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Mechanisms of the Biochemical Defense against Xenobiotics in Fish: *In vitro* and *in vivo* Investigations

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Mechanisms of the Biochemical Defense against Xenobiotics in Fish:
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Contents

General Introduction

Aquatic toxicology

It has been estimated that 50,000 to 100,000 foreign chemicals (xenobiotics) are currently used (Cooney 1995; Timbrell 1991). These include pesticides, environmental pollutants and industrial chemicals, many of which are toxic. Foreign chemicals are deliberately or unintentionally released to the environment. In consequence plants, animals and humans may get exposed to these compounds. The branch of toxicology that studies the noxious effects of natural or artificial substances on the living organisms that constitute the biosphere was defined as ecotoxicology by Truhaut in 1969 (see Truhaut, 1977; for more recent definitions see Newman, 1998). Within the science of ecotoxicology, aquatic toxicology is the sub-discipline that investigates toxic effects on aquatic organisms, e.g. algae, macrophytes, fish and invertebrates (Rand 1995).

Biochemical defense mechanisms

In organisms, biochemical defense mechanism exist which provide protection against toxic insults, particularly at low levels of exposure. It is generally assumed that these mechanisms emerged during the biological evolution as a defense against naturally occurring toxic compounds, such as combustion products or toxic metals, and the secondary metabolites of animals, plants and microorganisms (Roesijadi 1992; Stegeman & Hahn 1994; Walker & Mackness 1983). Biochemical defense mechanisms play an important role in determining an organism's susceptibility against the toxic action of anthropogenic xenobiotics. Following the uptake of a xenobiotic, and depending on the nature and the amount of the xenobiotic taken up, one possible scenario consists in its complete neutralization and/or elimination through the organism's biochemical defense. In an alternative scenario, however, this is not fully achieved, either because the organism cannot detoxify the xenobiotic, or because its capacity for detoxification is overloaded, so that a significant proportion of the xenobiotic may reach target sites and cause toxicity.

An understanding of the biochemical defense mechanisms in aquatic species will widen our mechanistic understanding of toxicity in these biota, and thus, hopefully, increase our ability to predict xenobiotic effects (Chambers & Carr 1995; Di Giulio et al. 1995; McKim & Nichols 1994). In particular, the mechanistic understanding of the biochemical defense in biota can help reveal the mechanisms of toxicity, assist with the identification of sensitive taxa and elucidate the structural features that distinguish hazardous compounds.

Another reason why biochemical variables associated with the anti-xenobiotic defense have attracted special attention in aquatic toxicology is that alterations in such variables can indicate xenobiotic exposure. For instance, xenobiotics may induce and/or inhibit detoxifying enzymes. The term 'biomarker' has been adopted to refer to the use of physiological,

biochemical and histological changes as indicators of exposure and/or effects of xenobiotics at the organismal or suborganismal level (Huggett et al. 1992; Rand et al. 1995).

Among the biochemical defense mechanisms existing in fish, different functional principles can be distinguished. Firstly, certain toxic compounds are scavenged through binding to specific proteins. This reduces the amount of the free form of the xenobiotic, and thus potential adverse effects. Examples of the action of xenobiotic scavengers include the binding of organophosphorus compounds by B-esterases (see below), that of activated epoxides by glutathion-*S*-transferases (Di Giulio et al. 1995) and that of metals by metallothioneins (Roesijadi 1992; Waalkes & Goering 1992). A second principle of biochemical defense is the enzymatic metabolism of xenobiotics, also called biotransformation (Buhler & Williams 1988; Di Giulio et al. 1995). Generally, biotransformation converts chemicals to less toxic products (detoxification). In some cases, however, biotransformation enzymes activate compounds with a low inherent toxicity to highly toxic metabolites (Buhler & Williams 1988; Sijm et al. 1997). Two phases of biotransformation are distinguished. In phase I reactions, polar functional moieties are introduced into the xenobiotic molecule (Di Giulio et al. 1995; Lech & Vodicnik 1985). An important group of phase I enzymes are the cytochromes P450 (see below). In phase II, the xenobiotic or its phase I metabolite is covalently bound to endogenous compounds such as glucuronic acid or glutathione sulfate by different types of transferases (Di Giulio et al. 1995; George 1994). A third principle of the biochemical defense consists of the mechanisms of the excretion of xenobiotics. The products of phase I and II biotransformation are readily excreted by the liver, kidney or gill (Buhler & Williams 1988). However, the cellular plasma membranes of the epithelia forming the boundaries to bile, urine or gill water, respectively, represent permeation barriers for the xenobiotic (metabolite) to be excreted. A major mechanism of the transfer of xenobiotics across plasma membranes during the renal and hepatobiliary excretion is the active transport by carrier proteins (Groothuis & Meijer 1996; Koepsell 1998; Pritchard & Bend 1991; Pritchard & Miller 1993). It has been suggested to consider such carrier proteins as a part of the integrated organismal defense against xenobiotics (Ishikawa 1992; Yeh et al. 1992). P-glycoproteins are an important groups of ATP-dependent carriers expressed in the liver and the kidney (see below). A fourth set of protective biochemical mechanisms consists of the defense systems against reactive oxygen species (Di Giulio et al. 1995). Biota living under aerobic conditions are confronted with reactive oxygen species arising as a byproduct of endogenous metabolism. Furthermore, the metabolism of certain xenobiotics can produce additional reactive oxygen species, e.g. through redox cycling or metabolic pathways that involve the activation of oxygen (Di Giulio et al. 1989; Lackner 1998). Antioxidant defenses essentially include antioxidants, such as water soluble reductants and fat soluble vitamins, and enzymes detoxifying reactive oxygen species and their metabolic products (Di Giulio et al. 1989).

It is noted here that some classes of proteins have multiple roles and cannot be clearly attributed to one of the above principles. For instance, depending on the nature of the

xenobiotic, B-esterases and glutathion-*S*-transferases may either irreversibly bind to the foreign molecule, or mediate its metabolic conversion, i.e. function either as scavengers or biotransformation enzymes (Chambers & Carr 1995; Di Giulio et al. 1995; Walker & Mackness 1983).

Aim of this work

During the past thirty years, considerable knowledge has accumulated concerning the biochemical and cellular mechanism underlying the interaction between fish and xenobiotics (reviewed in: Di Giulio et al. 1995; Myers et al. 1990; Newman 1998; Segner & Braunbeck 1996; Stegeman & Hahn 1994). However, different aspects of the biochemical defense in fish are still only poorly understood. The aim of the present work was to contribute to the understanding of selected unresolved aspects of the biochemical defense in fish.

The first thematic line of the present work deals with the enzyme **butyrylcholinesterase** which has been suggested to function as a scavenger of toxicants (Massoulié et al. 1993). Although butyrylcholinesterase may occur at high levels in fish tissues (Leibel 1988b; Stieger et al. 1989), it has yet attracted little attention in aquatic toxicology. The aim of this work was to characterize the butyrylcholinesterase in a teleost fish, three-spined stickleback (*Gasterosteus aculeatus*), and compare its response to organophosphate insecticides to that of the related enzyme, acetylcholinesterase. The results were expected to allow conclusions about the potential use of BChE as a biomarker of OP exposure.

The second thematic block of the present work is concerned with the effects of xenobiotics on **hepatic biotransformation enzymes** in fish. Hepatic piscine biotransformation enzymes have been the subject of a large number of studies, and a quite detailed knowledge exists, particularly about the biochemistry and molecular characteristics of cytochromes P450 (reviewed in: Buhler & Wang-Buhler, 1998; Goksøyr & Förlin, 1992; Stegeman & Hahn, 1994). Cellular in vitro systems have been used to investigate the effects of xenobiotics on hepatic biotransformation in fish, however, few studies have compared the patterns of responses observed in vitro with those seen in vivo (Monod et al. 1998; Segner 1998). The aim of this study was to validate the suitability of cultured isolated hepatocytes for the assessment of the xenobiotic effects on hepatic biotransformation enzymes in a comparative in vitro / in vivo study, using rainbow trout (*Oncorhynchus mykiss*) as a model species.

The third thematic line of this work deals with the transporter **P-glycoprotein** that has been proposed to function in renal and hepatic excretion. P-glycoproteins are transporters located in the cytoplasmic membrane that mediate the extrusion of lipophilic compounds from the cell (Bellamy 1996; Germann 1996). The presence of P-glycoproteins in different piscine organs, including the liver, has been demonstrated (Cooper et al. 1999; Hemmer et al. 1995). However, no studies exist concerning the hepatic function of P-gps in fish. The aim of the

present work was to characterize the P-glycoprotein in the liver of rainbow trout (*Oncorhynchus mykiss*) and to study its interaction with two model xenobiotics.

Below, the present knowledge on the three subjects of investigation, butyrylcholinesterase, hepatic biotransformation enzymes, and P-glycoprotein, is briefly reviewed, and the design of their investigation in the present study is outlined.

Butyrylcholinesterase

Cholinesterases (ChEs) accomplish the fast hydrolysis of choline esters. In vertebrates, two ChEs exist, acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, also called pseudo-cholinesterase or cholinesterase, EC 3.1.1.8) (Massoulié et al. 1993). The physiological role of AChE is the hydrolysis of the neurotransmitter acetylcholine at cholinergic synapses and neuromuscular junctions (Massoulié et al. 1993). AChE represents the target of the neurotoxic action of insecticidal organophosphates (OPs) and carbamates, which are also called anti-cholinesterase insecticides (anti-ChEs) (Fukuto 1987).

Apart from the role of some esterases as toxic targets, esterases can provide protection against xenobiotics by different mechanisms. On the one hand, esterases may cleave xenobiotic compounds containing ester bonds. For instance, A-esterases (Aldridge 1953) catalyze the hydrolysis of OPs (Chambers & Carr 1995; Landis 1991), while carboxylesterases (CaEs) cleave pyrethroid insecticides, phthalate esters and certain herbicides (Barron et al. 1999; Walker et al. 1996). On the other hand, esterases may act as scavengers of xenobiotic compounds. B-esterases have operationally been defined as esterases sensitive to OPs (Aldridge 1953) and comprise the ChEs and the CaEs. B-esterases dispensable for survival, such as CaE and BChE, can potentially provide a protection against OP inhibitors of AChE by getting themselves irreversibly inhibited by the OP compound, thus reducing the amount of the free inhibitor available for an attack on AChE (Chambers & Carr 1995; Massoulié et al. 1993; Maxwell 1992).

The properties and tissue distribution of BChE in fish are highly variable among species (Lundin 1962; Magnotti et al. 1994; Sturm et al. 1999). BChE often shows atypical properties in fish (Leibel 1988a; Lundin 1962; Lundin 1968; Toutant et al. 1985). Very little is known on the interaction of piscine BChE with xenobiotics. In some fish, BChE occurs at high levels in the skeletal muscle (Leibel 1988b; Stieger et al. 1989) and is extremely sensitive to OPs in vitro (Magnotti et al. 1994; Sturm et al. 1999).

In the present work, the interaction with OPs of the AChE and the BChE of the fish three-spined stickleback (*Gasterosteus aculeatus*) was investigated. In a first step, the AChE and the BChE from stickleback were characterized and their sensitivity to selected OPs recorded in vitro. Then, the response of stickleback AChE and BChE to OPs was investigated in the field in stickleback populations from streams differing in OP pollution (Chapter 1). Finally, the response of stickleback AChE, BChE and CaE to the OP parathion were observed following the exposure of stickleback in the laboratory (Chapter 2).

Biotransformation enzymes

Fish possess in principle the same classes of biotransformation enzymes as higher vertebrates, though significant differences may exist between fish and mammals concerning the levels and specificity of these enzymes. The most prominent phase I (i.e. non-synthetic) biotransformation enzymes in vertebrates are the cytochromes P450 (P450s, or CYPs). The ongoing molecular characterization of P450s in fish has revealed that they display an array of isoforms as complex as that observed in mammals (Buhler & Wang-Buhler 1998; Stegeman & Hahn 1994). The most important piscine phase II (i.e. conjugative) biotransformation pathways are the conjugation with glutathione and with glucuronic acid, while acetylation, amino acid conjugation and sulfation appear to play minor roles in fish (George 1994). The conjugation with glutathione, accomplished by glutathione-*S*-transferases, is the primary route of metabolism for electrophilic compounds, while nucleophilic compounds preferably undergo glucuronidation, accomplished by UDP-glucuronyltransferases (Clarke et al. 1991; George 1994; Nimmo 1987).

Cytochromes P450 form a large superfamily of heme thiolate proteins catalyzing the $NADPH/O_2$–dependent oxidation of endogenous compounds, such as fatty acids, steroids, prostaglandins and leukotrienes, and exogenous chemicals, such as plant metabolites, drugs, pesticides, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Gonzalez 1989; Nebert & Gonzales 1987). P450-dependent reactions are monooxygenase reactions in which one oxygen atom, originating from molecular oxygen, is inserted into the substrate (Gonzalez 1989). The broad substrate and reaction specificity of the P450-dependent monooxygenase system reflects the existence of multiple forms of P450. The present P450 nomenclature is based on primary amino acid sequences (Gonzalez 1989; Nebert & Gonzales 1987). Following the abbreviation 'P450' or 'CYP', gene families are designated with arabic numbers (or, in the older literature, roman numerals), followed by a capital letter indicating the subfamily and an arabic number denoting the individual gene (e.g., CYP1A1). In animals, 37 cytochrome P450s families are known to date, of which 16 exist in mammals and 8 in fish (Nelson 1998).

P450s have attracted special attention because they are important factors in the detoxification and/or toxic activation of xenobiotics. In mammals, the primary hepatic drug-metabolizing P450s are found among the members of the families 1 to 4 (Gonzalez 1989), all of which exist in fish (Nelson 1998). As in mammals, the CYP1A proteins are induced in fish by aromatic coplanar compounds which comprise various environmental chemicals, such as polychlorinated dibenzo-*p*-dioxins and -furans (PCDDs and PCDFs), polycylic aromatic hydrocarbons (PAHs), and certain polychlorinated biphenyls (PCBs) (Goksøyr & Förlin 1992). The measurement of CYP1A induction in feral fish is used in monitoring programs to diagnose the environmental exposure to such inducers (Foureman et al. 1983; Goksøyr & Förlin 1992; Payne et al. 1987). By contrast to CYP1A, other P450 isoforms strongly differ

between mammals and fish with respect to their inducibility (Buhler & Wang-Buhler 1998). Phenobarbital-type inducers, acting on CYP2Bs in mammals, have no effects in fish (Buhler & Wang-Buhler 1998; Lech & Bend 1980; Stegeman et al. 1990). Dosing of trout with glucocorticoids, inducers of the CYP3A family in mammals, led to inconsistent results (reviewed by Buhler & Wang-Buhler, 1998). Peroxisome proliferating agents, inducers of CYP4A in rodents (Nebert & Gonzales 1987), induce putative CYP2 isoforms in some fish, such as channel catfish (*Ictalurus punctatus*) and bluegill (*Lepomis macrochirus*) (Haasch et al. 1998a; Haasch et al. 1998b), but remain without effect in others, such as rainbow trout (*Oncorhynchus mykiss*) (Buhler & Wang-Buhler 1998).

The interaction of xenobiotics with cytochromes P450 can have profound toxicological consequences. On the one hand, piscine P450s, including CYP1A and CYP2K1, accomplish the activation of different protoxicants and procarcinogens (Buhler & Wang-Buhler 1998; Stegeman & Hahn 1994; Williams et al. 1998). Hence, an induction of the responsible CYP isoforms may result in adverse toxicological effects. Moreover, apart form induction, xenobiotics may cause the inhibition of biotransformation enzymes, or compete with endogenous substrates for cofactors. Because of the role of P450s in metabolism of endogenous compounds that have a regulative function, such as steroid hormones, all of the above xenobiotic interactions with P450s have the potential to result in severe metabolic disorders.

Cultured hepatocytes from teleost fish have successfully been used to study the interactions of foreign chemicals with biotransformation systems in fish (Monod et al. 1998; Pesonen & Andersson 1997; Segner 1998). Such studies, however, have largely focused on CYP1A and have rarely been validated by parallel in vivo studies. In Chapter 3 of the present work, two model water pollutants, the imidazole fungicide prochloraz and the detergent degradation product nonylphenol diethoxylate, were investigated concerning their effects on hepatic biotransformation variables in a comparative in vitro / in vivo approach, i.e. comparing the responses between cultured rainbow trout hepatocytes and juvenile rainbow trout (*Oncorhynchus mykiss*). The selected biotransformation variables included the levels of CYP1A, determined by a direct ELISA and as the catalytic activity 7-ethoxy-*O*-deethylase (EROD), and the hepatic metabolic profile of testosterone. The latter variable reflects the activities of constitutive P450s other than CYP1A and of UDP-glucuronyltransferase.

P-glycoprotein

P-glycoproteins (P-gps) are membrane transporters encoded by the highly conserved MDR (multidrug resistance) gene family (Bellamy 1996; Germann 1996) which forms a subgroup of the large ATP-binding cassette (ABC) protein superfamily (Higgins 1992). The overexpression of mdr1-type P-gps in certain tumors is one mechanism of the resistance of cancers to combination chemotherapy (multidrug resistance) (Bellamy 1996; Germann 1996). By contrast to P-gps of the mdr1 type, mdr2-type P-gps are not related to multidrug resistance

(Oude Elferink et al. 1996; Ruetz & Gros 1994). In addition to their occurrence in tumors, P-gps are also expressed in normal mammalian tissues. A high level of P-gp expression is found in the adrenal, while intermediate levels are found in small and large intestine, liver and kidney (Fojo et al. 1987; Thiebaut et al. 1987; Thiebaut et al. 1989). The localization of P-gp to the apical poles of epithelial cells in most of these organs suggests a physiological role in secretion and/or excretion. Further lines of evidence suggesting a role of mdr1-type P-gps in the defense against xenobiotics include the inducibility of these P-gps by xenobiotics (Burt & Thorgeirsson 1988; Schrenk et al. 1994; Schrenk et al. 1996) and by the observation that mdr1-type P-gp knock-out mice are phenotypically normal except that they show an increased and sensitivity to certain xenobiotics (Borst & Schinkel 1996; Schinkel et al. 1997).

Little is known on P-gps in lower vertebrates. In fish, the presence of genes showing homology to mammalian *P-gp* genes has been demonstrated (Chan et al. 1989). In immunohistochemical investigations using mammalian antibodies directed against conserved P-gp epitopes, the pattern of specific staining among tissues in fish resembles that in mammals (Hemmer et al. 1995). In Western analyses of fish liver extracts, mammalian P-gp antibodies detect a single band of 170 kDa, i.e. the approximate molecular weight of P-gps (Cooper et al. 1999). Functional studies of renal transport in fish further indicate the presence of a mdr1-like transport mechanism in proximal tubules (Miller 1995; Schramm et al. 1995; Sussman-Turner & Renfro 1995). No information is available, however, concerning functional aspects of P-gp in the liver of fish.

