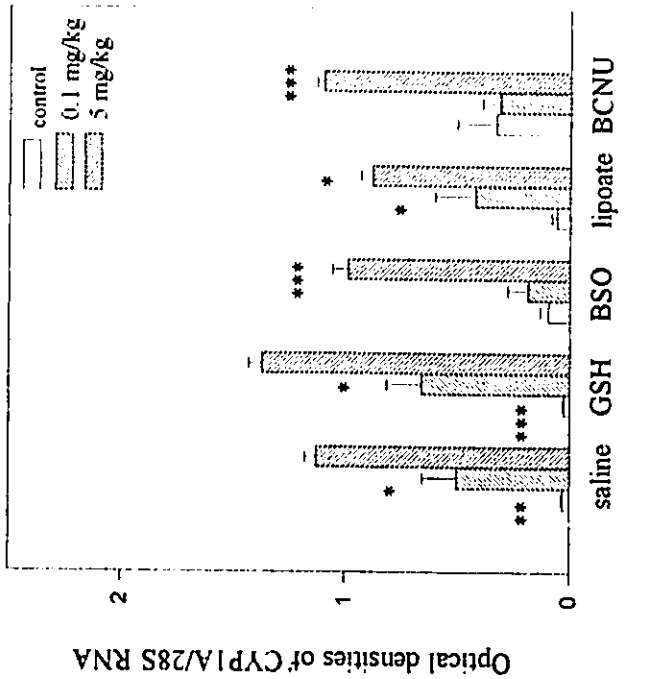


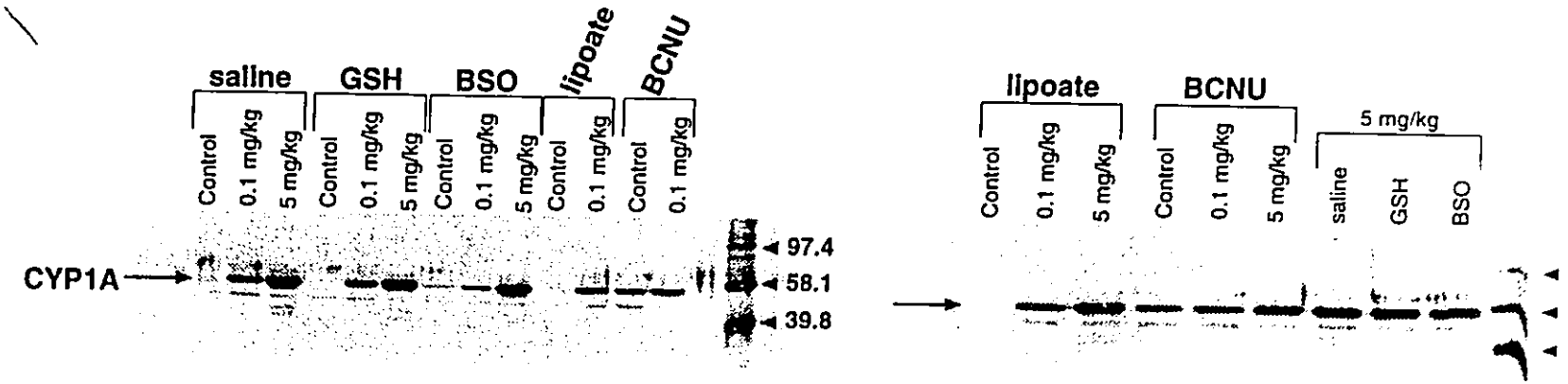
Fig. 8.1b

expression in all treatment groups studied (Figure 8.1a,b). TCB (5 mg/kg)-induced CYP1A/28S mRNA expression was potentiated in response to GSH supplementation. Such high concentration TCB-induced CYP1A/28S mRNA expression was lower in the lipoate- and BCNU-supplemented groups than in GSH-supplemented and BSO-treated trout (Figure 8.1c).

Compared with the respective treatment controls, the magnitude of TCB-induced CYP1A mRNA expression in the five different treatment groups (the respective TCB concentrations are indicated in adjacent parentheses) were as follows (Figure 8.1b): saline-treated, 17-fold (0.1 mg/kg) and 38-fold (5 mg/kg); GSH-supplemented, 27-fold (0.1 mg/kg) and 57-fold (5 mg/kg); GSH deficient, 2-fold (0.1 mg/kg) and 11-fold (5 mg/kg); lipoate-supplemented, 7.4-fold (0.1 mg/kg) and 16-fold (5 mg/kg); and BCNU-treated, unchanged (0.1 mg/kg) and 3.4-fold (5 mg/kg TCB). Thus the TCB-induction of CYP1A mRNA expression was markedly potentiated in response to GSH supplementation, and less induced in BSO-, BCNU- and lipoate-treated groups.

CYP1A protein expression. CYP1A protein expression responses in the different treatment groups were consistent with CYP1A mRNA expression data (Figure 8.2). Constitutive expression of CYP1A protein was low, and CYP1A protein was even less expressed in non-TCB GSH- or lipoate-supplemented controls. In contrast, BSO-treatment alone induced CYP1A protein expression. Moreover, CYP1A protein was markedly expressed in BCNU-treated controls. TCB (0.1 mg/kg) markedly induced CYP1A protein in all different treatment groups, but protein expression in BSO-treated (0.1 mg/kg) was notably weaker than in other treatment groups. CYP1A protein was further induced by 5 mg/kg TCB in all groups and no difference in induction in the different treatment groups was observed.