In the present work, the hepatic expression of P-glycoproteins in rainbow trout (*Oncorhynchus mykiss*) was examined by immunoblot and immuniohistochemical techniques. To study the function of P-glycoprotein in trout liver, the accumulation and efflux of the mdr1-type P-glycoprotein substrate rhodamine 123 was analyzed in rainbow trout hepatocytes in the presence and absence of inhibitors of mdr1 P-glycoproteins (Chapter 4). It was further investigated whether trout hepatic P-glycoprotein(s) interacted with selected environmental pollutants, the imidazole fungicide prochloraz and the detergent degradation product nonylphenol diethoxylate (Chapter 5). The interaction of these chemicals with P-glycoprotein function was investigated in vitro with cultured trout hepatocytes, while chemical effects on the hepatic expression of P-glycoprotein(s) in trout were studied in vivo, following the exposure of juvenile trout to sublethal concentrations of the model pollutants.

Outline of the present work

In **Chapter 1**, the cholinesterases are studied with special emphasis on butyrylcholinesterase in a teleost fish, three-spined stickleback (*Gasterosteus aculeatus*). The cholinesterases in the brain and skeletal muscle of stickleback are characterized and their response to inhibition by OP is examined in vitro and in the field. In **chapter 2**, the response of acetylcholinesterase and butyrylcholinesterase is examined following exposure of stickleback to the OP parathion under environmentally realistic conditions in the laboratory.

In **chapter 3**, the effects of selected environmental pollutants on piscine hepatic biotransformation enzymes are investigated in a parallel in vitro / in vivo approach, using rainbow trout (*Oncorhynchus mykiss*) as a model species. The aim of this study was to critically examine the suitability of cultured fish hepatocytes to predict the in vivo effects of xenobiotics on hepatic biotransformation enzymes, with emphasis of cytochromes P450.

In **chapter 4**, the hepatic expression of P-glycoprotein(s) in rainbow trout is examined with immunochemical techniques, and, on a functional level, in cultured rainbow trout hepatocytes with diagnostic substrates and inhibitors of P-glycoprotein. In **chapter 5**, two model environmental pollutants are investigated regarding their effects on the function and expression of hepatic P-glycoprotein(s) in fish.

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Chapter 1

Different Sensitivity to Organophosphates of Acetylcholinesterase and Butyrylcholinesterase from Three-spined Stickleback *(Gasterosteus aculeatus)*: Application in Biomonitoring

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Abstract—*Different cholinesterases from brain and body muscle from the fish three-spined stickleback (*Gasterosteus aculeatus*) were investigated concerning their potential use in the biomonitoring of organophosphate insecticides (OPs). Stickleback brain contained acetylcholinesterase (AChE) but no butyrylcholinesterase (BChE). Body muscle contained both AChE and BChE. In vitro, body muscle BChE was three orders of magnitude more sensitive than brain or body muscle AChE to the OPs paraoxon and dichlorvos. In 1998, cholinesterase activities were recorded in stickleback from eight streams differing in pesticide contamination. Automated on-site samplers that selectively collect water during runoff events were used to obtain water samples for pesticide analysis. The OP parathion was found in stream water sampled during runoff events in five cases. The variation of brain and body muscle AChE activity between streams was not related to water contamination by pesticides. Body muscle BChE was strongly decreased (>85%) in stickleback collected at all streams with previous parathion contamination events. A significant decrease in BChE (78%) occurred also, however, at one stream where other pesticides but no OPs were found. Because stickleback AChE and BChE differed strongly in their in vitro sensitivity and their response in the field, these enzymes should be considered separately in studies with fish.*

Keywords–Fish, Organophosphate, Monitoring, Acetylcholinesterase, Butyrylcholinesterase

INTRODUCTION

The physiological role of acetylcholinesterase (AChE, EC 3.1.1.7) is the cleavage of the neurotransmitter acetylcholine at cholinergic synapses and neuromuscular junctions, thereby terminating the neurotransmitter's effects on the postsynaptic membrane (Massoulié et al. 1993; Taylor & Brown 1994). The toxicity of insecticidal organophosphates and carbamates, also called the anticholinesterase insecticides (anti-ChEs; Fukuto 1987), is based on their inhibition of AChE, which results in interference with proper neurotransmission (Koelle 1975; Taylor & Brown 1994). During the past 30 years, anti-ChEs became increasingly common, partly because of the ban of organochlorine insecticides in many countries since 1970. Consequently, there has been concern about potential adverse effects of anti-ChEs on non-target species.

The measurement of AChE is well accepted as a method to diagnose exposure to anti-ChEs in vertebrates (Hill & Fleming 1982; Stansley 1993). In general, the degree of inhibition by anti-ChEs of AChE is related to the dimension of adverse toxic effects (Coppage & Mathews 1974; Murphy et al. 1968). The use of AChE to diagnose exposure of fish to anti-ChEs was suggested 40 years ago (Weiss 1959; Weiss 1964), so AChE represents one of the oldest biomarkers in fish. Although many studies have been published since then, some aspects of the use of fish AChE in environmental monitoring remain to be resolved. This is

partly due to pecularities of fish cholinesterases when compared to cholinesterases of higher vertebrates.

In addition to AChE, vertebrate tissues may contain the related enzyme butyrylcholinesterase (BChE, EC 3.1.1.8, also called pseudocholinesterase), the physiological significance of of which is unknown (Massoulié et al. 1993). The AChE and BChE, together also referred to as cholinesterase (ChE), display overlapping substrate and inhibitor specificities, but can be distinguished by the use of selective substrates and inhibitors (Silver 1974). In teleost fish, brain ChE appears to consist of AChE exclusively (Kozlovskaya et al. 1993), whereas body muscle ChE may either be comprised of AChE only or of a mixture of AChE and BChE, depending on the species (Kozlovskaya et al. 1993; Leibel 1988; Lundin 1962).

The characterization of fish ChE is complicated by the fact that fish BChEs often show atypical properties (Leibel 1988; Lundin 1968; Stieger et al. 1989; Toutant et al. 1985). The substrate most common for the assay of AChE, acetylthiocholine (AcSCh), is also cleaved by BChE (Silver 1974). In fish, BChE may show a high activity on AcSCh (Stieger et al. 1989; Toutant et al. 1985). Activities on AcSCh should therefore only be referred to as 'AChE' if the absence of BChE has been demonstrated (Habig et al. 1988; Szabó et al. 1992), or if BChE activity has been removed by a selective inhibitor prior to the assay of AChE (Sturm et al. 1999a). Otherwise, activities on AcSCh should be referred to as ChE (Silver 1974).

Fish BChEs vary in their sensitivity to organophosphates (OPs), being extremely sensitive in some species (Magnotti et al. 1994). It was recently found for three marine fish species that BChE was 1100- to 6500-fold more sensitive to OPs in vitro than the corresponding AChE (Sturm et al. 1999a), and it was suggested that fish BChE could represent a biomarker differing from AChE in sensitivity (Sturm et al. 1999a). Furthermore, if there is a presence of AChE and a much more sensitive BChE at the same time and the measured activity is mistakenly attributed to AChE alone, this could be misleading in the interpretation of results. The high toxicological significance of an inhibition of AChE is generally accepted; however, the significance of an inhibition of BChE remains obscure. Consequently, AChE and BChE should be considered separately, and a careful enzymological characterization should be undertaken before these enzymes are used in ecotoxicological studies.

The aim of the present study was to evaluate the combined use of fish AChE and BChE, as already suggested (Sturm et al. 1999a), for the biomonitoring of OPs. To achieve this, ChEs in brain and in muscle from stickleback were characterized and classified as AChE and/or BChE and conditions were defined for the selective assay of AChE and BChE. Then the sensitivity of stickleback AChE and BChE from brain and body muscle towards two anti-ChEs, paraoxon and dichlorvos, was characterized in vitro. Finally, brain AChE and muscle AChE and BChE were measured in stickleback collected from 8 small agricultural headwater streams in the surroundings of Braunschweig, Germany, during two occasions in 1998. The streams differed with respect to various factors, which determined the likelihood of pesticide

entry (type and intensity of agricultural use of the bordering areas, riparian vegetation, soil type, and incline of embankment), so that different levels of environmental exposure of stickleback to OPs and other pesticides were expected among streams. In a previous study in the same area (Sturm et al. 1999b), we found that body muscle ChE in three-spined stickleback *(Gasterosteus aculeatus)* appeared to be decreased at OP-contaminated streams; however, it was difficult to resolve this against the natural variability between streams (Sturm et al. 1999b). In addition to a more thorough investigation of cholinesterases in the present when compared to the previous study, the contamination of the streams was recorded at a higher temporal resolution and with a greater sensitivity. In the present study, water samples for pesticide analyses were collected by automated on-site samplers that selectively took samples during runoff events associated with precipitation (Liess et al. 1999). The patterns of enzyme activities among streams are discussed here in relation to the measured pesticide contamination of the streams and to the in vitro sensitivities of the ChEs. The results suggest that differentiation between AChE and BChE can strongly increase the information obtained in monitoring studies with fish.

MATERIALS AND METHODS

Chemicals

Acetylthiocholine iodide (AcSCh), acetyl-β-(methyl)thiocholine iodide (AcβMeSCh), propionylthiocholine iodide (PrSCh), butyrylthiocholine iodide (BuSCh), eserine hemisulfate, tetraisopropyl pyrophosphoramide (iso-OMPA), and 5,5'-dithiobis(2-nitrobenzoic acid) were obtained from Sigma-Adrich Chemical (Deisenhofen, Germany). Paraoxon-ethyl (paraoxon) and 2,2-dichlorvovinyl dimethyl phosphate (dichlorvos) were obtained from Dr. Ehrensdorfer Chemical, Augsburg, Germany.

Fish

Three-spined stickleback were selected as an indicator species because of their widespread occurrence as a dominant species in small streams in Europe (Illies 1966). Fish were collected in the field by electrofishing and transported to the laboratory in aerated cooled buckets. In the laboratory, fish were stunned by a blow on the head and killed by cervical dislocation. The whole brain and a piece of the trunk from the caudal region was collected for later analysis. Tissue samples were kept on ice during manipulation and stored at –20°C after freezing until the biochemical analyses. Collected adult stickleback had a body weight of 1.60 ± 0.45 g (0.87 - 2.81) (mean ± sd (range)) in June and a weight of 1.56 ± 0.38 g (0.82-2.80) in August. With fish of this size range there was no significant relationship between fish

size and ChE activities (not shown). Adult stickleback are nonmigratory during the summer, especially the territorial males. Initially, it was planned to restrict sampling to territorial males. Because it was not possible to obtain a sufficient number of males at some streams, however, females were also included in analyses. The sex of stickleback had no influence on the different ChE activities (not shown).

Biochemical Assays

Frozen tissues were thawed on ice. The body muscle tissue was separated from skin and bone. Cholinesterase was extracted from tissues by homogenization of the entire brain (~15 mg) or 50 - 150 mg of body muscle tissue in 1ml of buffer (0.1 M potassium phosphate, pH 7.4). The supernatant obtained after centrifugation of the homogenate (4°C, 10,000 × g, 15 min) was used as an enzyme extract. The determination of ChE activity (Ellman et al. 1961) and the characterization of stickleback ChE (Silver 1974) were carried out similarly as described before (Sturm et al. 1999a). For the determination of ChE, AChE or BChE activities, enzyme extracts were preincubated with 5,5'-dithiobis (2-nitrobenzoic acid) (0.5 mM final) in buffer for 5 min at 23°C in a 96-well microplate before the substrate (1 mM Acβ-MeSCh, 2 mM AcSCh, 1 mM PrSChE, or 1 mM BuSCh) was added and the change in absorbance at 405 nm was followed for 5 min. In some measurements, enzyme extracts were incubated (30 min, 23 °C) with inhibitors (eserine, iso-OMPA, dichlorvos, or paraoxon) before assays (see below). Assays were carried out in quadruplicate except in the determinations of the inhibition curves with iso-OMPA, dichlorvos, and paraoxon (see below). In these experiments, one replicate per fish and inhibitor concentration was used because the amount of extract obtainable from one stickleback was limited. Protein concentrations of enzyme extracts were measured by the coomassie blue G method (Stoschek 1990).

The selectivity of substrates and inhibitors for different fractions of cholinesterase (Silver 1974; Usdin 1970) is summarized in Table 1.1. ChE activity, i.e. the sum of AChE and BChE activities, was measured with AcSCh as the substrate. Because stickleback brain lacked BChE (see 'Results' and 'Discussion'), the ChE measurement represented AChE only in the brain. In stickleback body muscle, however, both AChE and BChE contributed to ChE activity (see Results and Discussion). To differentiate between AChE and BChE in body muscle, two parallel measurements were carried out with each sample. Prior to enzyme activity determinations, the sample was preincubated with 10^{-5} M iso-OMPA or in another set of replicates with the carrier ethanol (1% v/v). The activity observed without iso-OMPA preincubation represented the ChE activity and the residual activity after iso-OMPA-preincubation represented the AChE activity of the sample. The BChE activity was calculated as the difference between the ChE and AChE activities of a given sample. The BChE activity was determined in this way for all field-sampled fish. Because of the limited amount of tissue

Table 1.1. Enzymology of cholinesterases, and selectivity of substrates and inhibitors used in this study (Massoulié et al. 1993; Silver 1974; Usdin 1970).

Enzyme		Selective substrate [a]	Optimal substrate	Selective inhibitor
Cholinesterase (ChE) [b]	(EC 3.1.1.7 and/or EC 3.1.1.8)	AcSCh		eserine
Acetylcholinesterase (AChE)	EC 3.1.1.7	AcβMeSCh [c]	AcSCh	-
Butyrylcholinesterase (BChE, also called pseudocholinesterase or non-specific cholinesterase)	EC 3.1.1.8	BuSCh	BuSCh or PrSCh	iso-OMA

[a] AcSCh = acetylthiocholine iodide; AcβMeSCh = acetyl-β-(methyl)thiocholine iodide; BuSCh = butyrylthiocholine iodide; PrSCh = propionylthiocholine iodide

[b] 'Cholinesterase' is not a generic enzymological term but frequently (as here) used in the sense of 'AChE and/or BChE, but not other esterases'. It is noted here that in the older literature 'cholinesterase' is also used synonymous to BChE (EC 3.1.1.8)

[c] in fish, AcβMeSCh may not be selective for AChE (this report, Sturm et al. 1999a)

extract available from one stickleback, another approach to measuring BChE was used in the recording of inhibition curves with paraoxon and dichlorvos. In these experiments, BChE was measured using the specific substrate BuSCh (Table 1.1). For some fish (n = 29), body muscle BChE was measured in both ways, i.e. as the activity on BuSCh and as the difference in activity on AcSCh between iso-OMPA-treated and untreated aliquots of enzyme extracts. Body muscle BChE activities measured in these two ways were highly correlated ($r^2 = 0.896$; $p < 0.0001$); however, neither of these two estimates of BChE correlated with body muscle AChE activity ($p > 0.05$). This demonstrated the equivalence of the two methods used to measure BChE.

To estimate the sensitivity to in vitro inhibition by OPs of stickleback AChE and BChE, two OP insecticides were selected, paraoxon and dichlorvos. Paraoxon is the active metabolite of the common OP insecticide parathion (Boone & Chambers 1997) which was the most frequent anti-ChE in the investigation area. By contrast, dichlorvos does not require metabolic activation. To obtain in vitro inhibition curves of stickleback ChEs by OPs, the residual activity after preincubation of the enzyme with OPs was determined, using AcSCh and BSCh, respectively, as substrates for AChE and BChE. For measurement of body muscle AChE, iso-OMPA was included during the preincubation. Inhibitors were added from stock solutions in ethanol. Controls received equivalent amounts of ethanol (1% v/v final or less).

Investigation Area

The investigated streams were situated in an agricultural environment within a 70-km radius around Braunschweig, Lower Saxony, Germany. The streams had an average discharge of < 0.1 m^3/s and an average current velocity of < 0.35 m/s. The sites varied in type and the intensity of agricultural use of the areas bordering the streams, the width of the riparian buffer

strips, soil types and incline of the embankment. These variations led to differences in the likelihood of surface runoff , which is the major route of entry of insecticides into streams in the investigated area (Liess 1993). Selected physical and chemical parameters were recorded by standard methods, such as conductivity (recorded continuously), water depth, current velocity and water temperature (recorded weekly), pH, total and carbonate hardness, and concentrations of oxygen, ammonium, nitrite, nitrate and orthophosphate (recorded every 2 weeks). None of the general water quality parameters were related to AChE, ChE or BChE activities (not shown).

Water sampling and pesticide analysis

Two types of on-site water samplers, i.e., automated samplers and flood event samplers, designed to selectively collect water samples during runoff events, were used. Runoff during precipitation represents the major source of insecticide entry into the investigated streams. Under conditions of normal discharge, insecticides are usually not present in water at detectable levels. One sampler of each type was present at each of the streams. The automated samplers provided a measurement of the maximum pesticide contamination of suspension-free water by sampling the brief peak contamination levels during runoff events (Liess et al. 1999). Conductivity and water level were continuously monitored by probes integrated into the automated samplers. Water sampling was triggered by a decrease of conductivity greater than 0.5%/min or a increase in water level above that found in absence of precipitation (increase of 5–10 cm, depending on stream). The duration of the main contamination peak was assumed to be approximately 1 h. A detailed description of the dynamics of pesticide contamination during runoff events is provided elsewhere (Liess et al. 1999). The flood event samplers consisted of glass bottles with an inlet pipe for water and an outlet pipe allowing air to escape during sampling. The flood event samplers were installed in the streams, adjusting the position of the inlet pipe in such a way that water was sampled at peak water levels during runoff (5–10 cm above the water level, depending on stream, in absence of precipitation, the same as for the automated samplers). Both types of samplers were checked weekly. During most runoff events, both types of samplers collected water (not shown). Generally, there was a good correspondence of pesticides water concentrations between both types of samplers (not shown). When both samplers collected water samples at the same time from a given stream, the sample with the higher contaminant concentration was considered in this report.

The analyses of water samples were carried out at the Institute for Ecological Chemistry and Waste Analysis, University of Braunschweig, Braunschweig, Germany. Water samples were analyzed for trace concentrations of the organophosphate insecticide parathion-ethyl (parathion), the organochlor insecticide lindane, the synthetic pyrethoid insecticides fenvalerate and deltamethrin, the herbicides bifenox and pendimethalin, and

Table 1.2. Cholinesterase activity on different substrates in stickleback brain and body muscle (n=7).