Fig. 8.2. Western blots of anti-rat CYP1A polyclonal antibody immunoreactive protein from trout liver. Liver homogenates were pooled from 5 animals per treatment group and 20 μg of protein were separated by SDS page and electroblotted to nitrocellulose membranes. Blots were probed with polyclonal anti-rat CYP1A from rabbit and detected by chemiluminescent exposure of x-ray film. 2 Blots were made to allow comparisons between the various TCB-exposures of each of the 5 treatments and within one TCB-exposure between the 5 treatment groups. The arrow indicates the band which co-migrates with rat CYP1A. Arrowheads and numbers at right indicate molecular weight standards in kDa.


saline
GSH
BSO
lipoate
BCNU
Control
0.1 mg/kg
5 mg/kg
CYP1A
97.4
58.1
39.8
lipoate
BCNU
5 mg/kg
saline
GSH
BSO

Discussion

GSH status

Various PCB congeners enhance the production of reactive oxygen species (Amaro *et al.* 1996; Tithof *et al.* 1996) and thus sensitize cellular antioxidant defenses. TCB exposure modifies the tissue glutathione pool, and responses of tissue glutathione to the various treatments (Table 8.1). In non-TCB exposed controls, all treatments effectively altered tissue glutathione status. Lipoate supplementation efficiently enhanced hepatic GSH and TGSH levels. Lipoate has been consistently shown to elevate hepatic GSH content in mammalian species (Packer *et al.* 1995). BCNU is a DNA-alkylating and DNA-cross-linking agent with usage in treatment of brain tumors, lymphomas and malignant melanomas. As a result of GR inhibition, BCNU increases intracellular GSSG content (Becker & Schirmer 1995; Dröge *et al.* 1994). However, TCB exposure diminished the lipoate and BCNU effects on GSH status. Perhaps this is due to an interaction of TCB with lipoate or BCNU. Lipoate is rapidly reduced in tissues to dihydrolipoic acid, which is a potent reductant and spares oxidation of GSH (Handelman *et al.* 1994; Packer *et al.* 1995). The steady increase of GSSG content with TCB exposure in lipoate-supplemented trout is in contrast to our previous study, in which lipoate maintained a low GSSG/GSH ratio in TCB-exposed (1 mg/kg) rainbow trout (Chapter 8). However, the increased hepatic GSH content with high TCB exposure, as noted in saline- and BCNU-treated trout, is consistent with previous observations in this species (Chapter 5).

The significantly enhanced tissue availability of supplemented GSH in 0.1 mg/kg TCB exposed trout may suggest increased tissue demand for the thiol. GSH serves as a reservoir of cellular cysteine for protein synthesis (Meister 1991). In comparison, GSH dependent enzymes including

GPX, GR or glutathione S-transferase (GST) were little influenced by TCB exposure or the treatments with saline, GSH, BSO and lipoate (data not shown). However, the loss of BCNU effects on GSSG concentrations resulted from diminished inhibition of GR in TCB exposed trout. The present data show first evidence that TCB markedly modifies efficient alteration of tissue GSH status by the various treatments. In addition, GSH and BSO treatment effects on GSH status were further sensitive to the quantity of TCB exposure (0.1 vs 5 mg/kg). The therapeutic effects of treatments such as lipoate and BCNU may thus be altered in pollutant exposed organisms.

CYP1A transcription and catalytic activity

Biochemical activity of PCB congeners is thought to be primarily initiated through induction of Ah receptor-mediated signal transduction (Safe 1994). Enhanced CYP1A metabolism may increase production of ROS, which may result in oxidative DNA damage potentially leading to mutations and cancer (Park *et al.* 1996). In the absence of TCB, the various treatments to manipulate tissue GSH status resulted in altered expression of the CYP1A gene in trout (Figure 8.1). BCNU is chemically relatively unstable and is inactivated by denitrosation in the cytosol by GST. Additional denitrosation occurs in microsomes by cytochrome P450 enzymes, which are further induced by phenobarbital and dexamethasone (Weber & Waxman 1993). BCNU alone may activate cytochromes P450 by serving as a substrate, although BCNU effects on GSH redox status may be involved in cytochrome P450 induction. GSH deficiency induced by BSO appears to induce CYP1A mRNA expression and catalytic activity in the absence of TCB. GSH depletion by BSO is known to activate gene expression of conjugation enzymes in an AhR independent fashion (Shertzer *et al.* 1995). However, AhR is assumed to play multiple physiological roles in

the development of the liver and immune system (Fernandez-Salguero *et al.* 1995). An endogenous substrate for AhR has been postulated for constitutive gene expression such as CYP1A (Nebert 1994). GSH deficiency may interfere with such expression, providing further evidence for an apparent cross-talk between the GSH and P450 systems (Otto *et al.* 1996a). Such cross-talk is further supported by lower expression of CYP1A protein in GSH- and lipoate-supplemented controls, which both share increased GSH levels, compared with saline-treated controls (Figure 8.2).

We have previously observed that GSH deficiency by BSO treatment sufficiently suppressed TCB (1 mg/kg) induced CYP1A mRNA expression (Otto *et al.* 1996a). Low (0.1 mg/kg) TCB exposure marginally suppressed CYP1A mRNA and CYP1A protein expression in GSH deficient trout. However, exposure to 5 mg/kg TCB resulted in significantly higher hepatic CYP1A/28S mRNA expression in GSH-deficient (BSO) and GSH-supplemented groups than in lipoate- and BCNU-supplemented trout. Sensitivity of TCB induced CYP1A mRNA expression to depleted tissue GSH content (Otto *et al.* 1996a, and 0.1 mg/kg TCB exposure) may be overcome by increased TCB concentrations.

Interestingly, the increased CYP1A mRNA expression in 0.1 mg/kg and 5 mg/kg TCB exposed groups is accompanied by elevated hepatic GSSG levels in all treatments except GSH deficient (BSO) trout. Modulation of gene transcription by GSSG has been postulated, as the well described transcription factor NFκB is reported to be not only regulated by cellular GSH, but also GSSG levels (Dröge *et al.* 1994; Mihm *et al.* 1995). The present results cannot resolve whether GSSG is an influencing factor in CYP1A gene expression. A combination of factors including GSH and GSSG levels as well as TCB exposure concentrations may modulate CYP1A

transcription and gene expression. Whitlock *et al.* (1996) have described CYP1A1 as a model for the assessment of gene transcription; the sensitivity of CYP1A to glutathione status may strengthen this model.