Tissue	AcSCh	AcβMeSCh	PrSCh	BuSCh
	(nmol . min^{-1} . mg^{-1} protein) [a]			
Brain	10.7 ± 0.9	9.1 ± 0.8	3.3 ± 0.5	nd [b]
Body muscle	136 ± 0.17	1.15 ± 0.16	0.65 ± 0.1	0.57 ± 0.17

[a] Average ± SD
[b] Not detectable

the fungicides epoxiconazole, propiconazole, kresoximmethyl and azoxystrobin. These compounds were selected because they represent the most frequently used insecticides, herbicides, and fungicides, respectively, in the investigated area. Chemical data were determined once because of the limited amount of samples. The water samples were processed by solid-phase extraction using C18 columns (J.T. Baker, Griesheim, Germany). The measurements of pesticide concentrations were made with electron-capture gas chromatography (HP 5990, Series II; Hewlett-Packard, Avondale, PA, USA) and confirmed with gas chromatography /mass spectrometry using negative chemical ionization in a Varian 3400 gas chromatograph (Varian, Walnut Creek, CA, USA) with an HP 7673 autosampler that was directly capillary coupled to the quadrupole mass spectrometer SSQ 700 (Finnigan, Bremen, Germany), with a following quantification limit of 0.05 $\mu g\ l^{-1}$ (Liess et al. 1999).

Processing of data and statistics

The logit model was used to estimate median inhibition concentrations (IC50s) from in vitro inhibition data (Ashton 1972). The IC50 (expressed in M) is related to the bimolecular inhibition constant K_i (expressed in M^{-1} /min) according to the equation: $IC50 = -\ln 0.5/(K_i \,.t)$, with t being the reaction time in min (Johnson & Wallace 1987). Literature results reported as K_i or IC50 of a different reaction time were converted to recalculated IC50 values (t = 30 min) using the above equation.

The variation of different ChE activities in stickleback between streams was assessed by one-way analyses of variance (ANOVAs). After ANOVAs, multiple comparisons between streams were carried out by Hochberg's GT2 test. In all tests, the significance level was set at 0.05. The ANOVAs and associated tests were carried out with SPSS 7.5 for Windows (Statistical Product Service Solutions, Chicago, IL, USA). Enzyme activities were log transformed prior to analysis to increase the homogeneity of variances. The BChE activities were derived as the difference of two measurements (see above). Therefore, when the activity was practically zero, negative values close to zero occurred in some samples. To make a log transformation possible, a constant equal to the lowest negative activity rounded to the next

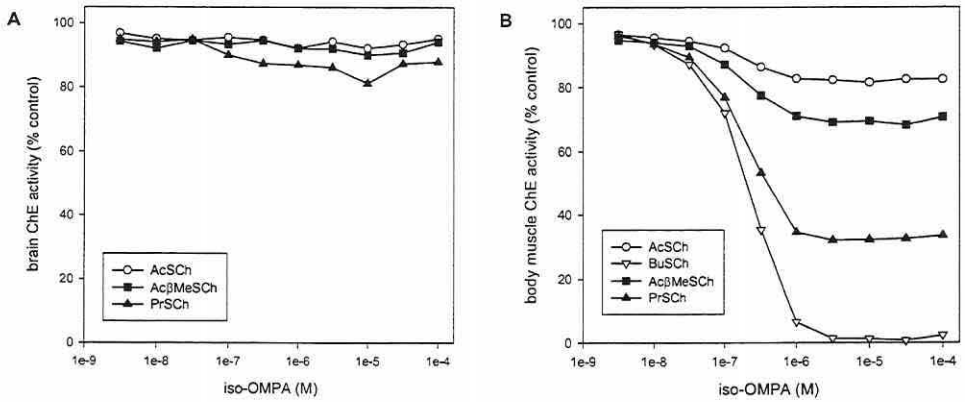

Fig. 1.1. In vitro inhibition effects of iso-OMPA on cholinesterase (ChE) activity in three-spined stickleback (*Gasterosteus aculeatus*) tissues: (A) body muscle (B) brain. Values represent the remainder of activity on different substrates (AcSCh = acetylthiocholine iodide, BuSCh = buryrylthiocholine iodide, AcβMeSCh = acetyl-β-(methyl)thiocholine iodide, PrSCh = propionylthiocholine iodide) after 30 min of incubation with 10^{-5} M iso-OMPA. Symbols represent mean values of at least three independent determinations on different fish, except with brain activities on AcβMeSCh and PrSCh which were based on two determinations. The coefficient of variation between observations was 6% or less (not shown for clarity).

integer (12 nmol/min/mg) was added to all BChE activities before log transformation and ANOVA.

RESULTS

Characteristics and tissue distribution of stickleback ChEs

For the characterization of stickleback ChE, selective substrates and inhibitors were used (Silver 1974; Usdin 1970) (Table 1.1). In brain and body muscle, activity on AcSCh was completely (>96%) inhibited by 10^{-5} M eserine (not shown), indicating that alkylthiocholine-cleaving nonspecific esterases were not present at significant levels. In brain, ChE activity on AcSCh and AcβMeSCh was higher than on PrSCh (Table 1.2). No activity on BuSCh could be detected. In body muscle, ChE activity was higher on AcSCh and AcβMeSCh than on BuSCh and PrSCh (Table 1.2). Brain ChE activity was not affected by iso-OMPA over a broad concentration range (Fig. 1.1A). The effect of iso-OMPA on body muscle ChE depended on the substrate. Concentrations of iso-OMPA of 3.16×10^{-6} M and higher completely inhibited body muscle ChE activity on BuSCh and partially inhibited activities on AcSCh, AcβMeSCh and PrSCh (Fig. 1.1B). An increase of the iso-OMPA concentration from 3.16×10^{-6} to 1×10^{-4} M had no additional effects on activities with AcSCh, AcβMeSCh and PrSCh (Fig. 1.1B).

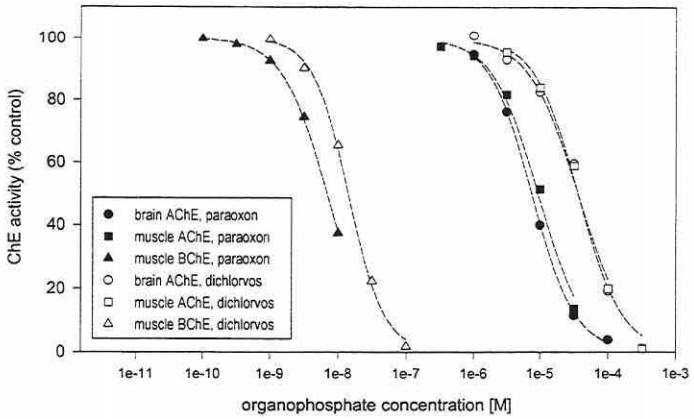

Fig. 1.2. In vitro inhibition of brain and body muscle acetylcholinesterase (AChE) and body muscle butyrylcholinesterase (BChE) by paraoxon and dichlorvos. Values represent the remainder of activity after 30 min of incubation of enzyme extracts with paraoxon or dichlorvos, respectively. Data are the mean of five independent determinations on different fish. The coefficient of variation between observations was 4% or less (not shown for clarity). Acetylthiocholine iodide and butyrylthiocholine, respectively, were used as the substrates for AChE and BChE. Prior to the assay of body muscle AChE, BChE activity was removed by 10^{-5} M iso-OMPA (see Materials and Methods).

In summary, an iso-OMPA–resistant ChE fraction could be distinguished from an iso-OMPA–sensitive ChE fraction in stickleback body muscle. The iso-OMPA–resistant fraction of body muscle ChE showed activity on AcSCh, AcβMeSCh and PrSCh, whereas the iso-OMPA-sensitive fraction of body muscle ChE showed activity on AcSCh, AcβMeSCh, PrSCh and BuSCh. The findings for body muscle are partly in conflict with the assumed specificities of the selective compounds used. Activity on AcβMeSCh, believed to be selective for AChE, should not be affected by iso-OMPA, believed to be selective for BChE (Table 1.1).

In vitro inhibition of stickleback cholinesterases by organophosphates

To estimate the sensitivity of stickleback ChEs to OPs, in vitro inhibition curves were recorded for two OPs, paraoxon and dichlorvos. Paraoxon was selected because it is the active metabolite of parathion, the main OP under use in the investigation area. Dichlorvos was selected to represent an insecticidal OP not requiring metabolic activation. With both paraoxon and dichlorvos, BChE was much more sensitive to OP inhibition than AChE (Fig. 1.2). The IC50s for BChE estimated from the curves in Figure 1.2 were 6.9×10^{-9} M and 1.4×10^{-8} M, respectively, for paraoxon and dichlorvos. The source of AChE (brain or body muscle) had no effect on its sensitivity to OPs (Fig. 1.2). The IC50s for paraoxon estimated from the curves in Figure 1.2 were 9.4×10^{-6} M and 7.5×10^{-6} M, respectively, for body muscle and brain AChE (not significantly different). For dichlorvos the IC50 was 3.6×10^{-5} M

for both body muscle and brain AChE. Accordingly, stickleback BChE was 1,090- to 1,360-fold more sensitive to paraoxon and 2,570-fold more sensitive to dichlorvos than stickleback AChE. For all ChEs tested, dichlorvos was a less potent inhibitor than paraoxon (Fig. 1.2).

Pesticide contamination of the investigated streams

The water sampling for the assessment of pesticide contamination of the streams started 1 week before the first potential insecticide applications were expected (April 10, 1998), and sampling continued until the end of this investigation (first week of August 1998). Water sampling occurred with two types of on-site samplers; one sampler of each type was present at each stream. Both types of samplers, automated samplers and flood event samplers, selectively collected stream water during precipitation events that led to runoffs (see Materials and Methods). Insecticide concentrations detected during runoff-associated contamination events by automated samplers are assumed to represent the peak contamination levels, being present for approximately 1 hour (Liess et al. 1999). Table 1.3 shows the contamination of water samples collected by the on-site samplers during a 7-week interval prior to the first fish sampling (April/May) and during the 8-week interval between the first and the second fish sampling (June/July). The number of samples collected by samplers at a given stream depended on the number of precipitation events sufficient to trigger sampling (see Materials and Methods). Despite the high sensitivity and temporal resolution of the methodology employed, streams 1 and 2 did not show detectable amounts of any of the pesticides analyzed (Table 1.3) and are therefore considered as reference streams. Streams 3 to 8 were contaminated with herbicides (bifenox, pendimethalin) and fungicides (kresoximmethyl, epoxiconazole, azoxystrobin, propiconazole). The organophosphate parathion was found at streams 7 and 8 before the June fish sampling and at streams 6 and 8 between the fish samplings in June and August (Table 1.3). Furthermore, there were two single records of the insecticide lindane at streams 7 and 8 (Table 1.3).

The lack of detectable pesticide contamination at streams 1 and 2 is in accordance with the characteristics of the bordering areas predicting a low probability of surface runoff (sandy soil, low incline of embankment, large width of riparian buffer strip and, at stream 1, low intensity of agriculture). In contrast, bordering areas of streams 3 to 8 were characterized by peaty to clay, sandy to clay, or clay soil, a high to medium incline of embankment, narrow to medium width of riparian buffer strip, and a medium to high intensity of agriculture.

Cholinesterase activities in field sampled fish

Activities of brain AChE and body muscle ChE, AChE and BChE of the sticklebacks from the investigated headwater streams and the results of analyses of variance (ANOVAs) are shown in Figure 1.3. Brain AChE activity did not differ significantly between the streams

Table 1.3. Pesticide contamination of the investigated streams. Pesticide concentrations in water samples obtained with runoff-triggered on-site samplers are summarized for a 7- and 8-week period, respectively, prior to the dates fo fish sampling in June and August.

			Pesticide concentrations[b]							
		number of	OP-insecticides	OC-insecticides	Herbicides		Fungicides			
Period	Stream	samples[a]	Parathionethyl	Lindane	Bifenox	Pendimethalin	Azoxystrobin	Kresoxim-methyl	Epoxiconazole	Propiconazole
Prior to June fish sampling[c]	1	1	nd[d]	nd	nd	nd	nd	nd	nd	nd
	2	4	nd	nd	nd	nd	nd	nd	nd	nd
	3	4	nd	nd	0.03-0.2 (2)	nd	0.2 (1)	0.03-0.2 (2)	0.2-0.3 (3)	nd
	4	4	nd	nd	nd	nd	0.3-0.6 (2)	0.1 (1)	0.3-0.8 (4)	nd
	5	8	nd	nd	0.04 (1)	nd	0.1-0.2 (2)	0.03-0.4 (1)	0.05-0.4 (5)	nd
	6	6	nd	nd	0.2 (1)	nd	0.2 (1)	0.05	0.1-0.7 (3)	nd
	7	8	0.3 (1)	nd	0.05-0.1 (2)	nd	0.07-0.4 (7)	0.08-0.2 (5)	0.1-0.7 (8)	0.08 (1)
	8	6	0.05-0.3 (3)	0.03 (1)	0.2 (2)	0.04 (1)	0.2-1.4 (3)	0.04-0.2 (3)	0.2-0.4 (4)	nd
Prior to August fish sampling[e]	1	4	nd	nd	nd	nd	nd	nd	nd	nd
	2	7	nd	nd	nd	nd	nd	nd	nd	nd
	3	6	nd	nd	nd	nd	nd	nd	nd	nd
	4	3	nd	nd	nd	nd	0.1-0.6 (3)	nd	0.08-0.4 (3)	0.1 (1)
	5	6	nd	nd	nd	nd	nd	0.3 (1)	0.06-0.1 (2)	nd
	6	7	0.2 (1)	nd	nd	nd	nd	nd	0.09 (1)	nd
	7	7	nd	0.2 (1)	nd	nd	0.07-0.3 (5)	0.06-0.3 (2)	0.2-0.8 (6)	0.06-0.08 (2)
	8	4	0.3 (1)	nd	nd	nd	0.2 (1)	0.1 (1)	0.07-0.6 (5)	0.7 (1)

[a] The number of samples depended on the occurrence of precipitation causing a change of water level and/or conductivity sufficient to trigger water sampling by samplers (see Materials and Methods). On-site samplers were active at all streams.

[b] Water concentrations (ranges) are given in µg/l, followed by the number of positive samples in parentheses. The synthetic pyrethroid insecticides deltametrin and esfenvalerat were not found in any of the samples.

[c] Period starting at April 10, 1998, and ending at June 4–9, 1998, depending on the stream.

[d] Not detected.

[e] Period starting at June 4–9, 1998, and ending at August 1–3, 1998, depending on the stream.

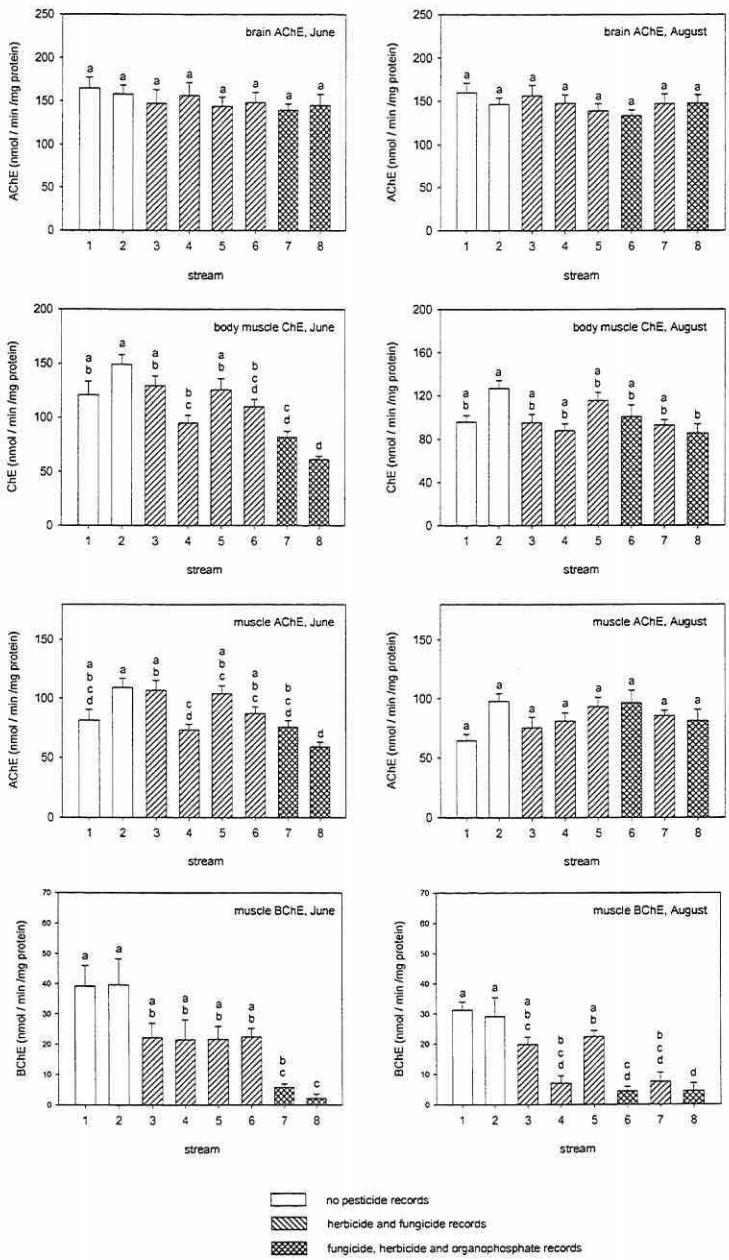

Fig. 1.3. Activity of different cholinesterase fractions in brain and body muscle tissues from sticklebacks sampled in June and August in the field (AChE = acetylcholinesterase, ChE = cholinesterase, BChE = butyrylcholinesterase). Bars represent the mean ± standard error of the enzyme activity observed on 9 – 10 fish. Within each diagram, mean activities represented by bars bearing the same letter did not significantly differ ($p < 0.05$ experimentwise). The filling pattern of bars indicate which types of pesticides were detected at the respective stream in a 7 to 8 week period prior to fish sampling (for details, see Table 3).

either in June or in August (Fig. 1.3). ChE activity in body muscle differed significantly between the streams in both June and August. In June, the body muscle ChE from sticklebacks was significantly decreased at streams 7 and 8, at which the OP parathion was found, but not at streams 3 to 6, at which herbicides and fungicides but no OPs were found, as can be seen by comparing these measurements to those at the reference streams 1 and 2 (Fig. 1.3). In August, none of the contaminated streams 3 to 6 showed stickleback body muscle ChE activities significantly different from that at both of the reference streams (Fig. 1.3). Body muscle AChE varied significantly between the streams in June but not in August. In June, no stream differed significantly from both reference streams in body muscle AChE activity (Fig. 1.3). Body muscle BChE varied significantly between the streams in both June and August. In June, body muscle BChE activity the OP-contaminated streams 7 and 8 was significantly decreased to that of reference streams (Fig. 1.3). In August, body muscle BChE was significantly decreased at streams 4, 6, 7 and 8 compared to reference streams 1 and 2 (Fig. 1.3). Within a 2-month interval prior to the August fish sampling, the OP parathion was found at streams 6 and 8, whereas no OPs were found for streams 4 and 7 in this time interval (Table 1.3). There was, however, a record of parathion at stream 7 in early May, i.e., approximately 3 months before the second fish sampling.