High TCB concentrations induced CYP1A mRNA and protein expression in all groups to a similar extent. The observed suppression of TCB induced EROD activity in lipoate-supplemented and high TCB-exposed GSH-deficient fish was not regulated by gene expression. With similar expression of CYP1A protein in these treatment groups, a post-translational regulation mechanism could establish a second stage for thiol regulation of CYP1A. Lipoate shares structural similarity to oltipraz, a dithiolthione, which has been shown to enhance hepatic GSH content and to suppress 3-methylcholanthrene induced EROD activity (Langouët *et al.* 1995; van Lieshout *et al.* 1996). Inhibition of CYP1A has been suggested to involve nitric oxide and heme oxygenase (Kim *et al.* 1995). The protein-bound heme iron of cytochromes is the target for destruction by nitric oxide and increased degradative heme oxygenase activities (Kim *et al.* 1995). The cytochrome P450 apoprotein remains intact, although catalytic activity is lost without the prosthetic heme group. Moreover, GSH supplementation in 5 mg/kg TCB exposed trout, significantly potentiated hepatic EROD activity compared with the respective saline treated group, in line with previous observations (Otto *et al.* 1996a). Present data in combination with our previous observations indicate that regulation of TCB induced CYP1A by glutathione occurs at two stages, first at the AhR mediated transcription of the CYP1A gene and second at the catalytic activity, most likely by a post-translational mechanism.

Lipoate, N-acetylcysteine (NAC) and oltipraz are increasingly used in chemoprevention (Smith & Gupta 1996; Packer *et al.* 1995). Inhibition of catalytic CYP1A activity would decrease

formation of DNA-adducts by P450 metabolites or DNA damage as a result of cytochrome P450 produced ROS (Park *et al.* 1996). The precise mechanisms of chemopreventive agents is little known. Lipoate, NAC and oltipraz are pro-GSH agents and their chemopreventive action has not been directly distinguished from their effects on GSH levels. Exogenous GSH is an efficient delivery agent of GSH to tissues in fish, but not in mammals (Otto *et al.* 1996a). Fish may serve as models to elucidate whether such inhibition or delayed onset of carcinogenesis is due to synergism of these agents with GSH, inhibition of CYP1A catalytic activity or possibly other mechanisms involved.

In summary, efficient manipulation of tissue thiol status provides a sensitive tool in testing the regulation of cytochrome P450 metabolism by tissue glutathione status. TCB exposure concentrations modify the effectiveness of the various treatments. Treatment with thiols, BSO and BCNU already in the absence of TCB influences constitutive expression of CYP1A. In the presence of TCB, thiols influence CYP1A metabolism at two processing stages, at the gene expression and/or at a posttranslational level. Present results further support a cross-talk between thiols and cytochrome P4501A and identify another transduction pathway of antioxidant regulated gene expression.

Chapter 9. GENERAL DISCUSSION

Glutathione biochemistry has been studied for more than a century, but present research has shown that numerous gaps remain in our knowledge of this ubiquitous tripeptide thiol (Meister 1988; 1995; Dröge *et al.* 1994). This thesis demonstrates that glutathione metabolism plays important roles in detoxication processes along with drug metabolizing enzymes. For the first time, basic features and differences of the piscine GSH system are pointed out and compared with our present (mammalian) knowledge. Further evidence is provided that tissue GSH influences major xenobiotic detoxication pathways.

9.1. GSH dependent defenses in teleosts exposed to PCBs

9.1.1. Induced pathways in various tissues

Xenobiotic metabolic responses have been studied for more than two decades in aquatic organisms (for review see Stegeman & Kloepper-Sams 1987; Lindström-Seppä 1990; Roy 1994; Stegeman & Hahn 1994). Although the implications of xenobiotic exposure on antioxidant defenses have been studied in aquatic species (Winston & Di Giulio 1991; Roy 1994), the abilities of PCB congeners to cause changes in the prooxidant/antioxidant balance of teleost species are little known (Thomas & Wofford 1993; Rudneva-Titova & Zherko 1994). Antioxidant defenses are significantly affected in PCB exposed teleosts and their alterations occur concomitantly with changes in activities of phase I and II enzymes in bullheads and trout (Chapters 2,5). Although the feral bullheads were only analyzed for PCB congeners, several other xenobiotics including PAHs, pesticides, dioxins etc. are likely involved in eliciting such biochemical changes (Chapter 2), which

are as extensive as in trout exposed to a single i.p. injection of TCB (Chapter 5). Severalfold induction of hepatic EROD activity with chemical exposure is a known response of such a widely used biomarker (Lindström-Seppä 1990; Stegeman & Hahn 1994).

The sensitivities of tissue SOD, CAT and GPX activities to chemical exposure are still an issue of debate, with reported increases (Rodríguez *et al.* 1993) or unchanged activities (Mather-Mihaich & Di Giulio 1991; Otto *et al.* 1994). SOD and CAT are strictly involved in the removal of superoxide and H_2O_2 and may show limitations in sensitivities to xenobiotic exposure (Aust *et al.* 1993). However, SOD and CAT along with GPX and GR increased in white muscle of TCB exposed trout (Chapter 5). The detoxication processes between the different tissues, particularly liver and kidney versus muscle appear to be different. Although liver is a major detoxication organ and a primary target for PCB toxicity (Safe 1994), induced cytochrome P450 activities may alter antioxidant defenses. Cytochrome P450-catalyzed hydroxylations of PCBs to chlorinated hydroxybiphenyl metabolites (Amaro *et al.* 1996) may give rise to various ROS (see Fig. 1.2.). Uncoupling P450 reactions increase superoxide and H_2O_2 byproducts. The induction of SOD and CAT in liver and kidney may provide limited protection from several types of ROS produced (Fig. 1.2.). Protection by the GSH system (GST, GPX, GR and GSH) in these tissues may be more efficient. Whereas muscle, with a considerably lower antioxidant machinery than found in other tissues (Chapters 2-5) and negligible cytochrome P450 metabolism (Stegeman & Hahn 1994), may increase its protection to xenobiotic stress by induction of all available antioxidant defenses. These are speculative points on a yet unresolved issue by which mechanism PCBs or other xenobiotics induce changes in the prooxidant/antioxidant balance in tissues of organisms.