DISCUSSION

Characteristics of stickleback cholinesterases

Stickleback brain ChE showed the properties of AChE (Table 1.1) in all aspects investigated, i.e., it hydrolysed AcβMeSCh but not BuSCh and was resistant to inhibition by iso-OMPA (Table 1.2, Fig. 1.1). We therefore conclude that only AChE, but not BChE was present in brain. The presence of both AChE and BChE in stickleback body muscle is suggested by the activity on both AcβMeSCh and BuSCh (Table 1.2). Furthermore, in body muscle, an iso-OMPA–sensitive fraction of ChE can be distinguished from an iso-OMPA–resistant fraction (Fig. 1.1). However, the sensitivity of the activity on AcβMeSCh towards iso-OMPA in body muscle (Fig. 1.1) is in conflict with the assumed specificities of the diagnostic compounds (Table 1.1).

It was found in earlier studies that certain fish ChEs combine properties of (mammalian) AChE and BChE (Leibel 1988; Lundin 1968; Stieger et al. 1989; Toutant et al. 1985). There has been some controversy concerning how to classify such atypical fish ChEs (Leibel 1988; Lundin 1968; Stieger et al. 1989). Recent progress in the genetics of ChEs revealed that two genes encoding ChEs exist in higher vertebrates, AChE and BChE (Massoulié et al. 1993). Alternative splicing gives rise to different molecular forms of both AChE and BChE; however, the specificities towards substrates and inhibitors are consistent within either group

(Massoulié et al. 1993; Taylor & Radic 1994). All of the atypical fish ChEs known to date were found to occur together with AChE (Leibel 1988; Lundin 1962; Stieger et al. 1989; Toutant et al. 1985). Therefore, these atypical ChEs most probably represent BChEs with somewhat deviant characteristics (Massoulié et al. 1993; Toutant et al. 1985). Consequently, the two fractions of stickleback body muscle ChE distinguished by iso-OMPA can be classified as AChE (iso-OMPA–resistant) and BChE (iso-OMPA–sensitive). We postulate that stickleback BChE atypically cleaves AcβMeSCh.

In vitro inhibition of stickleback ChEs

The paraoxon IC50 found for AChE in stickleback of 9.4×10^{-6} M (this study) is similar to values for rainbow trout (*Oncorhynchus mykiss*) (2.0×10^{-5} M) and fathead minnow (*Pimephales promelas*) (3.0×10^{-6} M) (Johnson & Wallace 1987). The paraoxon IC50 for stickleback BChE (6.9×10^{-9} M, this study) is in the higher range of literature values, which range from 1×10^{-10} M (red snapper, *Lutjanus campechanus*; Magnotti et al. 1994, recalculated) to 1×10^{-8} M (Florida pompano, *Trachinotus carolinus*; Magnotti et al. 1994, recalculated). In stickleback, the in vitro sensitivity to the selected OPs paraoxon and dichlorvos of BChE is much higher than that of AChE. This suggests that a more or less complete inhibition of BChE will precede an inhibition of AChE after exposure to OPs. However, it must be stressed here that the effects of OPs may be modified by several physiological factors and therefore cannot be predicted on the basis of in vitro sensitivities of ChEs alone. Phosphorothionate compounds as parathion require metabolic activation to their toxic oxon analogs, believed to be mainly mediated by hepatic cytochrome(s) P450 (Boone & Chambers 1997). On the other hand, OPs may be detoxified by different enzymes including cytochrome(s) P450, organophosphorus acid anhydrases (also called A-esterases) (Landis 1991) and aliesterases (also called carboxylesterases) (Boone & Chambers 1997; Straus & Chambers 1995). Nevertheless, if, after uptake of OPs and interaction with the above processes, OPs (or their oxon metabolites) reach body muscle tissue, a differential inhibition of AChE and BChE is predicted by the highly different in vitro sensitivities of AChE and BChE.

The physiological function of BChE in fish is unknown (Magnotti et al. 1994). It was suggested that one function of BChE in higher vertebrates is to protect AChE by scavenging ChE inhibitors (Amitai et al. 1998; Massoulié et al. 1993). The much higher sensitivity of body muscle BChE than AChE to certain OPs in the few fish species systematically studied (Sturm et al. 1999a, this report) suggest that BChE may have a similar protective function in fish.

Cholinesterase activities in stickleback from the field and relation to contamination of habitat

The mechanistic basis of the inhibition of ChEs by organophosphate compounds is the irreversible phosphorylation of the enzyme, resulting in deactivation of the enzyme (Usdin 1970). The recovery of decreased ChE activities resulting from OP exposure depends on de novo synthesis of the enzyme and usually takes several weeks in fish (da Silva et al. 1993; Straus & Chambers 1995; Weiss 1959). During the recovery of mosquitofish (*Gambusia affinis*) after exposure to chlorpyrifos, brain AChE recovered to control levels within 45 d, while body muscle AChE was still decreased by 80% after 60 d of recovery (Carr et al. 1997). Therefore, it was expected that the enzyme activities in stickleback populations would integrate the sum of contamination events with OPs from several weeks prior to fish sampling rather than reflect only recent contamination events.

Both brain and body muscle AChE from stickleback showed no apparent relation to the contamination of the habitat. By contrast, body muscle BChE was strongly decreased at all streams that had parathion proofs prior to the sampling of fish (streams 7 and 8 for June fish sampling and streams 5 and 8 for August fish sampling). This suggests that the decreases of BChE reflect parathion exposure of stickleback at these streams. At stream 7, in which no parathion was found after June, the decreased BChE in August could possibly still reflect OP contamination found before the June sampling (Table 1.3). In August, however, BChE was also strongly decreased in fish from streams 4, at which no parathion was found.

The inhibition of BChE at stream 4 could reflect exposure to anti-ChE compounds, which were not detected with the methodology used. The water sampling strategy, using on-site samplers, was designed to get a representative picture of the peaks of insecticide contamination, which are associated with precipitation events. Although this sampling strategy reveals insecticide contamination at a much higher probability than alternative approaches (Liess et al. 1999), it cannot be excluded that minor insecticide entry into the streams had occurred under conditions that did not trigger water sampling. The OP parathion was the only anti-ChE compound analyzed. This reflects that, during the past 5 years, parathion was the anti-ChE used by far most frequently in the investigation area. In the recommendations of the agricultural association (Landwirtschaftskammer, Hannover, Germany) for crops cultivated in the investigation area, parathion was the anti-ChE mentioned most frequently in 1998; however, in some cases, other anti-ChEs were recommended (OP demethon-S-methyl and insecticidal carbamate pirimicarb). Hence, it cannot be excluded that anti-ChE compounds other than parathion had occurred in the investigated streams. More anti-ChEs will be considered in further studies. The detection limit for parathion was 0.05 µg/L in this study. Reported threshold water concentrations of parathion capable of inhibiting AChE in fish range from 0.1 to 1.0 µg/L (Weiss 1964). While the sensitivity of the analytical methodology appears appropriate to detect OP concentrations that may have caused inhibition of fish AChEs, this might not be true for BChE. The much higher in vitro sensitivity of BChE

than AChE to OPs suggests that thresholds concentrations of OPs to effect inhibition may be lower for BChE than for AChE.

Apart from the possibility that some contamination with parathion or other anti-ChEs had remained undetected, decreased BChE in stickleback could potentially also reflect exposure to other pesticides. Not taking into account stream 4 in August (where BChE was strongly decreased; see above), it is striking that BChE was slightly, albeit nonsignificantly, decreased at all other streams at which no OPs but herbicides and fungicides were found (streams 3–6 in June and streams 3 and 5 in August; Fig. 1.3). It was reported that compounds other than anti-ChE can inhibit fish AChE (Davies & Cook 1993; Gill et al. 1990). However, the concentrations required to elicit effects on AChE are comparatively high. Nevertheless, several herbicidal and fungicidal compounds were found at all contaminated streams, and more frequently than the OP parathion (Table 1.3). Consequently, exposure of stickleback to these compounds was probably higher than to parathion. More research is needed before the potential of non–anti-ChE pesticides to inhibit fish BChE can be judged.

Potential use of BChE as a biomarker

The results of this study suggest that, in certain fish species, BChE may react more sensitively to OPs than AChE and should be considered as a biomarker. The presence of BChE in the body muscle of fish has been known for a long time (Clos & Serfaty 1957; Lundin 1962), as has the high sensitivity of some fish BChE for certain OP compounds (Lundin 1968). However, body muscle BChE was scarcely, if at all, considered in ecotoxicological studies with fish. This may partly reflect that BChE does not occur in the body muscle in salmonid and cyprinid fish (Clos & Serfaty 1957; Lundin 1962), which comprise many common test species. However, high concentrations of BChE were found in body muscle tissue of pleuronectid and percid fish (Magnotti et al. 1994), many of which are used in biomonitoring programs (Sturm et al. 1999a; Bocquené et al. 1993). Before BChE can be applied as a biomarker, basic aspects have to be addressed, such as the dose–response relationships for the inhibition of BChE by different OPs and the time course of the recovery of BChE activity after exposure. There were no indications that stickleback populations showing decreased BChE activities were adversely affected by OPs (J. Wogram, personal communication), and BChE may merely represent a marker of OP exposure. Short-term contamination of agricultural streams with OPs, however, may have dramatic effects on aquatic macroinvertebrate populations, which may last for 3 to 6 months (Liess & Schulz 1999; Schulz & Liess 1999). The measurement of BChE in an abundant indicator species as stickleback could be used as a cost-effective screening tool to diagnose water contamination by OPs to protect sensitive aquatic non-target species.

CONCLUSIONS

Butyrylcholinesterase was more sensitive to organophosphate insecticides than AChE in stickleback and should be considered as a potential biomarker. In studies with fish AChE or BChE, the specificity of assay conditions should be carefully checked. To consider AChE and BChE separately appears to be the more appropriate approach in ecotoxicological studies with fish compared to the use of nonselective assay conditions that measure the sum of AChE and BChE (ChE).

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Chapter 2

Effects of parathion on acetylcholinesterase, butyrylcholinesterase and carboxylesterase in three-spined stickleback (*Gasterosteus aculeatus*) following short-term exposure

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Abstract—*The sensitivity of butyrylcholinesterase (BChE) toward the inhibition by the organophosphorus insecticide (OP) parathion was compared to that of other esterases in the fish three-spined stickleback. A previous study (Sturm et al. 2000, Environmental Toxicology and Chemistry 19, in press, chapter 1 of this work) suggested that stickleback BChE represented a more sensitive biomarker of OP exposure than acetylcholinesterase (AChE). In the present study, stickleback were exposed under environmentally realistic conditions, i.e. using a short duration of exposure (1 h) and low parathion concentrations (0.01 µg/l; 0.1 µg/l and 1.0µg/l). Following exposure, stickleback were maintained in clean water for 48 h (recovery), allowing the metabolic activation of parathion. After recovery, the activities of BChE (body muscle, gills, liver), AChE (brain, body muscle, gills) and carboxylesterase (CaE, liver) were determined. The exposure of stickleback to 1.0 µg/l parathion caused a significant inhibition of BChE in body muscle (~30%) and liver (~60%) and a nonsignificant decrease of gill BChE activity (~30%), while it had no effects on the remainder of esterase activities. Lower concentrations of parathion (0.1 µg/l; 0.01 µg/l) had no effect on any of the esterases measured. The results confirm the high sensitivity of stickleback BChE to OPs suggested earlier.*

Keywords—Fish Organophosphate Monitoring Acetylcholinesterase Butyrylcholinesterase

INTRODUCTION

Organophosphorus (OPs) compounds constitute a major class or insecticides presently under use. An advantage of OPs is their low environmental persistence. The high toxicity of OPs, however, represents a hazard for non-target species. Significant amounts of insecticides enter running freshwater systems through the surface runoff from agricultural areas (Ghadiri & Rose 1991). The peak concentrations of runoff-borne insecticides in stream water are in the low µg/l range and present only for short time intervals (hours) (Liess et al. 1999). Despite the transient nature of insecticide contamination in streams, it can cause severe, long lasting population effects on aquatic invertebrates communities (Liess & Schulz 1999; Schulz & Liess 1999). Appropriate monitoring strategies are required to protect sensitive non-target species against the adverse effects of OPs and other insecticides. Automated, runoff-triggered water sampling techniques have been developed to identify insecticide contamination of streams (Liess et al. 1999). Such approaches, however, are expensive in terms of apparatus, maintenance labor and chemical analyses.

Biomarkers, i.e. biochemical, physiological or histological changes indicating xenobiotic exposure and/or effects (Huggett et al. 1992), can afford cost-effective screening tools in the monitoring of pollutant exposure and effects. The measurement of acetylcholinesterase (AChE, EC 3.1.1.7) activity in fish is well accepted as a method to diagnose exposure to OPs (Weiss 1959; Weiss 1964). OPs exert their toxic action through the irreversible inhibition of

AChE (Usdin 1970). Accordingly, the recovery of AChE activity after inhibition by OPs depends on the *de novo* synthesis of the enzyme and usually takes weeks in fish (Straus & Chambers 1995; Weiss 1959). In addition to AChE, butyrylcholinesterase (BChE, EC 3.1.1.8) and carboxylesterase (CaE, also called aliesterase, EC 3.1.1.1) are further types of esterase also irreversibly inhibited by OPs. Both BChE (Sturm et al. 2000, chapter 1 of this work) and CaE (Barron et al. 1999) have been suggested as biomarkers of OP exposure in fish. In a previous study, we showed that in three-spined stickleback (*Gasterosteus aculeatus*) the BChE was more than 1000-fold more sensitive to selected OPs in vitro than the AChE (Sturm et al. 2000, chapter 1 of this work). Stickleback from OP-contaminated streams showed strongly decreased activities of the BChE in body muscle, but no difference in AChEs activities of brain or body muscle, when compared to populations from pristine reference streams (Sturm et al. 2000, chapter 1 of this work). This suggested BChE as a more sensitive biomarker of OP exposure than AChE.

The aim of the present study was to characterize the effects of the OP parathion-ethyl on stickleback esterases under controlled laboratory conditions, using environmentally realistic exposure scenarios. In addition to BChE, AChE and CaE were also recorded. The duration of exposure (1 h) and the pesticide concentrations resembled the conditions met during typical runoff events. The results confirm the high sensitivity of stickleback BChE as a biomarker of OP exposure.

MATERIALS AND METHODS

Chemicals

Acetylthiocholine iodide (AcSCh), butyrylthiocholine iodide (BuSCh), tetraisopropyl pyrophosphoramide (iso-OMPA), and 5,5'-dithiobis(2-nitrobenzoic acid) were obtained from Sigma-Adrich Chemical (Deisenhofen, Germany). Parathion-ethyl (parathion) was purchased as the technical formulation E 605 forte® from Bayer AG, Leverkusen, Germany.

Fish

Juvenile three-spined stickleback (*Gasterosteus aculeatus* L.) were obtained from a pristine stream in the surroundings of Braunschweig, Germany, by electrofishing. For acclimatization, stickleback were maintained in a 600 l tank in well-aerated tap water. Stickleback were fed *Chironomus riparius* larvae from laboratory culture. Maintenance and experiments with stickleback took place in a climate chamber set at 15°C. The water temperature, pH, oxygen concentration and conductivity was measured daily; concentrations of ammonia, nitrite and nitrate every two days.

Exposure experiments

Groups of 6 stickleback were exposed for one hour in 18 l tanks to parathion at the following nominal concentrations: 0.01 µg/l, 0.1 µg/l or 1.0 µg/l. Twice the desired amount of parathion was dissolved in tap water using sonication. At the begin of the exposure, 6 l of the double-concentrated solution was added to the tanks, which contained 6 l of tap water and stickleback, and immediately mixed. Tanks with control stickleback received water only. After exposures, stickleback were briefly bathed in clean water and transferred to 50 l maintenance tanks, in which they were allowed to recover in tap water for 2 days. No overt signs of toxicity were observed. Fish treated with 1.0 µg/l parathion appeared to feed slightly slower than fish in the control groups. After the recovery period, stickleback were stunned by a blow on the head and killed by cervical dislocation. Tissues were excised immediately, rinsed in ice-cold buffer (0.1 M potassium phosphate, pH 7.4), introduced into 1.5 ml Eppendorf containers containing a known volume of ice-cold buffer, frozen, and stored at –80°C pending analysis.

Biochemical analyses

Frozen tissue samples were thawed on ice and homogenized in a Potter device. The supernatant obtained after centrifugation of the homogenate (4°C, 10,000 × g, 15 min) was used as an enzyme extract. The determination of AChE and BChE activity (Ellman et al. 1961) was carried out similarly as described before (Sturm et al. 2000). Briefly, enzyme extracts and reagents (final concentrations: potassium phosphate, 0.1 M, pH 7.4; AcSCh or BuSCh, respectively, 1 mM; 5,5'-dithiobis(2-nitrobenzoic acid), 0.5 mM) were introduced into the wells of a 96-well microplate and the change in absorbance at 405 nm was followed. Assays were carried out in triplicate and at room temperature. For the determination of AChE or BChE activities, respectively, AcSCh or BuSCh were used as a substrate. Prior to measurements of AChE in tissues containing BChE activity, reaction mixtures lacking substrate were incubated with 10^{-5} M iso-OMPA for 30 min prior to assay to inhibit BChE. Protein concentrations of enzyme extracts were measured by the coomassie blue G method (Stoschek 1990). The in vitro sensitivity of different esterases was determined as described earlier (Sturm et al. 2000, chapter 1 of this work). In brief, enzyme extracts were incubated with different concentrations of paraoxon at room temperature in the presence of all assay components except of the substrate. After 30 min, the respective substrate was added and the remainder of activity recorded as described above.

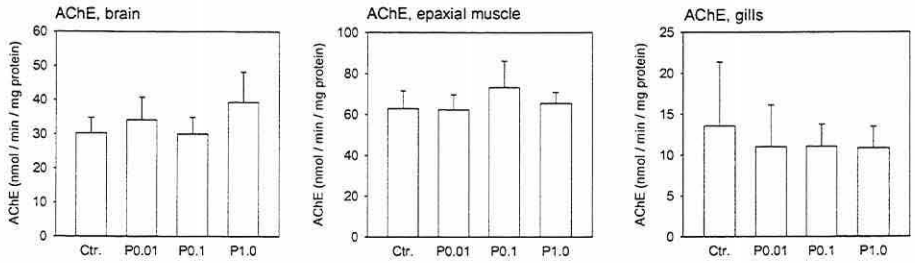

Fig. 2.1. Acetylcholinesterase (AChE) activity in tissues of three-spined stickleback following short-term exposure (1 h) to parathion (P0.01, 0.01 µg/l; P0.1, 0.1 µg/l; P1.0, 1.0 µg/l; Ctr, untreated control group) and recovery (48 h). Values are the mean and standard deviation of enzyme activities of 6 fish. No significant differences were observed between treatments ($\alpha = 0.05$).

Analysis of parathion

At the beginning of the one-hour exposure peroid, water was sampled for later analysis of parathion concentrations. The analyses of water samples were carried out at the Institute for Ecological Chemistry and Waste Analysis, University of Braunschweig. The water samples were processed by solid-phase extraction using C18 columns (J.T. Baker, Griesheim, Germany). The measurements of pesticide concentrations were made with GC/ECD (gas chromatograph HP 5990, Series II; Hewlett-Packard, Avondale, PA, USA) and confirmed with GC/MS using negative chemical ionization in a Varian 3400 gas chromatograph (Varian, Walnut Creek, CA, USA) with an HP 7673 autosampler which was directly capillary coupled to the quadrupole mass spectrometer SSQ 700 (Finnigan, Bremen, Germany), with a following quantification limit of 0.05 $\mu g\ l^{-1}$ (Liess et al. 1999).

Statistical analyses

Results are reported as means ± SD. Mean values were compared between parathion-treated and control groups with Student's T-test.

RESULTS

Juvenile three-spined stickleback (*Gasterosteus aculeatus*) were exposed for 1 h to the OP insecticide parathion (nominal concentrations of 0.01 µg/l; 0.1 µg/l and 1.0 µg/l). For the two higher concentrations, it was confirmed in chemical analyses that the actual concentrations of parathion in the water were in the range of 70 to 80% of the nominal concentrations. Following exposure and 48 h of recovery of stickleback, AChE and BChE

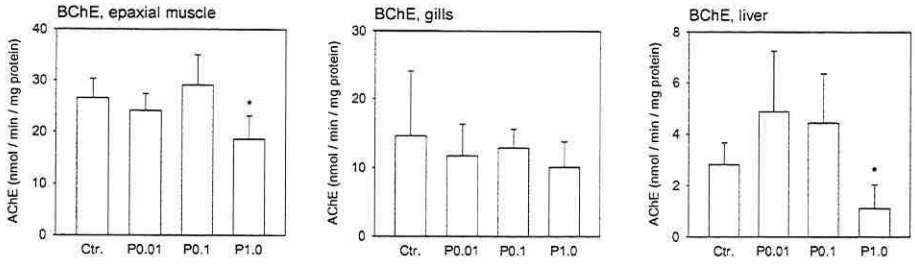

Fig. 2.2. Butyrylcholinesterase (BChE) activity in tissues of three-spined stickleback following short-term exposure (1 h) to parathion (P0.01, 0.01 µg/l; P0.1, 0.1 µg/l; P1.0, 1.0 µg/l; Ctr, untreated control group) and recovery (48 h). Values are the mean and standard deviation of enzyme activities of 5 to 6 fish. Significantly different from corresponding control group: * $P<0.05$.

activities were recorded in brain, body muscle, liver and gills. AChE was detectable in all tissues investigated except liver, while BChE activity was present in all tissues investigated except brain. In addition, CaE was determined in liver, a tissue known to contain high amounts of this enzyme.

In all of the tissues investigated, AChE activity remained unaffected by parathion exposure (Fig. 2.1). BChE was significantly decreased in liver (~60%) and body muscle (~30%) of stickleback exposed to 1.0 µg/l parathion. The degree of BChE inhibition in gills was similar to that in body muscle (~30%) for stickleback exposed to this concentration, however, the difference to controls was not significant for gill BChE (Fig. 2.2). Exposure to the two lower concentrations of parathion (0.01 µg/l and 0.1 µg/l) had no effect on BChE activities (Fig. 2.2). Liver CaE was not changed upon exposure to parathion (Fig. 2.3).

The in vitro sensitivity of stickleback AChE, BChE and CaE to paraoxon is shown in Table 2.1. AChE from brain or body muscle showed median inhibition concentrations (IC50s) for paraoxon of 7.5×10^{-6} M and 9.4×10^{-6} M, respectively, being approximately three orders of magnitude less sensitive than BChE from body muscle or liver (IC50s of 6.7×10^{-9} M and 3.0×10^{-9} M, respectively). The sensitivity of both AChE and BChE was similar between the tissues investigated. CaE in liver showed a paraoxon IC50 of 5.6×10^{-7} M, and thus was 13- and 17-fold more sensitive to paraoxon in vitro, respectively, than brain and body muscle AChE.

DISCUSSION

In the present study, 1 µg/l parathion caused a significant decrease of BChE activity in the liver and body muscle of the stickleback while BChE in gills remained unchanged (Fig. 2.2). The relative decrease of BChE activity was higher in liver than in body muscle and gills.

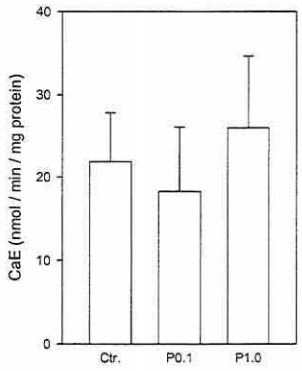

Fig. 2.3. Liver carboxylesterase (CaE) activity of three-spined stickleback following short-term exposure (1 h) to parathion (P0.1, 0.1 µg/l; P1.0, 1.0 µg/l; Ctr, untreated control group) and recovery (48 h). Values are the mean and standard deviation of enzyme activities of 5 fish. No significant differences were observed between treatments ($\alpha = 0.05$).

Table 2.1. In vitro inhibition of different esterases in three-spined stickleback by paraoxon.

Enzyme[a]	Tissue	IC50[b] of paraoxon (M)
AChE	Brain[c]	$7.5\ (1.9) \times 10^{-6}$
	Epaxial muscle[c]	$9.4\ (2.7) \times 10^{-6}$
BChE	Epaxial muscle[c]	$6.7\ (2.2) \times 10^{-9}$
	Liver	$3.0\ (1.0) \times 10^{-9}$
CaE	Liver	$5.6\ (2.4) \times 10^{-7}$

[a] AChE, acetylcholinesterase (EC 3.1.1.7); BChE, butyrylcholinesterase (EC 3.1.1.8); CaE, carboxylesterase (EC 3.1.1.1).
[b] Median inhibitory concentrations with SE in parentheses.
[c] Data from Sturm et al. 2000 (Chapter 1 of this work).

Phosphorothionate OPs, such as parathion, are poor inhibitors of AChE/BChE and require metabolic activation to their oxon analogues, which are highly potent inhibitors. The activation of phosphorothionates is believed to be mediated by cytochrome P450s (Boone & Chambers 1997) which are present at high levels in liver, but lack or exhibit very low levels in muscle and gills (Lindström-Seppä et al. 1981). Accordingly, the higher relative effects of parathion in liver (~60% inhibition) when compared to body muscle and gills (~30% inhibition) may reflect higher paraoxon concentrations in liver due to the activation of parathion in this tissue.

In an earlier field study, the BChE in the body muscle of feral stickleback was strongly decreased (~85%) at streams with previous proofs of parathion contamination during runoff at water concentrations of 0.2 to 0.3 µg/l (Sturm et al. 2000, chapter 1 of this work). The maximum concentrations of parathion in water during runoff-associated contamination events may be as high as 6 µg/l (Liess et al. 1999), but remain in most cases below 1 µg/l (Liess et

al. 1999; Sturm et al. 2000, chapter 1 of this work). Under the exposure conditions of the present report (1 h), the threshold concentration of parathion for the inhibition of stickleback BChE is between 0.1 µg/l and 1.0 µg/l, i.e. in the range of environmental concentrations. Hence, this study supports that the decreased BChE activity observed in stickleback from streams with low levels of OP contamination reflects OP exposure. The inhibition of stickleback BChE observed earlier in the field (Sturm et al. 2000, chapter 1 of this work), however, was more pronounced than that following 1 h of exposure to 1.0 µg/l parathion in this study, although the parathion concentrations in the field were lower. Different factors could explain this. On the one hand, the measured field concentrations may have underestimated environmental exposure. Due to the sampling technique used, values in the earlier study represent the estimated average concentrations during the one-hour peaks of run-off borne contamination events (Liess et al. 1999; Sturm et al. 2000, chapter 1 of this work). However, low levels of exposure also may have occurred before and after the contamination peak. Moreover, food-borne exposure may have also occurred. On the other hand, the difference between stickleback in the laboratory and the field may reflect differences in susceptibility. Because of the limited availability of adult stickeback, juvenile animals have been used in this study while adults were sampled in the previous field study (Sturm et al. 2000, chapter 1 of this work). Different biochemical factors, including cytochrome P450 dependent enzymes and protective esterases, are involved in the toxicology of anti-ChEs (Boone & Chambers 1997); and these factors may have differed between fish of different age.

In vitro, stickleback BChE was more than 1000-fold more sensitive than AChE to paraoxon, the active metabolite of parathion, while the paraoxon sensitivity of CaE exceeded that of AChE by a factor of 13 to 17 (Table 2.1). The lower in vitro sensitivities of AChE and CaE when compared to BChE suggest that the parathion concentrations required to inhibit AChE and CaE in stickleback are much higher than those required for BChE inhibition. Published studies suggest that comparatively high OP concentrations are required to inhibit piscine AChEs and CaEs following short term (hours) exposures. Weiss (1959) exposed different species of fish to 1.0 mg/l parathion for 1.5 hours and studied the time course of AChE activity during recovery. After 48 h of recovery, the brain AChE activity in exposed fish was 80% below that of control fish in largemouth bass (*Micropterus salmoides*), while it was 50%, 60% and 50% below control activity, respectively, in bluegills (*Lepomis macrochirus*), golden shiners (*Notemigonus crysoleucas*) and goldfish (*Carassius auratus*). Similarly, in a study with channel catfish (*Ictalurus punctatus*) (Straus & Chambers 1995), 4 h of exposure to 1.25 mg/l parathion resulted in a decrease of AChE in different tissues by 90%, while liver CaE was inhibited by 68% when compared to untreated controls. In conclusion, the measurement of AChE in fish appears less suitable for the monitoring of OPs when the exposure is expected to be very short-termed (hours). In such exposure scenarios, BChE should be more appropriate biomarker.

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Chapter 3

Xenobiotic effects on hepatic biotransformation enzymes in trout: Comparative in vitro / in vivo-assessment of prochloraz and nonylphenol diethoxylate using cultured hepatocytes as a model system

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Abstract—*The suitability of cultured rainbow trout hepatocytes as a model system for the assessment of xenobiotic effects on hepatic biotransformation enzymes in fish was examined. The effects of two model water pollutants, the imidazole fungicide prochloraz and the alkylphenolic compound nonylphenol diethoxylate (NP2EO), on biotransformation variables were investigated in a comparative in vitro / in vivo approach. Biotransformation enzymes were measured in cultured hepatocytes following exposure to xenobiotics in vitro, or in the liver of juvenile rainbow trout (Oncorhynchus mykiss) exposed in vivo. The patterns of biochemical responses to the model pollutants were generally similar between in vitro and in vivo investigations. Levels of cytochrome P4501A (CYP1A) protein and the catalytic activity of the CYP1A-dependent enzyme 7-ethoxyresorufin-O-deethylase (EROD) were induced in vitro after 24 h of exposure to 0.37 mg/l prochloraz. In vitro, higher prochloraz concentrations induced only the levels of CYP1A, but not of EROD activity above control levels. In vivo exposure of juvenile trout to 100 µg/l prochloraz resulted in an induction of CYP1A and EROD after 7 and 14 days, while 10 µg/l prochloraz had no effects. In vitro, the 6β- and 16β-hydroxylation of testosterone was significantly decreased by 0.37 to 1.13 mg/l prochloraz, while in vivo these variables were significantly inhibited after exposure to 100 µg/l prochloraz for 7 and 14 days. NP2EO did not affect EROD activity in vitro. In vivo, EROD activity and CYP1A remained unchanged following 7 days of exposure to 100 µg/l or 400 µg/l NP2EO. NP2EO (4.6 to 15.4 mg/l) inhibited the 16β-hydroxylation and glucuronidation of testosterone in vitro. In vivo, 7 days of exposure to 100 to 400 µg/l NP2EO resulted in a significant inhibition of the 6β- and 16β-hydroxylation of testosterone. The good qualitative correspondence between in vitro and in vivo results indicates that studies using trout hepatocytes allow the identification of biochemical targets of xenobiotic effects in fish liver. However, more research is needed before quantitative predictions, e.g. of effective concentrations, can be made from in vitro investigations.*

Keywords —fish, liver cell, steroid hydroxylation

INTRODUCTION

Fish are exposed to diverse anthropogenic water pollutants, originating from various point and non-point sources. Especially hydrophobic organic pollutants are readily bioaccumulated by fish. After uptake, a major factor in determining the fate of pollutants in the organism is their enzymatic biotransformation (Kleinow et al. 1987; Sijm et al. 1997). Biotransformation enzymes are subdivided into those of phase I, which catalyze non-synthetic reactions, and those of phase II, which catalyze the conjugation of the xenobiotic (or its phase I metabolite) with endogenous compounds such as glutathione or glucuronic acid. Generally, biotransformation converts xenobiotics to less toxic and more excretable products. In some cases, however, biotransformation pathways activate pollutants with a low inherent toxicity to

highly toxic metabolites (Chambers & Carr 1995; Sijm et al. 1997). In vertebrates, the highest rates of biotransformation are usually found in liver (Lindström-Seppä et al. 1981). Cytochromes P450 (P450, CYP) constitute a large superfamily of heme thiolate proteins that mediate the NADPH/O_2 dependent oxidation of certain endogenous compounds and xenobiotics, such as plant metabolites, drugs, pesticides, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Gonzalez 1989; Nebert & Gonzales 1987). P450s accomplish a major part of phase I biotransformation (Di Giulio et al. 1995; Lech & Vodicnik 1985). In fish, P4501A is the major xenobiotic-inducible P450 family (Stegeman & Hahn 1994), being induced by various environmental chemicals, such as polychlorinated dibenzo-*p*-dioxins and -furans (PCDDs and PCDFs), polycylic aromatic hydrocarbons (PAHs), and certain polychlorinated biphenyls (PCBs) (Goksøyr & Förlin 1992). Cytochromes P450 are also involved in the metabolism of steroid hormones (Gonzalez 1989). Xenobiotics that alter the activity of such P450 isozymes may therefore have adverse effects on reproduction.

Because of the central role of biotransformation in xenobiotic accumulation and toxicity, the need to consider biotransformation in environmental risk assessment has recently been stressed (Sijm et al. 1997). However, a generally agreed methodological framework to assess the interaction of biotransformation enzymes with xenobiotics is still lacking, particularly for aquatic species. One aspect contributing to this situation is the lack of test systems suitable for rapid screening. Suborganismal in vitro systems allow for the testing of a high number of samples and hence may provide effective tools in this context. Concerning the investigation of hepatic biotransformation in fish, cultured hepatocytes represent a promising in vitro system. During the last ten years considerable progress was achieved in the primary culture of fish hepatocytes (reviewed in: Mommsen et al., 1994; Monod et al., 1998; Segner, 1998). The use of adequate substrates for cell adherence, such as extracellular matrix components, was shown to be a critical factor in the preparation of rainbow trout hepatocyte monolayers that show life spans of 4 days or longer and maintain liver-specific physiological functions (Blair et al. 1990; Miller et al. 1989; Rabergh et al. 1995). The life span and maintenance of liver-specific functions in rainbow trout hepatocytes can be prolonged in culture systems allowing more complex, three-dimensional intercellular interactions, e.g. the aggregate culture system or coculture systems with fibroblast cell lines (Cravedi et al. 1996; Flouriot et al. 1993; Scholz et al. 1998). Such systems, however, are more difficult to maintain and less validated than the monolayer system, which therefore is more frequently employed in physiological and toxicological studies. Ecotoxicological studies using fish hepatocytes have, among other things, focused on the induction of biotransformation enzymes (Masfaraud et al. 1992b; Miller et al. 1989; Pesonen & Andersson 1991; Scholz & Segner 1999) and metabolites formed from certain xenobiotics (Cravedi et al. 1999). Hardly ever, however, have the findings of such investigations been validated by parallel in vivo studies.

In the present work, the response of hepatic biotransformation enzymes to selected xenobiotics has been investigated in a comparative in vitro / in vivo approach, using juvenile

rainbow trout and cultured trout hepatocytes. Two compounds have been selected as model water pollutants, the imidazole fungicide prochloraz and the alkylphenolic compound nonylphenol diethoxylate. Prochloraz is widely used as an agricultural fungicide. The action of imidazole fungicides is based on the inhibition of a cytochrome P450–dependent step in ergosterol synthesis (Wilkinson et al. 1972). Imidazole fungicides further inhibit a broad spectrum of other cytochrome P450–dependent enzyme activities, including those of steroid and xenobiotic metabolism (Levine & Oris 1999; Sheets et al. 1986). In addition to inhibition, prochloraz also induces cytochrome P450(s) in rodents (Laignelet et al. 1989), birds (Johnston et al. 1989) and fish (Bach & Snegaroff 1989). Alkylphenol polyethoxylates (APnEO; number of ethoxylate units, n = 1-40) are widely used non-ionic surfactants (Takada & Eganhouse 1998). Certain environmental degradation products of APnEOs, namely alkylphenols and alkylphenol di- and monoethoxylates, show a higher toxicity (Servos 1999) and environmental persistence than the parent compounds (Maguire 1999). In addition, some APnEO degradation products are weak estrogenes, eliciting estrogen–dependent responses in fish (Nimrod & Benson 1996; Servos 1999). Moreover, alkylphenolic compounds have been shown to inhibit cytochrome P450-dependent xenobiotic and steroid metabolism in mammals and fish (Arukwe et al. 1997; Hanioka et al. 1999; Lee et al. 1996b). In our study, cytochrome P4501A (CYP1A), measured as immunodetectable protein and as 7-ethoxyresorufin-*O*-deethylase (EROD) catalytic activity, and the metabolite spectrum of testosterone were selected as biotransformation variables. CYP1A represents the major inducible hepatic cytochrome P450 in fish, while the metabolite spectrum of testosterone reflects the activity of different constitutive hepatic cytochrome P450s and the phase II enzyme UDP-glucuronyltransferase. The model pollutants were investigated for their effects on biotransformation variables following exposure of cultured trout hepatocytes in vitro or juvenile rainbow trout in vivo, respectively. The results suggest that investigations using cultured hepatocytes can qualitatively predict xenobiotic effects on hepatic biotransformation in fish.