Chapters 2 and 5 show that the antioxidant status of white muscle of trout and bullhead play

significant roles in the overall defense of an organism during xenobiotic exposure. Phase I and II enzyme responses are mainly examined in the liver, and to some extent in kidney, intestine, heart or gill (George 1994; Stegeman & Hahn 1994). Conditions which elevate tissue prooxidant forces, *e.g.* during physical exercise, have been consistently reported to cause alterations of GSH status and antioxidant defenses in mammalian skeletal muscle (Ji 1995; Sen 1994, 1995). White muscle can load xenobiotics to high quantities (Schell *et al.* 1993; George *et al.* 1995; Chapters 2,3), induction of detoxication processes at site may aid to ameliorate the overall effect of xenobiotics on health status. Xenobiotics are primarily thought to enter teleost tissues through ingested food or by transfer across the gills (Makarewicz *et al.* 1993; Otto *et al.* 1996b). Although antioxidant responses in gill tissues were not analyzed in Chapters 2 and 5, Chapters 3,4,6 and 7 indicate that this tissue contains an active GSH system as well as SOD and CAT activities, which may participate in detoxication or excretion of xenobiotics (Mommsen 1984).

Exposure to PCBs or other contaminants may induce preferentially different conjugation pathways in trout and bullheads (Chapters 2,5). George and coworkers (Clarke *et al.* 1991; George 1994) have suggested that glucuronidation in fish species is the quantitatively more important detoxication route for xenobiotics. Exposure to xenobiotics in rainbow trout resulted in significant changes of hepatic and renal UDPGT activities, whereas GST activities in these tissues were not or little affected, which is consistent with other studies on this species (Lindström-Seppä 1990; Otto *et al.* 1994; Huuskonen *et al.* 1996; Chapter 5). However, the significant induction of GST activities in tissues of bullhead (Chapter 2) may indicate a preferred route for xenobiotics through conjugation with GSH. Such enhanced conjugation appears to function at the expense of tissue TGSH content (Chapter 2). Other reports (Kirby *et al.* 1995; Gallagher *et al.* 1996) further

support that representatives of this teleost order (Cypriniformes), which include in addition to bullhead, channel catfish (a bullhead relative) and white sucker, efficiently eliminate PAHs by GSH conjugation. GSH conjugation was, therefore, suggested to contribute to the resistance to environmental hepatocarcinogenesis in these species (Kirby *et al.* 1995; Gallagher *et al.* 1996). Tissue GST activities are markedly higher in black bullheads than in rainbow trout (Chapter 4) and it appears that muscle, along with liver and kidney, is involved in conjugation of xenobiotics (Chapter 2). Glucuronidation is a microsomal process and is primarily occurring in the liver, but UDPGT activities have been detected in kidney, intestine and gill of some species (George 1994). Muscle with approximately 80% of total body weight has not been reported to show glucuronidation activities. Xenobiotic conjugation via glutathione may also involve tissues that have high basal GST activities, as does white muscle in bullheads (Chapters 2,4). Also cytochrome P4501A activities are highest in the liver concomitantly with highest conjugation activities (George 1994; Stegeman & Hahn 1994). Liver somatic index is greater than double in bullheads compared with rainbow trout (Chapters 2,4), detoxication functions of liver over whole body may thus be higher in bullheads. The high GSH conjugation activities in liver as well as in most tissues may further aid in overall detoxication capacity in bullheads. Efficient elimination of xenobiotics in bullheads is further supported by the fact that muscle from St. Lawrence River bullheads (Chapter 2) contains markedly lower total PCB contents than hatchery reared rainbow trout, which are raised in clean water (Chapter 3). The induction of EROD and GST activities may contribute to a lower PCB accumulation in the St. Lawrence River bullheads.

Brown and black bullheads are related species and are known to hybridize (Scott & Crossman 1973). Both species show like channel catfish and white sucker higher tolerance to polluted

waters than other species, including rainbow trout. The view that survival in polluted systems is a result of biochemical adaptation has been proposed. That is higher pollution tolerance in aquatic macrophytes has been reported to result primarily by increased peroxidase activity and/or glutathione dependent detoxication (Roy 1994). Suggested detoxication routes of bullheads and trout are outlined in Figure 9.1. Increased protection by GSH conjugation in the whole organism of teleosts may play an important role in such pollution tolerance and should be considered for ecotoxicological and aquacultural studies.

9.1.2. Environmental implications

Exposure of rainbow trout to high PCB contamination resulted in high tissue PCB concentrations, but relatively unchanged xenobiotic metabolism and antioxidant defenses (Chapter 3). The lack of a biochemical response to the PCB loading in the same fish is further supported by unaltered physiological parameters (bone calcium, muscle water, plasma cortisol content) and carbohydrate metabolism (Cano 1996). The PCB congeners freed by dredging are not all coplanar or monoortho coplanar PCBs and mediation of biochemical effects through the Ah receptor may occur only to a limited extent. Sensitive indicators or biomarkers such as EROD activity are thus not fully effective. Toxic effects of PCBs are primarily thought to be mediated through the Ah receptor (Safe 1994) and approaches using the toxic equivalency

Fig. 9.1. Hypothetical model of possible detoxication pathways activated in rainbow trout and (brown and black) bullheads exposed to PCBs. Continuous arrows indicate direct effect, dashed arrows possible direct effects. Dotted lines indicate possible influence by factors described at left hand side (i.e. continuous expose to complex PCB mixtures inhibit biochemical adaptive response, short-time interval post PCB exposure to assess biochemical response). See list of abbreviations for parameters and text for further detail. * Factors as described by Safe 1994.

Xenobiotic (PCB)
entry via
-ingestion
-absorption by gills
-absorption by skin?