MATERIALS AND METHODS

Chemicals

Prochloraz (N-propyl-N-[2-(2,4,6-trichlorphenoxy)-ethyl]imidazol-1-carboxamid, 99 % pure) was obtained from Riedel-de-Haën. Prochloraz (95 % pure) was a generous gift from Agrevo UK limited. Testosterone was purchased from NEN products (Boston, MA). Its radiochemical purity was at least 99% as indicated by radio-TLC and radio-HPLC analyses and its specific activity was 2.2 GBq/mmol. Technical nonylphenol diethoxylate (NP2EO) was obtained from Aldrich (Igepal® CO-210). 7-ethoxyresorufin, resorufin, medium 199

containing Hank's salts (catalogue number M3274), fetal calf serum, L-glutamine, penicillin-streptomycin, ethyl-4-aminobenzoate were obtained from Sigma. Riedel-de-Haën, Aldrich and Sigma products were purchased from Sigma-Aldrich, Deisenhofen, Germany. Trypan blue and defatted bovine serum albumin were obtained from Serva, Heidelberg, Germany. Collagenase D was obtained from Roche, Mannheim, Germany. All other chemicals were of analytical grade.

Hepatocyte culture and in vitro exposure

Hepatocytes were isolated from female trout (250-350 g) by collagenase perfusion of the liver (Mommsen et al. 1994). After isolation, hepatocytes were separated from cell debris by three washing steps with modified Hank's minimal essential medium containing 1% defatted bovine serum albumin (Mommsen et al. 1994), each followed by 3 min of centrifugation at 50 × g and 4°C. After washing, hepatocytes were resuspended in medium 199 (M199) supplemented with 0.9 g/l HEPES, 0.35 g/l $NaHCO_3$, 0.1 g/l $CaCl_2$, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. During manipulation after isolation and prior to seeding, hepatocytes were kept on ice. Cell viability was assessed by the exclusion of a solution of 0.175% trypan blue in phosphate buffered saline and was at least 80%. Hepatocyte suspensions were brought to a density of 1.5×10^6 viable cells/ml in M199 containing 5% fetal calf serum and seeded into 24-well or 6-well cell culture plates or, 60 mm petri dishes (Falcon Primaria, Becton and Dickinson). 24-well cell culture plates received 0.4 ml, 6-well plates 2 ml, and petri dishes 5 ml hepatocyte suspension per well. Before use, culture dishes were coated with a commercially available matrix protein (Matrigel, Collaborative Biochemical). Fetal calf serum was added to the medium only during the first 24 h of culture to increase adherence of cells. After seeding, hepatocytes were allowed to recover and form adherent monolayers for at least 12 h before use in experiments. The culture took place in an incubator at 15°C and 80% relative atmospheric humidity. To determine the range of concentrations causing acute toxic effects, hepatocytes precultured for 24 h after isolation were exposed to the model pollutants, prochloraz and NP2EO. Dimethyl sulfoxide (DMSO) was used as a solvent carrier (0.1 % final concentration). After 24 h of exposure, the leakage of lactate dehydrogenase (LDH) was determined as a cytotoxicity endpoint as decscribed before (Scholz et al. 1998). Using hepatocytes derived from different fish (n = 3 to 5), LDH determinations were carried out for solvent carrier controls and at least five concentrations per chemical. The lowest observed effect concentrations of prochloraz and nonylphenol diethoxylate, respectively, were 140 µM and 100 µM. The model pollutants were tested for their effects on biotransformation metabolism in an analogous way at lower, non-toxic concentrations. Exposures periods were for 24 h except where noted otherwise. When longer exposure periods were used, the media were changed daily.

Fish

In in vivo experiments, juvenile rainbow trout (*Oncorhynchus mykiss*) of both sexes were used. The fish were obtained from a local hatchery and maintained in the INRA facilities for at least 2 weeks before use in experiments. The average body weight of trout was 51 g in experiment 1 (March 1998) and 72 g in experiment 2 (November 1998). The trout were maintained in a flow-through of dechlorinated, aerated city water and fed a commercial trout diet.

Exposure experiment 1

Experiment 1 was carried out to assess sublethal biochemical effects of the fungicide prochloraz on trout. Fish were exposed for 7-14 days. In addition to a solvent carrier control (0.03% ethanol), experimental treatments included two prochloraz concentrations, 10 µg/l and 100 µg/l, and a mixture treatment of prochloraz (100 µg/l) and NP2EO (33µg/l). Per treatment, three 200 l tanks, housing 20 fish each, were set up. After introduction to tanks, fish were allowed to acclimate for 7 days before the beginning of exposures. Each of the 200 l tanks received a water flow-through of dechlorinated, aerated city water of 0.14 l / min. At the start of exposures, toxicants were added to the water from 1000-fold concentrated solutions in 30% ethanol (200 ml per tank). To maintain toxicant concentrations during exposures, 0.14 ml/min of 1000-fold toxicant solutions in 30% ethanol were delivered by a peristaltic pump. During the whole experiment, fish were fed a commercial trout diet at 1% body weight per day. Five fish per tank (15 per treatment) were sampled at days 7 and 14 of exposures.

Exposure experiment 2

Experiment 2 was carried out to assess sublethal biochemical effects of the surfactant NP2EO on trout. Exposure of aquatic animals to toxic contaminants under field conditions is usually to a complex mixture of compounds, and often accompanied by elevated levels of nutrients. To check whether the effects of NP2EO were altered in the presence of other waterborne compounds, both city water and the water of the moderately contaminated river Le Couesnon (area of Rennes, France) were used as the dilution water. The experimental treatments consisted of two concentrations of NP2EO in city water (100 µg/l and 400 µg/l) and one in river water (400 µg/l), and control treatments of fish maintained in either city water or river water. Fish were exposed in 200 l tanks. 50 % of the water/toxicant solution was renewed daily. After introduction, fish were allowed to acclimate for 7 days. During the experiment, fish were fed a commercial trout diet at 0.5% of body weight. Fish sampling took place at day 7 after the beginning of exposures.

Determination of model pollutants water concentrations

Water samples containing prochloraz or NP2EO were extracted with dichloromethane and analyzed by high-performance liquid chromatography as reported by Cravedi et al. (submitted) except that the system was equipped with an UV detector set at 296 nm and 260 nm, respectively.

Sample treatment prior to biochemical analyses

Fish from in vivo exposures were killed by an overdose of ethyl-4-aminobenzoate. The total body length and weight was recorded. Fish were opened ventrally and the liver cleared of blood by perfusion with ice-cold Ringer's solution via the portal vein. The liver was removed, weighed, quick-frozen in liquid nitrogen and stored at –80°C. Microsomes were prepared from liver tissue or from hepatocytes as described before (Scholz et al. 1997). Approximately 400 mg of liver tissue or 8×10^6 cells were required per determination. The analyses of EROD activity (see below) in hepatocytes were carried out either on microsomes or directly on cells disrupted by freezing-thawing. In the latter case, hepatocytes were cultured and exposed in 24-well plates. At the end of the experiments, the medium was sucked off, the cells were washed with PBS and the culture plates shock-frozen in liquid nitrogen and stored at -80°C until analysis.

Biochemical Assays

7-Ethoxyresorufin-*O*-deethylase (EROD) activity in cultured hepatocytes was measured by a modification of the method of Kennedy et al. (1995). The reaction was allowed to proceed for 10 min at 25°C, at a final NADPH concentration of 0.19 mM. EROD activity in hepatic microsomes of rainbow trout was determined using the method of Burke & Mayer (1974). CYP1A was measured immunochemically by a direct ELISA as described before (Scholz et al. 1997). Protein concentrations of microsomes were measured by the method of Lowry et al. (1951). Testosterone hydroxylation by liver microsomes was determined by incubation of microsomes with 100 µM labelled testosterone, NADPH and a NADPH-generating system containing glucose 6-phosphate and glucose 6-phosphate dehydrogenase, for 2 h at 25°C, as described by Cravedi et al. (1995). The glucuronidation of testosterone in microsomal incubation were carried out for 1h at 25°C as previously reported (Cravedi et al. 1995). The reaction mixture contained 1 mg microsomal protein, 2 mM UDP-glucuronic acid and 250 µM [^{14}C]-testosterone. Incubation of testosterone with hepatocytes was performed as previously described (Cravedi et al. 1998) except that labeled testosterone was added in DMSO (0.1% v/v final) to the culture medium. Control incubations received equivalent amounts of DMSO only. Testosterone biotransformation was measured after incubation with microsomes and isolated hepatocytes by separation and quantification of metabolites present

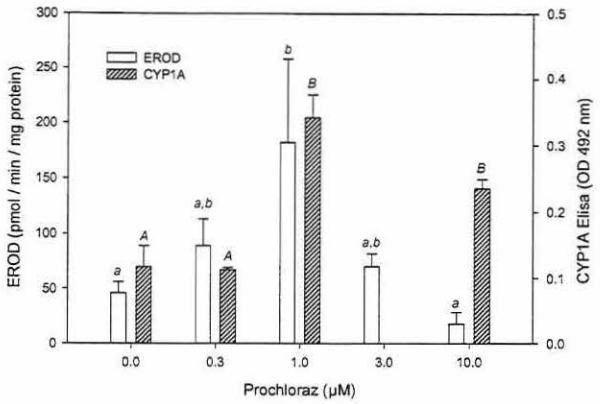

Fig. 3.1. EROD activity and CYP1A protein in rainbow trout hepatocytes treated with non-cytotoxic concentrations of prochloraz for 24 h. Values are means and S.E. of 4 observations on cell cultures from different fish (EROD) or of observations on 3 cell cultures derived from one fish (CYP1A). Within each biochemical variable, means with different letters are statistically different ($P < 0.05$).

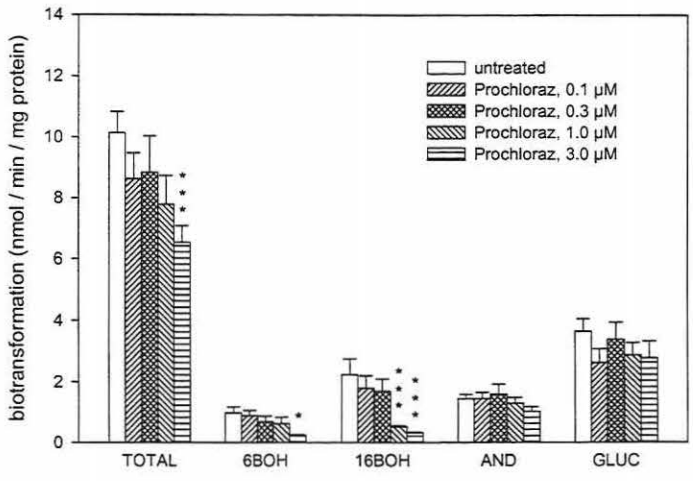

Fig. 3.2. Biotransformation of testosterone in cultured rainbow trout hepatocytes following 48 h of exposure to prochloraz. Values are the means and S.E. of 6 observations on cell cultures derived from different fish. Within each reaction rate (6BOH, 6β-hydroxylation; 16BOH, 16β-hydroxylation; AND, androstenedione formation; GLUC, testosterone glucuronidation), asterisks indicate whether means significantly differed from those in untreated cells (* $P < 0.05$, *** $P < 0.001$).

in the medium, as described by Wood et al. (1983). Metabolites were identified by comparison with authentic standards and quantified using radioactivity monitoring.

Determination of radioactivity

The determination of radioactivity was done by liquid scintillation counting in a Tricarb 2200CA liquid scintillation counter (Packard Instrument Co., Downers Grove, IL) after use of Ultima Gold (Packard Instrument Co.) for the cocktail. HPLC was performed on a Hewlett Packard 1100 system equipped with a reverse phase Kromasil C18 column (180 x 4.6, 5µm, Interchim, Montluçon, France). A flow one / beta-radioactivity flow detector (Packard Instrument Co.) was used for ^{14}C monitoring.

Statistics

Except where noted otherwise, the effects of chemical treatments on biochemical markers were tested by one-way analyses of variance (ANOVAs) followed by multiple comparisons using the Student-Newman-Keuls test. In some cases data were log transformed prior to tests to increase homogeneity of variances. With experiments on hepatocytes, repeated-measures ANOVAs were used, thus matching the observations made on cells obtained from the same fish.

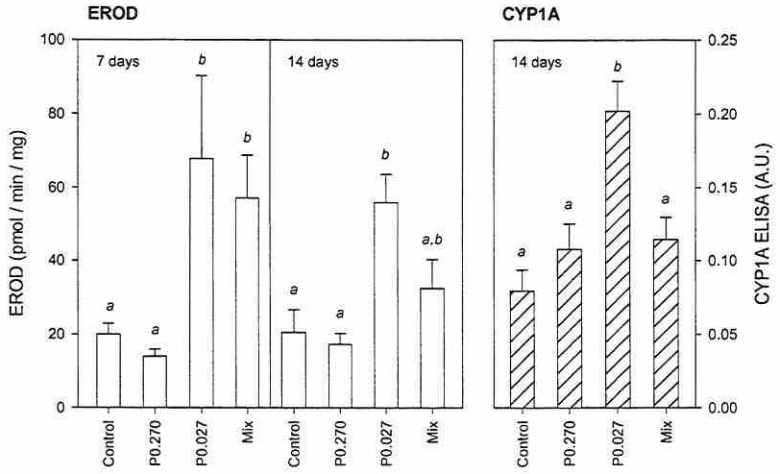

Fig. 3.3. Hepatic EROD activity and CYP1A protein in juvenile trout exposed for 7 days (D7) or 14 days (D14) to dechlorinated city water alone (C) or containing sublethal concentrations of selected pollutants (P10, 10 µg/l prochloraz; P100, 100 µg prochloraz; Mix, 100 µg/l prochloraz and 33 µg/l nonylphenol diethoxylate). Values are means and S.E. (EROD, n = 13 to 15; CYP1A, n = 9 to 12). Within either of both EROD and CYP1A, values marked with the same letter are not statistically different ($P < 0.05$).

RESULTS

Effects of prochloraz on cultured hepatocytes in vitro

Following 24 h of primary culture after isolation, rainbow trout hepatocytes were exposed for 24 h to non-cytotoxic concentrations of prochloraz (0.11 to 3.76 mg/l, equivalent to 0.3 to 10 µM). Prochloraz affected CYP1A-dependent EROD activity in a biphasic manner (Fig. 3.1), with a significant EROD induction at 0.37 mg/l, but no apparent induction at lower or higher concentrations of prochloraz. By contrast, the expression of CYP1A, measured by a direct ELISA, was significantly increased following exposure to prochloraz at concentrations of 0.37 and 3.76 mg/l. Significant decreases of the 6-β- and the 16-β-hydroxylation and the total biotransformation of testosterone were observed following exposure of hepatocytes to prochloraz concentrations in the range of 0.37 to 1.13 mg/l (Fig. 3.2).

Effects of prochloraz on juvenile trout in vivo

Juvenile trout were exposed to prochloraz for 7 to 14 days. Nominal and measured prochloraz water concentrations are shown in Table 3.1. Particularly at the higher concentration of prochloraz, the measured concentration was markedly lower than the nominal concentration, apparently reflecting adsorption of prochloraz by tubes of the dosing system, feces, food and fish. In the presentation of biochemical responses below, we will refer to nominal concentrations. Exposure to 10 µg/l prochloraz did not cause significant changes of hepatic EROD activity or CYP1A levels (Fig. 3.3). A significant induction of hepatic EROD activities and CYP1A concentrations was observed after exposure of trout to 100 µg/l prochloraz. In trout exposed to a mixture of prochloraz (100 µg/l) and NP2EO (33 µg/l), EROD activity was significantly induced after 7 days, but only slightly and non-significantly increased after 14 days (Fig. 3.3). With respect to the microsomal metabolism of testosterone, the exposure of juvenile trout to 100 µg/l prochloraz, and to a mixture of 100 µg/l prochloraz and 33 µg/l NP2EO, significantly inhibited the total oxidative metabolism, the 6β- and the 16β-hydroxylation of testosterone (Fig. 3.4, A and B). Moreover, exposure to the mixture of prochloraz and NP2EO significantly induced testosterone glucuronidation after 7 days (Fig. 3.4A), but not after 14 days (Fig. 3.4B).

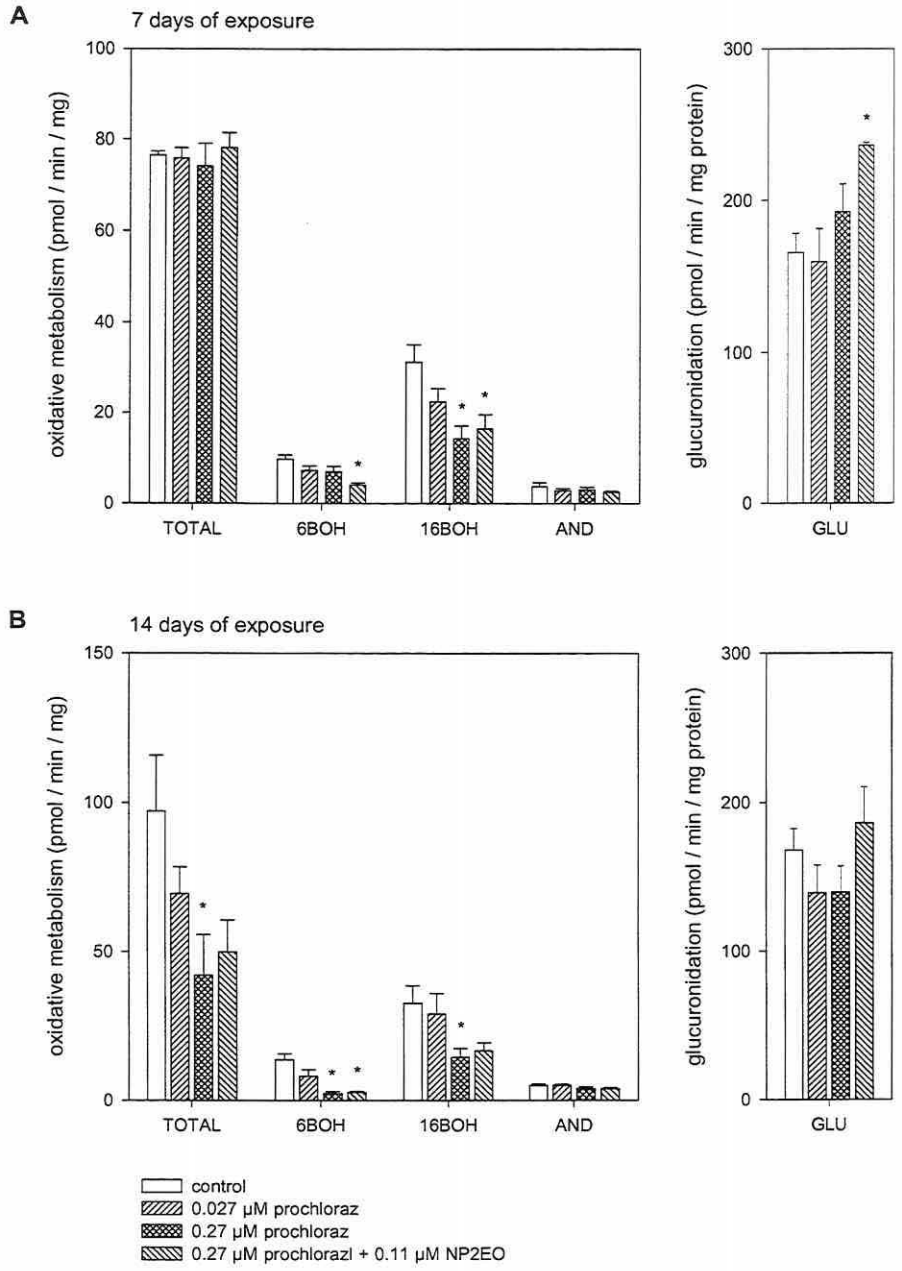

Fig. 3.4. Hepatic testosterone biotransformation in juvenile trout after exposure to sublethal concentrations of prochloraz, or a mixture of prochloraz and nonylphenol diethoxylate (NP2EO), for 7 days (A.) or 14 days (B). Values are the mean and S.E. of testosterone reaction rates (6BOH, 6β-hydroxylation; 16BOH, 16β-hydroxylation; AND, androstenedione formation; GLUC, testosterone glucuronidation) of 5 fish (except for GLUC where n = 4). Within each diagram and for each reaction, asterisks indicate whether means differed from those in untreated control fish (* $P < 0.05$).