CYP1A → **GST** *(most tissues)* ← *synthesis?*

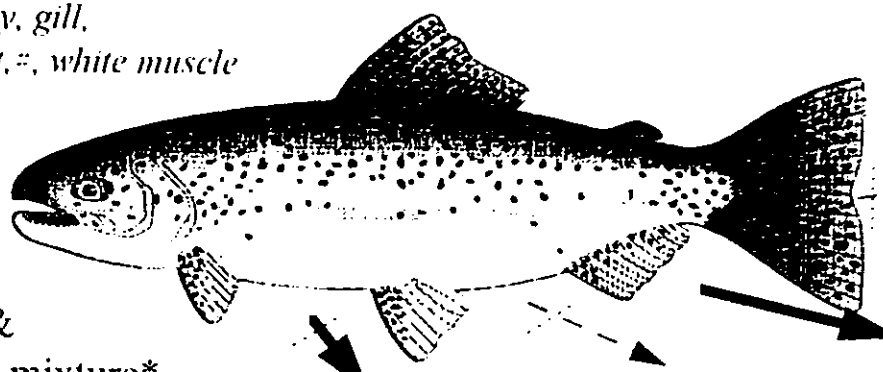
(liver, kidney, other) UDPGT *(liver, kidney, other?)*

GSH *(most tissues)*

GPX
SOD?
CAT?

tissues of interest:
liver, kidney, gill,
gut≠ , heart,≠, white muscle

biochemical &
toxic responses
of PCBs depend on:
-chlorinated content &
purity of commercial mixture*
- animal species*
- age of animal*
- route & duration of exposure*
- time interval post exposure



CAT
SOD *(White muscle, other)*
GPX

GSH *(most tissues)*

CYP1A → **UDPGT**

(liver, kidney, other) *(liver,kidney, other)*

GST *(liver, other)*

GR, ***synthesis?***

factor (TEF) are limited due to antagonistic interactions between PCB congeners (Harper *et al.* 1995). Although no significant changes could be observed from the multiple parameters assayed (Chapter 3; Cano 1996), the high PCB contents accumulated in trout muscle indicate that the health status of the fish may be altered or the trout are highly tolerant to PCBs.

It may be argued that this impaired inducibility of parameters is a species dependent response. Results from a preceding study showed that caging black bullheads and rainbow trout at the GM site without dredging for 35 days in the previous fall had no effects on trout and a 40 % increase in bullhead hepatic EROD activities. GST activities and antioxidant responses were little responsive in both species (Moon *et al.* 1996; Otto *et al.* 1996b). Such lack of biochemical response could be a result of possible antagonistic effects of congeners in such complex PCB mixtures. However, well described AhR agonists such as TCB have little effect on tissue antioxidant defenses during short term studies (Chapters 6-8). In contrast, the same single TCB exposure causes significant alterations of GSH contents and antioxidant enzymes in various tissues of trout (Chapter 5). These studies differ primarily by the time interval post TCB exposure. Using a similar protocol of TCB injection, significant biochemical alterations of vitamin A metabolism in rainbow trout were only observed 8 weeks and not 7 days post TCB injections (Gilbert *et al.* 1995). Similarly, a lack of biochemical response was observed 4 days post xenobiotic exposure, but GGT enzyme activation occurred 14 weeks post injection in TCDD intubated rats, although EROD activities were induced at both time periods (Fan & Rozman 1995). Such studies clearly indicate that various xenobiotics have a sustained effect on an organism, which should not be neglected for the assessment of toxicity studies of PCBs and related compounds. Continuous exposure to high PCB contaminated areas may also impair the

inducibility of biochemical parameters (Chapter 3). Caging of contaminated fish in clean waters for a few weeks and analysis of biochemical parameters may give further insight into the mode of xenobiotic induction.

Rainbow trout caged at the control site also significantly bioconcentrated PCB congeners with caging time (Chapter 3). Although total PCB contents are still relatively low, prolonged caging for several months may increase PCB levels. Members of the Mohawk community have had a long-term interest to commence aquacultural projects at this site and nearby less polluted areas (Buttner *et al.* 1996). The accumulation of pollutants as well as fish health should be carefully evaluated when large scale projects begin.

9.2. Features of piscine glutathione metabolism

The use of GSH dependent enzyme changes in teleosts has been proposed for environmental monitoring studies and their evaluation as biomarkers (Winston & Di Giulio 1991; Förlin *et al.* 1995). Basic studies summarized observations on basal GSH status and enzyme activities (for review see Otto 1992; Filho *et al.* 1993; Lemaire *et al.* 1993), tissue GSH turnover (Gallagher *et al.* 1992) and several species comparisons (Hasspieler *et al.* 1994 a,b; Di Giulio *et al.* 1995). Piscine GSH metabolism resembles that of mammals to a great extent, the few differences found (Chapters 4,6,7) should be further evaluated for toxicological and aquacultural studies. Although the present studies did not provide any activities of GSH synthesizing enzymes, exposure to TCB has been consistently shown to increase hepatic GSH content (Chapters 5,7,8). Regeneration of GSH by GR may also contribute markedly to such elevated levels (Chapter 5; Fig. 9.1). However, increased synthesis in liver under xenobiotic stress could be a prime mechanisms. Liver could then

export larger quantities of GSH to other tissues, especially muscle under stress by ROS (e.g. by xenobiotics). Such a mechanism is widely described in mammals (Deneke & Fanburg 1989; Ji 1995). Hepatic GSH synthesis is favoured by higher availability of cysteine, which can also be formed from methionine by the transsulfuration pathway in the liver (DeLeve & Kaplowitz 1991). The transsulfuration pathway may be equally active in teleosts, and teleost liver may play a similar important role for GSH export in whole body GSH homeostasis. However, the different distributions of tissue GSH contents (Chapter 6) support differences compared with the mammalian system.

Activities of glutathione reductase (GR) appear to be of major importance in rainbow trout (Chapters 4,5). TCB exposure results in marked shifts in the GSSG/GSH ratio (Chapters 7 and 8). Regeneration of GSH via GR prevents tissue loss of GSSG and ensures availability of amino acid components of GSH, provided sufficient NADPH is present for GR activity. The marked inducibility of GR under sustained xenobiotic stress (Chapter 5) invites further study. In contrast, bullhead contains markedly lower GR, but higher GPX, activities than trout (Chapter 4). The active conjugation with GSH in bullhead (Fig. 9.1) consistently withdraws GSH from the system, active GSH synthesis is thus of prime importance for tissue GSH replenishment. Markedly active GSH synthesis in this species may be further supported by observations of Di Giulio and coworkers (Gallagher *et al.* 1992), who showed little suppression of hepatic GSH contents by BSO alone in the bullhead relative, the channel catfish.