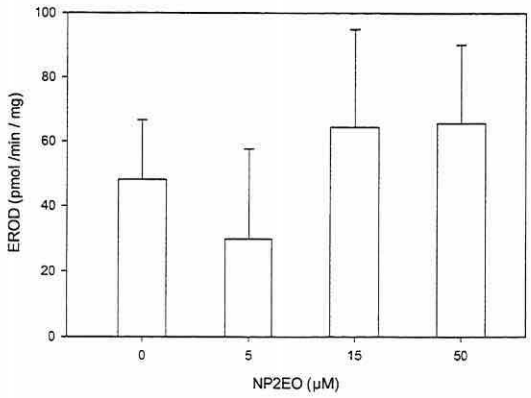

Fig. 3.5. EROD activity in rainbow trout hepatocytes after treatment with nonylphenol diethoxylate. Cells were exposed to nonylphenol diethoxylate (NPE1.5, 1.5 mg/l; NPE4.6, 4.6 mg/l; NPE15.4, 15.4 mg/l), or remained untreated (Contr). Values are means and S.E. of 4 determinations using cells derived of 2 fish. No significant differences were observed.

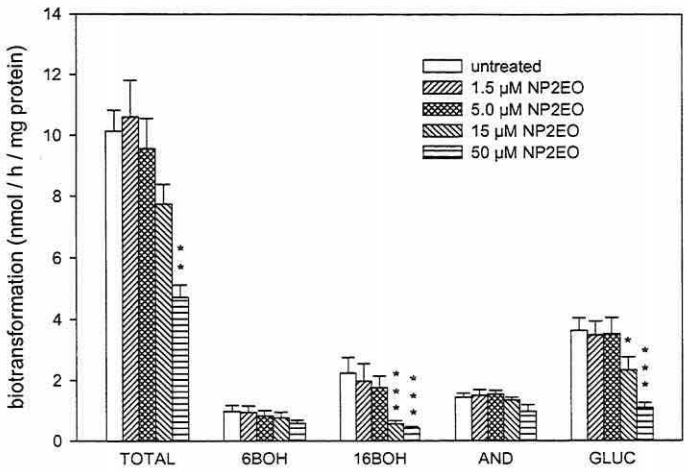

Fig. 3.6. Biotransformation of testosterone in cultured rainbow trout hepatocytes following 48 h of exposure to nonylphenol diethoxylate (NP2EO). Values are the means and S.E. of 6 observations on cell cultures derived from different fish. Within each reaction rate (6BOH, 6β-hydroxylation; 16BOH, 16β-hydroxylation; AND, androstenedione formation; GLUC, testosterone glucuronidation), asterisks indicate whether means significantly differed from those in untreated cells (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Table 3.1. Measured water concentrations of prochloraz and nonylphenol diethoxylate (NP2EO) in experiments with juvenile rainbow trout.

Experiment	Treatment	Dilution water	Measured water concentrations (µg/l)
1	untreated control	City water	na[a]
	Prochloraz, 10 µg/l	City water	7.5
	Prochloraz, 100 µg/l	City water	41.7
	Prochloraz, 100 µg/l + NP2EO, 33 µg/l	City water	42.9 nd[b]
2	untreated control	City water	na
	untreated control	River water	na
	NP2EO, 100 µg/l	City water	50
	NP2EO, 400 µg/l	City water	215
	NP2EO, 400 µg/l	River water	84

[a] Not applicable.
[b] Not determined.

Effects of NP2EO on cultured hepatocytes in vitro

In in vitro investigations, trout hepatocytes were exposed to NP2EO concentrations ranging between 0.5 and 15.4 mg/l (0.5 to 50 µM). The treatment of trout hepatocytes with NP2EO did not affect EROD activity (Fig. 3.5). However, exposure to NP2EO in the range of 4.6 – 15.4 mg/l significantly decreased the 16β-hydroxylation, the glucuronidation and the total biotransformation of testosterone in trout hepatocytes (Fig. 3.6).

Effects of NP2EO on juvenile trout in vivo

The nominal and measured NP2EO water concentrations from the in vivo exposure of juvenile trout are shown in Table 3.1. The effects of NP2EO are presented below, referring to the nominal water concentrations. In juvenile trout exposed to NP2EO, no significant changes of EROD activity or CYP1A levels were observed (Fig. 3.7). Exposure of juvenile trout to 400 µg/l NP2EO in both city water and river water caused significant inhibition of the total oxidative metabolism, the 6β- and 16β-hydroxylation of testosterone (Fig. 3.8). To a lesser degree, these activities were also decreased in trout exposed to 100 µg/l NP2EO (significant only for the 16-β-hydroxylation, Fig. 3.8).

DISCUSSION

Effects of prochloraz

The increase of cytochrome P4501A (CYP1A) levels following exposure to prochloraz, observed both in vitro and in vivo in this study (Figs. 1; 3) indicates that this imidazole fungicide induces *CYP1A* gene expression in rainbow trout. Imidazoles (Kobayashi et al. 1993), including prochloraz (Rivière et al. 1985), have been reported to induce CYP1A in mammals. The induction of *CYP1A* gene expression by xenobiotics is usually mediated through the binding of the inducer to a cytosolic regulator protein, the arylhydrocarbon receptor (AhR), and subsequent gene activation (Hahn 1998). The induction of CYP1A in trout by prochloraz also appears to follow this mechanism, as the AhR antagonist α-naphthoflavone blocked the induction of EROD activity in trout hepatocytes by prochloraz (Sturm & Segner, unpublished data). By contrast, CYP1A induction by benzimidazoles may also involve non-classical, AhR-independent pathways (Daujat et al. 1992; Gleizes-Escala et al. 1996).

The type of response of CYP1A levels and EROD activity observed with prochloraz, i.e., decreasing EROD activities at higher concentrations of the inducer, together with a further increase of (or maintenance of induced) levels of CYP1A, has been observed for a number of

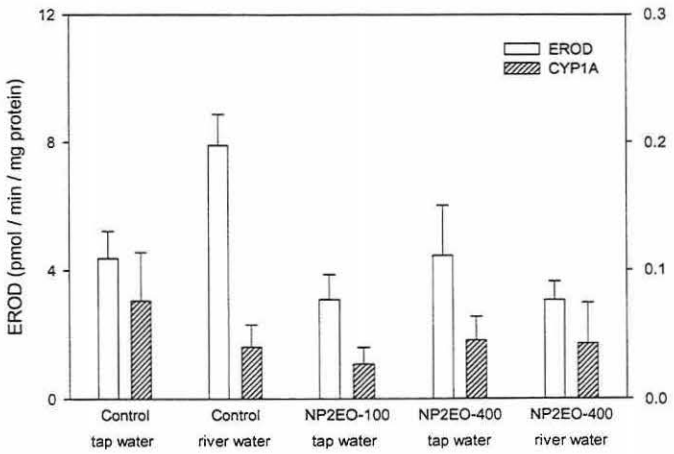

Fig. 3.7. Hepatic EROD activity and CYP1A protein in juvenile trout exposed for 7 days to dechlorinated city water (C-cw), river water (C-rw), nonylphenol diethoxylate dissolved in dechlorinated city water (100 µg/l, NPE100-cw; 400 µg/l, NPE400-cw) or nonylphenol diethoxylate dissolved in river water (400 µg/l, NPE400-rw). Values are means and S.E. (n = 10). No statistically significant differences were observed ($P < 0.05$).

CYP1A-inducers, including PCBs and PAHs, and is generally understood as an inhibition of EROD catalytic activity by the inducer (Behrens et al. 1999; Gooch et al. 1989; Hahn et al. 1996). Inhibitory effects by P450-inducers or other chemicals are of particular relevance with respect to the use of EROD activity as a biomarker in environmental monitoring (Goksøyr & Förlin 1992). Unless CYP1A levels are also determined, an interference of chemicals with enzymatic activity may mask induction, and thus pollutant exposure.

Imidazole fungicides have been reported to effect inhibition of cytochromes P450 in fish following non-competitive (Levine et al. 1999; Snegaroff & Bach 1989), competitive (Snegaroff & Bach 1989) and non-competitive mixed type mechanisms (Levine & Oris 1999). In the in vivo experiments of the present study, exposure of trout to the higher concentration of prochloraz only provoked a moderate degree of EROD induction (2.7 to 3.4-fold, Fig. 3.3). In earlier in vivo studies, total cytochrome P450 levels in trout treated with prochloraz in food-borne exposures or via i.p. injection were consistently increased; however, at the level of EROD activity the effects of prochloraz were variable, ranging from inhibition to moderate (2.7-fold) induction (Bach & Snegaroff 1989; Snegaroff & Bach 1989). This shows that an inhibition of trout EROD activity by prochloraz, observed only in vitro in this study, can also occur in vivo.

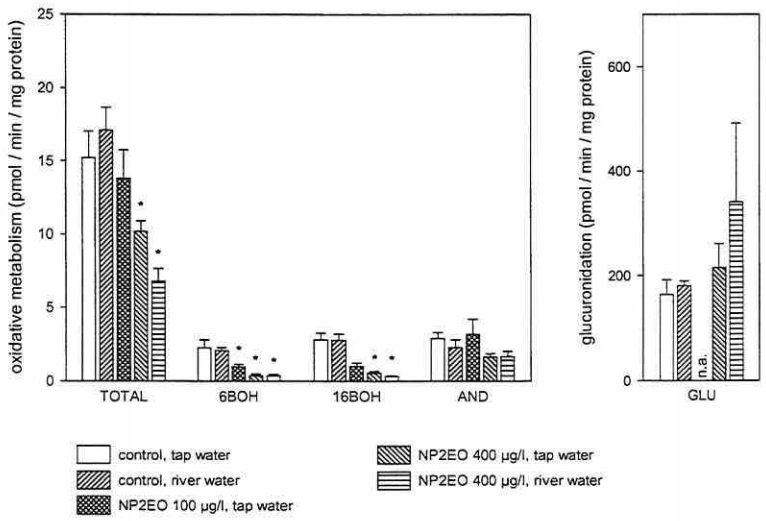

Fig. 3.8. Hepatic testosterone biotransformation in juvenile trout after exposure to sublethal concentrations of igepal for 7 days. Values are the mean and S.E. of testosterone reaction rates (6BOH, 6β-hydroxylation; 16BOH, 16β-hydroxylation; AND, androstenedione formation; GLUC, testosterone glucuronidation) of 5 fish (except for GLUC where n=4). Within each diagram and for each reaction, asterisks indicate whether means differed from those in untreated control fish (* $P < 0.05$).

With respect to testosterone metabolism, prochloraz caused an inhibition of the 6β- and 16β-hydroxylations both in vitro (hepatocytes) and in vivo (Figs. 2; 4). These biotransformation reactions are catalyzed in rainbow trout by the constitutive hepatic cytochrome P450s LMC5 and, to a somewhat lesser extend, LMC2 (Miranda et al. 1989). LCM2 and LCM5 most probably are the products of the genes *CYP2K1* and *CYP3A27*, respectively (Buhler & Wang-Buhler 1998). The inhibition of testosterone hydroxylation observed in this study is in line with the general inhibitory effects of prochloraz on cytochrome P450-dependent metabolism. Monod et al. (1993) showed the inhibition of trout ovarian aromatase activity by different imidazoles including prochloraz. Altogether, this report and others indicate that imidazole fungicides have the potential to interfere with steroid hormone metabolism in fish.

Effects of nonylphenol diethoxylate

Alkylphenol ethoxylates (APnEO) are widely used as detergents and/or surfactants (Servos 1999). The environmental degradation of APnEOs involves the stepwise removal of ethoxylate units from the polyethoxylate chain, giving rise to alkylphenol ethoxylates and alkylphenol carboxylates with less polyethoxylate units, and, finally alkylphenols (Giger et al. 1984; Nimrod & Benson 1996). These APnEO degradation products are detectable in many freshwater systems, and may reach high concentrations (Ahel et al. 1993; Blackburn & Waldock 1995). Alkylphenols and alkylphenol mono- and diethoxylates are weak estrogens, eliciting various estrogen-dependent responses in mammals and fish (Nimrod & Benson 1996; Servos 1999). In fish, the exposure to such alkylphenolic compounds can cause the induction of the yolk protein precursor vitellogenin and of eggshell proteins (zona radiata proteins) in male and juvenile fish (Arukwe et al. 1999; Jobling et al. 1996). Normally, these proteins are estrogen-dependently expressed only in females during oogenesis. Furthermore, alkylphenolic compounds have also been shown to inhibit testicular growth (Jobling et al. 1996) and to cause histological aberrations in fish gonads (Christiansen et al. 1998), including intersex (testis-ova) (Gray & Metcalfe 1997).

The majority of studies on the effects of alkylphenolic compounds and other xenoestrogens in fish have looked at the induction of estrogen-dependent responses. However, the roles of natural estrogens do not only include inductive, but also suppressive effects on gene expression. Hence, suppressive effects on the expression of certain genes could also be caused by xenoestrogens. Prespawning female salmonids display high levels of the estrogen estradiol, together with high levels of vitellogenin (Scott & Sumpter 1983), and, compared to male and juvenile fish, lowered levels of different CYP isoforms and P450-dependent catalytic activities (Förlin & Haux 1990; Hansson & Gustafsson 1981; Larsen et al. 1992). Studies, in which juvenile or hypophysectomized fish were treated with different hormones provide evidence that the down regulation of cytochromes P450 in females during maturation is caused by estradiol (Buhler & Wang-Buhler 1998; Pajor et al. 1990; Vodicnik & Lech

1983). For this reason, it is an intriguing question whether xenoestrogens may also be able to cause a 'feminization' of the biotransformation metabolism.

In the present study, the alkylphenolic compound NP2EO had no effect on hepatic EROD activity in rainbow trout, neither in vitro nor in vivo (Figs. 5; 7). NP2EO, however, inhibited the 6β- and 16β-hydroxylation of testosterone both in vitro and in vivo (Figs. 6; 8). Similar effects of putative xenoestrogens on P450 isoforms and/or P450-dependent catalytic activities have been found in other studies with salmonid fish (Arukwe et al. 1997; Buhler & Wang-Buhler 1998). In a study on hepatic biotransformation in Atlantic salmon (*Salmo salar*), 4-nitrophenol injected i.p. caused, at the lowest dose tested (1 mg/kg), an increase of progesterone 6β-hydroxylation, while it inhibited EROD and progesterone 6β-, 16β - and 17α-hydroxylase activities at the highest dose tested (125 mg/kg) (Arukwe et al. 1997). In the same study, the hepatic expression of CYP1A was slightly but significantly decreased by the entire range of administered 4-nitrophenol doses, while putative CYP2K and CYP3A isoforms were only decreased at the highest dosage used (Arukwe et al. 1997). Similarly, in a study with juvenile rainbow trout, three putative xenoestrogens (methoxychlor, 4-(*tert*-octyl)phenol and biochanin A) caused, at i.p. doses capable of inducing vitellogenin synthesis, the inhibition of P450-dependent laurate hydroxylation and a markedly repressed expression of CYP2M1, CYP2K1, and CYP3A27 in the liver (Buhler & Wang-Buhler 1998). The hepatic 6β- and 16β-hydroxylation of testosterone in rainbow trout are mediated by the P450 forms CYP3A and CYP2K1 (see above). The decrease in these steroid hydroxylation rates caused by NP2EO both with hepatocytes and in vivo in the present study could, on the one hand, reflect a suppression of constitutive P450 forms, as it was observed with other xenoestrogens in the studies quoted above. On the other hand, the lowered testosterone hydroxylation in hepatocytes and juvenile trout treated with NP2EO could also be due to a direct inhibiton of P450 isoforms. In rat liver microsomes, NP inhibits EROD (Lee et al. 1996a) and steroid hydroxylase activities (Lee et al. 1996b). Hanioka et al. (1999) reported the inhibition of various cytochrome P450-dependent enzymes from rat liver – including testosterone hydroxylase and 7-ethoxycoumarin-*O*-deethylase – by octylphenol and octylphenol mono- and diethoxylates. This indicates that certain APnEOs and APnEO degradation products can inhibit cytochrome P450.

Suitability of cultured trout hepatocytes as a system to study xenobiotic effects on biotransformation

Isolated fish hepatocytes have been suggested as a model in aquatic toxicology since they represent cells which, firstly, are central to systemic metabolic regulation in the intact organism, secondly are significantly involved in the metabolism of xenobiotics, and thirdly often represent the specific target of toxic chemicals (Baksi & Frazier 1990; Hinton et al. 1999; Monod et al. 1998; Pesonen & Andersson 1997; Segner 1998). In primary cultures of hepatocytes, biotransformation enzyme activities remain rather stable and inducible for

periods up to 5 days, even under simple monolayer culture conditions (Monod et al. 1998; Pesonen & Andersson 1997; Segner 1998), which is a pre-requisite for obtaining results relevant to the in vivo situation. The most convincing applications of hepatocytes to date are studies aiming at the identification of mechanisms of xenobiotic action (e.g., Anderson et al. 1996; Miller 1993; Navas & Segner 1999; Rabergh & Lipsky 1997) and studies concerning the hepatic metabolic profile of xenobiotics (e.g., Masfaraud et al. 1992a; Pesonen & Andersson 1991). By contrast, the quantitative extrapolation of the in vitro findings to the in vivo situation remains a matter of debate.