The activities of GR, GPX and SOD in gill are markedly influenced 6 days post TCB exposure and suggest prime importance of this tissue in detoxication. Gill GSH content further appears to be less susceptible to fluctuations, e.g. by massive GSH manipulation either by depletion (BSO) or

GSH supplementation, than liver or kidney (Chapters 6,7). The stronger responses of liver and kidney to BSO or exogenous GSH may be more as a result of the high synthesis of GSH in liver and high renal GGT activities. The negligible cytochrome P450 metabolism in gills of teleosts may be compensated for by conserved GSH status and antioxidant enzymes.

The efficient GSH delivery of GSH to fish tissues (Chapters 6-8) appeals for interest in using fish models in various research fields (toxicology, pharmacology, carcinogenesis, aquaculture etc.). Direct effects of pro-GSH agents (lipoate, NAC, etc.) under stresses could be compared to a direct GSH (GSH supplementation) effect. Fish are presently the only reported species (Otto *et al.* 1996a; Chapters 7,8) which allow a direct comparison of such agents vs. a directly GSH enhanced system. Moreover, with the increased investigation of immunological functions of GSH (Dröge *et al.* 1994), GSH supplementation may be efficiently used in the aquacultural industry. Several pathological states, which impair the immunological system and are extensively reported in salmonid aquaculture (Thorarinsson *et al.* 1994), may be ameliorated in tissues with enhanced GSH contents.

This GSH delivery to tissues raises questions of possible mechanisms for effective uptake of GSH by fish cells. Cultured mammalian epithelial and endothelial cells use exogenous GSH to reduce cystine to cysteine, which is rapidly transported by GGT and used as a substrate for intracellular GSH synthesis (Deneke *et al.* 1995). Tissue GSH content increased in mammals only by administration of GSH mono (glycyl) methyl or ethyl esters (Meister 1991). It remains to be resolved whether GSH uptake in fish is a result of GGT activities or some other transport mechanism(s). A unique transport system for GSH is found in mammalian mitochondria (Meister 1995). Mitochondria contain a separate GSH pool from the cytosol and GSH redox enzymes, but

lack GSH synthesizing enzymes (Griffith & Meister 1985). There is rapid exchange of GSH between the cytosol and mitochondria, which is characterized by a slow net transport and more rapid exchange transport (Meister 1995). However, the precise mechanism of this transport system is little understood and suggested mechanisms include diffusion of GSH into mitochondria through a channel and transport via a high affinity system dependent on ATP or subject to rapid exchange between cytosol and mitochondria (Garcia-Ruiz *et al.* 1995). Rapid increases of intracellular GSH were observed in rainbow trout and black bullhead isolated hepatocytes a few minutes after GSH supplementation (D. Shanghavi, D. Otto, C. Sen, T. Moon unpublished observations). Although the uptake mechanism(s) for GSH into fish cells is presently unknown, commercial availability of GSH spiked fish feed may raise the immunological resistance to diseases in cultured fish species. Such GSH enriched feed may favour improved health of black bullhead, yellow perch and rainbow trout intended for larger scale aquacultural projects at less polluted sites in the St. Lawrence River (Buttner *et al.* 1996).

9.3. Role of tissue glutathione in cytochrome P4501A metabolism

The view that specific ROS function as second messengers in biological systems is relatively new (Baeuerle *et al.* 1996). Of major interest is the redox regulation of the transcription factors NFκB and AP-1, which have been extensively reviewed (Dröge *et al.* 1994; Baeuerle *et al.* 1996; Sen & Packer 1996). However, the stimulation of AP-1 is opposite to NFκB (Meyer *et al.* 1993). Whereas DNA binding and transactivation by AP-1 is induced by a variety of antioxidants including N-acetylcysteine and thioredoxin, NFκB is activated by H_2O_2. This activation is inhibited by these antioxidants and lipoate (Meyer *et al.* 1993; Sen & Packer 1996). The

activation of NFκB by H_2O_2 has been further suggested to be mediated by intracellular calcium (Sen *et al.* 1996). These findings have introduced a novel function for antioxidants, the redox regulation of gene transcription (Sen & Packer 1996). Whether other transcription factors or transduction pathways are subject to such redox regulation is presently not known.

The TCB induced CYP1A RNA expression (Chapters 7,8) is thought to be solely mediated by TCB binding to the Ah receptor in mammals and fish (Hahn *et al.* 1993; Hahn & Chandran 1996). TCDD has the highest binding affinity for AhR known and it has been questioned, why cells contain a receptor for substances which are human made and of relatively recent origin (Whitlock *et al.* 1996). An endogenous substrate for AhR has been proposed for constitutive gene expression such as CYP1A (Nebert *et al.* 1994) and likely other genes which contain AhRE in their 5'-flanking region (Lai *et al.* 1996). Such endogenous substrate has yet to be identified (Puga *et al.* 1992b). Interestingly, basal expression and catalytic activity of CYP1A is markedly influenced by tissue glutathione status (Chapter 8). Whether BCNU induces CYP1A mRNA expression by ligand binding to AhR or mediated by the alteration of cellular redox status is not known. This cytostatic drug has been analyzed for its ability to induce DNA cross-links and its usage for chemotherapy (Becker and Schirmer 1995; Ali-Osman *et al.* 1996). The additional mechanisms of BCNU actions which include GR inhibition and carbamoylation of GSH may be involved in the cytostatic effect of BCNU, although this has not been reported. Several therapeutic agents such as BCNU, oltipraz, N-acetylcysteine and lipoate alter cellular GSH status (Becker & Schirmer 1995; McLellan *et al.* 1995; van Lieshout *et al.* 1996). However their side actions or interactions with other intracellular pathways is little known; *i.e.* activation or inhibition of cytochrome P450 metabolism may modulate a part of their efficiencies.

Although basal CYP1A metabolism is altered by GSH manipulations, exposure to TCB has contrastive effects (Chapter 8). GSH deficiency induces CYP1A mRNA, but BSO treatment suppresses the inducibility of CYP1A by TCB (Chapter 7,8). Nothing is yet known about the mechanism of action of cellular glutathione status on CYP1A gene expression. Glutathione status may affect the AhR transduction pathways at several steps, the inactive AhR/HSP90 complex, ligand binding to AhR, binding of AhR/ARNT heterodimer to DNA, the transcriptional process or mRNA stability (Fig. 9.2). The *in vitro* sensitivity of DNA binding of AhR/ARNT to redox conditions has been previously shown (Ireland *et al.* 1995). Regions upstream of the human and murine CYP1A1 genes have been identified which are involved in negative regulation of CYP1A1 transcription (Nebert 1994). DNA binding of transcription factors such as NFκB is sensitive to glutathione status (Mihm *et al.* 1995). However, high TCB exposure (5 mg/kg) abolishes an affect of GSH status on CYP1A RNA expression (Chapter 8). Such offsetting effects of TCB may suggest that not necessarily DNA binding, but preceding steps of AhR transduction (ligand binding, translocation) may be affected by glutathione status. However, glutathione status may interfere at multiple steps of AhR transduction and CYP1A gene transcription. These are a series of speculations, which need be further investigated. Figure 9.2 presents a hypothetical model of the regulation of CYP1A

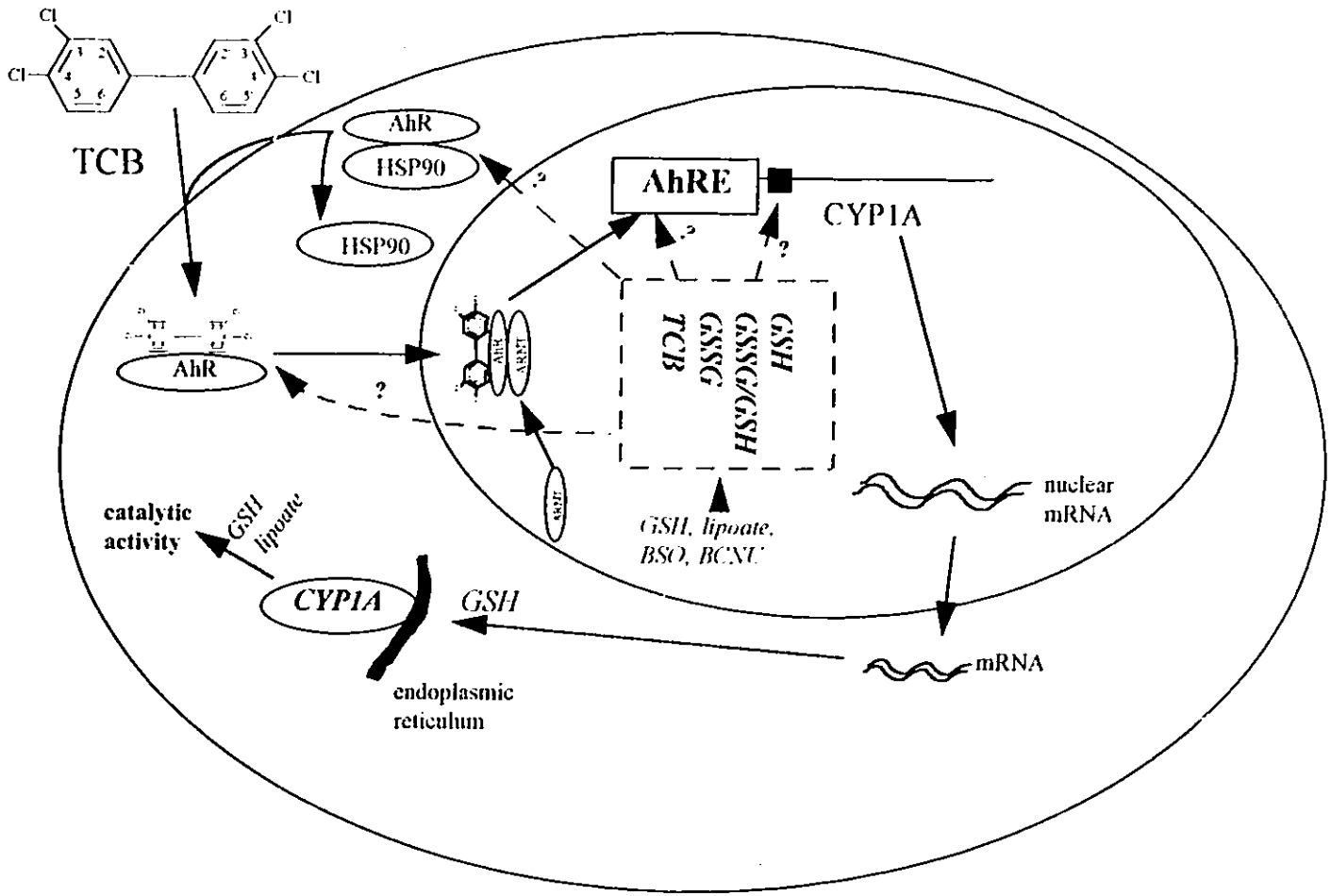


Fig. 9.2. Hypothetical figure of possible mechanisms by which glutathione status influences basal or TCB induced cytochrome P4501A Factors in dashed box may influence AhR complex with HSP90, the liganded AhR or the DNA binding of AhR/ARNT to AhRE leading to altered gene expression. Another possibility for redox sensitive regulation would be a sequence upstream of CYP1A that interferes with negative regulation of the gene (solid box), which is reported for mammalian species (Nebert 1994). These sites of possible redox-sensitivity have currently no or little empirical evidence (Ireland *et al.* 1995) (thus indicated by question marks and dashed lines) Elevated GSH content may increase CYP1A protein expression and/or catalytic activity. Lipoate influences CYP1A catalytic activity See list of abbreviations for parameters.

metabolism by glutathione.

Lipoate suppressed CYP1A expression in 1 mg/kg TCB exposed trout (Chapter 7), but not in 0.1 or 5 mg/kg TCB exposed fish (Chapter 8). The experimental design differed little between Chapters 7 and 8, yet it appears there are differences in the hepatic redox status between both experiments. Whereas hepatic GSSG levels and GSSG/GSH ratio were low in BSO treated and lipoate supplemented groups which showed suppressed CYP1A expressions (Chapter 7), GSSG contents and GSSG/GSH ratio markedly increased in TCB-exposed lipoate-supplemented fish (Chapter 8). Some reports suggest that transduction pathways could be regulated by GSSG along with GSH. NFκB activity appears to be sensitive to cellular GSSG contents (Galter *et al.* 1994; Mihm *et al.* 1995). At low GSSG levels, NFκB cannot be optimally activated, whereas high GSSG levels inhibit the DNA binding activity of NFκB (Dröge *et al.* 1994). Such sensitivity to GSSG may be lost by high concentrations of TCB (BSO-treated trout in Chapter 8). Whether GSSG is a possible candidate for CYP1A expression or AhR transduction is not resolved. Present data, however, suggest that GSSG along with GSH content and ligand concentrations may be important for CYP1A RNA expression. Pharmacological and toxicological experiments should thus investigate cellular glutathione or thiol redox status and not only GSH content.

The inhibition of TCB induced EROD activities in BSO-treated and lipoate-supplemented trout (Chapters 7,8) suggests a second step for regulation of CYP1A metabolism by glutathione. Increased GSH contents significantly potentiate EROD activity (Chapters 7,8). Yet GSH status alone may not be responsible for this step. Heme oxygenase is induced by cellular oxidative stress conditions (Keyse & Tyrell 1989) and plays an essential role in heme catabolism by cleaving heme from heme containing substances such as cytochromes P450. Also increased nitric oxide has been

associated with such protein-bound heme destruction (Kim *et al.* 1995). Whether these parameters are influenced by tissue BSO treatment or lipoate supplementation is not known. Oltipraz has been suggested to behave as a competitive inhibitor to EROD activity (Langouët *et al.* 1996). However, the precise actions of lipoate or oltipraz on EROD activity need to be investigated.

Present results (Chapters 7,8) further confirm a previously proposed cross-talk between the glutathione and the cytochrome P450 systems (Otto *et al.* 1996a). There may be other transduction pathways that are also sensitive to the tissue GSH or thiol pool. A cross-talk between AhR and steroid mediated transduction has been described (Harper *et al.* 1994b; Khara & Saatcioglu 1996). We have previously shown that cortisol metabolism is altered in TCB exposed rainbow trout (Vijayan *et al.* 1996). Plasma cortisol concentrations are significantly elevated in TCB-exposed GSH-supplemented rainbow trout (T. W. Moon, unpublished data). Such preliminary results invite further studies on other redox sensitive transduction pathways. Suitable models for such studies are teleost fish which make exogenous GSH available to tissues.

An important question which needs to be addressed remains. What biochemical changes occur first with PCB exposure? Safe (1994) suggests that biochemical activities of PCBs are primarily initiated through AhR signal transduction. Present results assessed AhR mediation only indirectly by induction of CYP1A metabolism. CYP1A catalytic activity was induced by PCBs in most studies (Chapters 2,5,7,8), and short-term exposures observed as first biochemical alterations induced CYP1A metabolism (Chapters 7,8). AhREs are found in a number of genes (Lai *et al.* 1996) and ligand bound AhR may induce gene expression of several other genes, which may be important for the fate of PCBs in an organism. Antioxidant enzymes are little affected in short-

term assessment of PCBs (Chapters 6-8). However, present results showed that tissue TGSH content and/or glutathione redox status are altered in PCB exposed teleost fish during short- and long-term assessments (Chapters 2,3,5,7,8). These changes may occur simultaneously with CYP1A induction and determine the ultimate inducibility of CYP1A metabolism by PCBs.

9.4. Conclusions

This series of investigations suggests that:

- contaminant exposed bullheads from the St. Lawrence River adapt to chemical loading by increased EROD and GST activities. At sufficient substrate availabilities of GSH, conjugation with glutathione serves as an efficient detoxication pathway that may enable enhanced pollution tolerance.
- continuous exposure to complex PCB mixtures impairs inducibility of catalytic activities of drug metabolizing and antioxidant enzymes. Similarly, short-term assessment of TCB effects shows little alterations of conjugation and antioxidant enzyme activities. Time interval post PCB exposure should allow assessment of immediate and sustained xenobiotic effects.
- teleost species depend on an active endogenous antioxidant machinery which underlies age and/or maturation related changes.
- long-term effects of single TCB exposure induces complex changes in antioxidant defenses and xenobiotic metabolism. Glucuronidation may be a preferred conjugation pathway in TCB exposed trout. Glutathione reductase activity is of major importance for maintenance of tissue glutathione pool in the reduced state.

- hepatic and extrahepatic tissues (kidney, gills, muscle) are included in detoxication processes in PCB exposed fish. White muscle is likely to play an important role in whole body glutathione homeostasis under basal and stress conditions.
- exogenous glutathione is an efficient delivery agent of GSH to tissues. Tissue enhancement of GSH content may play vital roles in overall antioxidant protection and immunological resistance in cultured fish species.
- tissue GSH status is of prime importance for TCB induced hepatic cytochrome P4501A metabolism. GSH deficiency by BSO and lipoate supplementation suppresses TCB induced CYP1A mRNA expression. GSH supplementation potentiates CYP1A possibly at the protein level or catalytic activity.
- basal cytochrome P4501A metabolism is markedly influenced by hepatic GSH status. Regulation of CYP1A by glutathione status may occur at two processing stages, at a transcriptional level and/or at a posttranslational level. GSSG along with GSH and TCB amounts may be influencing factors.

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