In the present work, a combined in vitro / in vivo evaluation of the effects of the model pollutants prochloraz and NP2EO on biotransformation enzyme activities in trout liver was made. As discussed above, the results generally showed a good correspondence between in vitro and in vivo experiments. However, a variable not fitting in this general picture was testosterone glucuronidation, accomplished by UDP-glucuronyltransferase. Prochloraz had no effect on testosterone glucuronidation in vitro (Fig. 3.2). In vivo, however, this enzymatic variable was increased following 7 days of exposure to 100 µg/l prochloraz alone (not significant) and in combination with 33 µg/l NP2EO (significant) (Fig. 3.4, A). In vitro, NP2EO significantly inhibited testosterone glucuronidation (Fig. 3.6), which remained unchanged, however, following exposure to NP2EO in vivo (Fig. 3.8). Generally, the response of phase II biotransformation enzymes to inducers proceeds slowly in fish, often requiring 1 to 2 weeks until induction becomes apparent (Andersson et al. 1985; Zhang et al. 1990). Moreover, phase II enzymes in fish show maximal induction rates of only about twofold (Andersson et al. 1985; Zhang et al. 1990). Therefore, a lack of response in short-term in vitro exposures, as observed with prochloraz in this study, is not surprising. The measurement of glucuronidation in microsomes, the methodology used with samples from in vivo experiments in this study, requires the addition of exogenous UDP-glucuronic acid. By contrast, with hepatocytes the metabolites of testosterone were determined in the medium after incubation of the intact cells, so glucuronidation relied on endogenous levels of UDP-glucuronic acid. Consequently, the inhibition of testosterone glucuronidation by NP2EO in vitro could either reflect inhibition of the enzyme, or could have been due to a depletion of intracellular UDP-glucuronic acid. Such a depletion of UDP-glucuronic acid would have remained without effect on the glucuronidation rates measured in microsomes, and is a possible explanation for the discrepancy between in vitro and in vivo results with NP2EO for testosterone glucuronidation.

When cellular in vitro systems are used as experimental models, it is a central question whether the observed responses occur at the same or at a different sensitivity when compared to the whole animal. In vivo, significant responses of CYP1A, EROD and testosterone 6β- and 16β-hydroxylation were observed after exposure to 100 µg/l prochloraz, while in vitro, these variables responded not below 0.37 mg/l prochloraz. Similarly, NP2EO caused a significant decrease in testosterone 16β-hydroxylation in vivo at 100 µg/l, while this effect

was observed in vitro not below 4.6 mg/l NP2EO. Consequently, on the basis of nominal concentrations, hepatocytes reacted 3.7 to 46 times less sensitively than trout with respect to effects on different biotransformation enzymes. However, it must be borne in mind that comparing water and medium concentrations means looking at functionally different levels. Fish readily bioaccumulate many waterborne hydrophobic compounds, resulting in blood plasma concentrations that exceed water concentrations by factors of 10^2 to 10^3 (Oikari & Kunnamo-Ojala 1987). In vivo, hepatocytes are exposed to xenobiotics via the blood. Therefore, it appears more appropriate to compare blood plasma and liver tissue concentrations of xenobiotics in vivo, respectively, with medium and cellular concentrations of xenobiotics in vitro. However, such values are in part difficult to determine; and it was beyond the scope of the present study to estimate these concentrations. Experiments with ^{14}C-prochloraz (Sturm and Cravedi, unpublished data) showed that when hepatocytes were treated with 2.5 µM prochloraz, the radioactivity in cells reached a steady state within 2 h. At this point of time, the free concentration of prochloraz and/or metabolites had dropped to 1.76 µM and the radioactivity in cells exceeded that in the medium 50-fold. This demonstrates that hepatocytes significantly accumulate xenobiotics from the medium, and in consequence actual xenobiotic concentrations in the in vitro assay may be overestimated by nominal concentrations.

In the present study, a good correspondence was observed in the response of different cytochrome P450-dependent variables to xenobiotic treatment observed in vitro with hepatocytes and in vivo with juvenile trout. In the few other comparative studies that have performed such an assessment of metabolic enzyme responses in fish liver, a good qualitative correspondence of hepatic enzyme responses has also been observed between in vitro / in vivo investigations (Monod et al. 1998). Our findings on enzyme responses confirm that there is a generally good comparability of xenobiotic metabolites formed by liver cells in vitro and in vivo (Monod et al. 1998).

Altogether, the available evidence indicates the principal suitability of the in vitro liver model as predictor of in vivo enzyme responses, although the response may differ in specific instances (Cravedi et al. 1999). More studies will be needed to extend the available empirical data base, particularly in order to clarify if in vitro and in vivo responses are also comparable quantitatively.

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Chapter 4

Expression and functional activity of P-glycoprotein in cultured hepatocytes from rainbow trout (*Oncorhynchus mykiss*)

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Abstract—*P-glycoproteins encoded by multidrug resistance 1 (*mdr1*) genes are ATP-dependent transporters located in the cytoplasmic membrane which mediate the extrusion of hydrophobic compounds from the cell. In primary cultures of isolated rainbow trout hepatocytes, we characterized a mdr1-like efflux mechanism of the teleost liver. Using the monoclonal antibody C219 that recognizes a conserved epitope of P-glycoproteins, the presence of immunoreactive protein(s) of 165 kDa in trout liver and cultured hepatocytes was shown in immunoblots. Immunohistochemistry stained bile canaliculi in vivo, while immunocytochemistry stained the entire plasma membrane in cultured isolated hepatocytes. During primary culture, the levels of P-glycoprotein expression determined in immunoblots strongly decreased with time in culture. Compounds known to interfere with P-glycoprotein-dependent transport (verapamil, vinblastine, doxorubicin, cyclosporin A) increased the accumulation of the mdr1 substrate rhodamine 123 by hepatocytes and decreased the efflux of rhodamine 123 from hepatocytes. This indicates the presence of a mdr1-like mechanism in teleost liver and suggests its function in the biliary excretion of hydrophobic compounds.*

Keywords—fish, liver cell

INTRODUCTION

The excretion via bile constitutes an important mechanism in the elimination of xenobiotics, particularly for organic compounds exceeding a molecular weight of 400 Da (Arias et al. 1993). In fish, the significance of the biliary route in the excretion of hydrophobic chemicals is well documented (Krahn et al. 1987; Oikari & Kunnamo-Ojala 1987). For instance, after exposure of fish to effluents, the total bile concentrations of chlorophenolic compounds and/or their metabolites exceeded their blood plasma concentrations by 10^2 to 10^3, indicating an effective hepatocellular concentration step (Oikari & Kunnamo-Ojala 1987). Similar concentration gradients of xenobiotics are found between blood and bile in mammals (Arias et al. 1993). Bile is formed at the bile canaliculi, i.e. tubular structures that form the most proximal part of the biliary tree. The membranes surrounding the canaliculi are specialized (apical) poles of adjacent hepatocytes, which contain carrier proteins for transport of chemicals from hepatocytes into bile (Arias et al. 1993; Oude Elferink et al. 1995). The small dimension and the delicate nature of the bile canaliculus have hampered direct mechanistic studies of the transfer of compounds from the hepatocyte into bile.

Recently, molecular biological approaches have successfully been employed to identify and characterize different carrier proteins present in the canalicular plasma membrane of the mammalian hepatocyte (reviewed in: Kamisako et al. 1999; Oude Elferink et al. 1995). Several canalicular transport proteins belong to the ATP-binding cassette (ABC) protein superfamily (Kamisako et al. 1999). One group among these ATP-dependent proteins are the

P-glycoproteins (P-gps), membrane transporters which are encoded by the highly conserved MDR (multidrug resistance) gene family (Bellamy 1996; Germann 1996). Two genes encoding P-gps are known in humans (*MDR1* and *MDR2*; Roninson et al. 1986),whereas three have been described in rodents (*mdr1a, mdr1b* and *mdr2*; Hsu et al. 1989). *Mdr1* genes can confer multidrug resistance in transfection studies with full length cDNAs (Devault & Gros 1990; Van der Bliek et al. 1988) and encode P-gps that mediate the ATP-dependent extrusion of a broad spectrum of hydrophobic chemicals. Overexpression of mdr1-type P-gps in certain tumors is associated with their resistance to a broad spectrum of structurally and functionally unrelated hydrophobic drugs (multidrug resistance) (Bellamy 1996; Germann 1996). By contrast to *mdr1* genes, *mdr2* genes code for phospholipid transporters and are not related to multidrug resistance or decreased drug accumulation (Smit et al. 1993). In addition to their occurrence in tumor cells, *mdr* genes are also expressed in normal mammalian tissues (Fojo et al. 1987; Thiebaut et al. 1987; Thiebaut et al. 1989). The *mdr1* genes are expressed at high levels in adrenal cortex, renal proximal tubules, the canalicular pole of hepatocytes, small and large intestinal mucosal cells, and pancreatic ductules (Fojo et al. 1987; Thiebaut et al. 1987). Lower levels of expression of MDR1 exist in other tissues, including the capillary endothelial cells of the brain and testis (Thiebaut et al. 1989). MDR2 is predominantly expressed in the canalicular pole of hepatocytes and shows only a minor extrahepatic expression (Smit et al. 1993).

Different reports have demonstrated the presence of P-gp(s) in teleost fish. In immunohistochemical studies, the distribution of conserved P-gp epitopes among tissues in fish resembled that in mammals (Hemmer et al. 1995). In Western analyses of liver extracts from teleost fish, a mammalian P-pg antibody recognized a band of 170 kDa, the approximate molecular weight of mammalian P-gps (Cooper et al. 1999). Partial coding sequences of two genes showing high homology to *mdr* genes have been identified in a genomic DNA library from a pleuronectid teleost (Chan et al. 1992). Functional studies of P-gp-like proteins in fish, however, have been restricted to the teleost kidney, in which the presence of a P-gp-like mechanism of the cell-to-lumen transport of hydrophobic compounds has been demonstrated (Miller 1995; Schramm et al. 1995; Sussman-Turner & Renfro 1995). No studies are available to date, however, concerning the involvement of P-gp(s) in liver transport in fish.

The aim of this study was to characterize hepatic P-glycoprotein in teleost fish, using rainbow trout (*Oncorhynchus mykiss*) as a model. By immunochemical methods, employing antibodies directed against conserved P-gp epitopes, the presence of P-pg(s) was shown in trout liver and cultured hepatocytes. To characterize hepatic P-gp(s) in trout on a functional level, we used isolated hepatocytes from trout, in which we studied the accumulation and efflux of rhodamine 123, a fluorescent substrate for mammalian mdr1-type P-gp, in the presence and absence of inhibitors of mdr1 proteins and other hepatic transport systems. To our knowledge, this is the first functional investigation of hepatic P-gp in fish. Such research appears important to gain fundamental insights into the mechanisms of hepatobiliary excretion

in lower vertebrates and the function and regulation of their P-gp(s). The physiology of hepatobiliary excretion in fish could differ from that in higher vertebrates, since the anatomical organization of the biliary system in teleosts markedly differs from that in mammals (Hinton et al. 1999). Moreover, the study of hepatic P-gp(s) in fish has implications for the ecological risk assessment of toxic compounds, and the use of fish as surrogate species in toxicological testing.

MATERIALS AND METHODS

Chemicals and cell culture material

Rhodamine 123, verapamil, vinblastine, tetraethylammonium chloride, doxorubicin, cyclosporin A, ethyl-4-aminobenzoate, streptomycin-penicillin and L-glutamine were obtained from Sigma, Deisenhofen, Germany. Collagenase D was purchased from Boehringer, Mannheim, Germany. Defatted bovine serum albumine (BSA), NADH and polyvinylidene difluoride membranes were from Serva, Heidelberg, Germany. Fetal calf serum (FCS) was obtained from Biochrom, Berlin, Germany. Primaria cell culture dishes and Matrigel were obtained from Becton-Dickinson, Heidelberg, Germany. Chamber slides (2 wells per slide type) were from Nalge Nunc International, Naperville, IL, USA. The monoclonal antibody C219 was purchased from Centocor, Malvern, PA, USA and the polyclonal antibody PC03 from Calbiochem-Novabiochem, Bad Soden, Germany. Prestained SDS-PAGE protein standards were purchased from BIO-RAD, München, Germany. Glycergel mounting medium, horseradish-peroxidase or biotin coupled secondary antibodies, horseradish peroxidase-coupled streptavidin, and FITC-coupled streptavidin were obtained from DAKO A/S, Glostrup, Denmark. The ECL system was from Amersham, Braunschweig, Germany.

Animals

Female rainbow trout of a weight between 250 and 350 g were obtained from a local trout farm. Trout were maintained at 15°C in tanks receiving a flow-through of well-aerated, charcoal-filtered city water and fed a commercial trout diet (Trouvit, Milkivit, Burgheim, Germany).

Isolation and Primary Culture of Trout Hepatocytes

Hepatocytes were isolated as described by Mommsen et al. (1994). In brief, following anesthetization with ethyl-4-aminobenzoate and the injection of 200 U of heparin, trout were ventrally opened and the liver perfused in situ via the intestinal vein. The liver was perfused

for 10 min with solution 1 (137 mM NaCl, 5.4 mM KCl, 0.81 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.33 mM Na_2HPO_4, 5.0 mM $NaHCO_3$, 10 mM HEPES, pH 7.63), followed by 25 min of perfusion with solution 2 (0.008% collagenase D and 1% defatted bovine serum albumin dissolved in solution 1), followed by 5 min of perfusion with solution 1. Perfusion solutions were temperated to 15°C and bubbled with air before use. After perfusion, the liver was removed and minced in ice cold solution 3 (1% defatted bovine serum albumin and 1.5 mM $CaCl_2$ in solution 1). The resulting suspension was filtered through a set of nylon screens (250 µ, 100 µ, 50µ). Cells were collected by centrifugation (3 min, 50 *x* g). The pellet was washed three times with ice-cold solution 3. After the last washing step, cells were resuspended in modified M199 (Sigma M-3274, supplemented to final 3.5 mM HEPES, 4.1 mM $NaHCO_3$, 3.4 mM $CaCl_2$, 2 mM L-glutamine, 10 U/ml penicillin, 10 µg/ml streptomycin). Cell viability was assessed by trypan blue exclusion and was at least 80%. The hepatocytes were seeded at a density of 0.34 x 10^6 viable cells / cm^2 in modified M199 containing 5% FCS onto culture dishes previously coated with Matrigel (0.1 mg/ml protein applied at 110 µl per cm^2 of culture dish surface). Hepatocytes were allowed to attach for at least 12 hours in an incubator set at 15°C and subsequently the medium was replaced with fresh, serum-free modified M199. Further changes of the medium were performed daily.

Immunoblot analyses

Cytoplasmic membrane fractions of 300 mg of liver tissue or 30 x 10^6 hepatocytes per sample were isolated by sucrose gradient centrifugation according to Simpson et al. (1983). To serve as a positive control, cytoplasmic membrane fractions were prepared analogously from male bovine adrenal. The buffers used for homogenization and centrifugation contained 1 mM phenylmethylsulfonyl fluoride. Ten µg of protein per lane, determined according to Lowry et al. (1951), were subjected to electrophoresis through 7.5% SDS-polyacrylamide gels (Laemmli 1970). Proteins were then transferred to polyvinylidene difluoride membranes by semidry-blotting (Khyse-Andersen 1984) using a continuous buffer system (48 mM Tris, 39 mM glycine, 0.038% (w/v) SDS and 15% (v/v) methanol, pH 9.0). P-gp was detected by using (a) the primary monoclonal antibody (mAB) C219 (Georges et al. 1990) (b) the primary polyclonal antibody PC03. PC03 was raised against a conserved sequence in the C-terminal cytoplasmic P-glycoprotein region (SALDTESEKVVQEALDKAREG). Peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibodies, respectively, were used as the appropriate secondary antibodies. Protein bands were visualized by enhanced chemoluminescence using the ECL system. After densitometry, the staining intensity of trout immunoreactive bands were expressed relative to that of the P-pg band obtained from a known amount of a bovine adrenal preparation.

Immunohistochemistry

Trout livers were fixed in Bouin's fluid for 24 h, paraffin embedded, sectioned and deparaffinized. Trout hepatocyte adherent cultures were fixed for 15 min in phosphate-buffered (0.01 M, pH 7.2) 3.7% formaldehyde, washed twice with PBS/glycine (0.01 M phosphate, 0.123 M NaCl, 0.1 M glycine, pH 7.2), permeabilized with absolute methanol at –20°C for 15 min and washed twice with PBS/glycine. The following incubations took place in a moist chamber and were at room temperature except where noted otherwise. Samples were rinsed four times between incubations with PBS/glycine. Liver sections and hepatocytes were incubated for 30 min with 5% non-fat dry milk/PBS (0.01 M phosphate, 0.123 M NaCl, pH 7.2), and incubated overnight at 4°C with mAB C219 (2 µg/ml) in 5% non-fat dry milk/PBS. After rinsing, liver sections and hepatocytes were incubated for 2 hours with biotin-conjugated goat-anti-mouse IgG (1:400) in 5% non-fat dry milk/PBS. Following rinsing, liver sections were incubated for 3 hours with horseradish peroxidase-coupled streptavidin (1:300) in PBS, whereas hepatocytes were incubated for 3 hours with FITC-coupled streptavidin (1:100) in PBS. Liver sections were then rinsed and treated with substrate (diaminobenzidine / H_2O_2 mixture) for 10 min, rinsed in tap water and mounted. Hepatocytes were rinsed with PBS and mounted.

Accumulation and efflux of rhodamine 123 and doxorubicin

Trout hepatocytes cultured for 24 h after isolation were used in experiments on the accumulation or the efflux of the fluorescent substrates of mammalian mdr1-type P-gps, rhodamine 123 (Rh123) and doxorubicin (Efferth et al. 1989; Germann 1996). Verapamil, vinblastine, cyclosporin A and doxorubicin were used as inhibitors of P-gp (Germann 1996). Tetraethylammonium was used as a substrate of hepatic type I cation transport systems (Oude Elferink et al. 1995). Incubations took place at 15°C. In accumulation experiments, the medium of hepatocyte monolayers was replaced by serum-free modified M199 containing the indicated fluorescent P-gp substrate alone, or in combination with the indicated inhibitor. After variable times of incubation, the medium was removed, the hepatocyte monolayer washed with PBS, and the culture dish stored at –20°C for later determination of intracellular levels of the respective P-gp substrate. In efflux experiments, the medium was removed from hepatocyte monolayers and replaced by 5.25 µM Rh123 in serum-free modified M199. After 2 h of incubation, the medium was removed, and the cells were washed with PBS. One replicate per experiment was removed and stored at –20°C for the determination of the accumulated levels of intracellular Rh123 ('before efflux'). The remaining replicates were incubated for the indicated times with serum-free modified M199 alone, or in combination with the indicated inhibitors. After incubation, the medium was removed and culture dishes

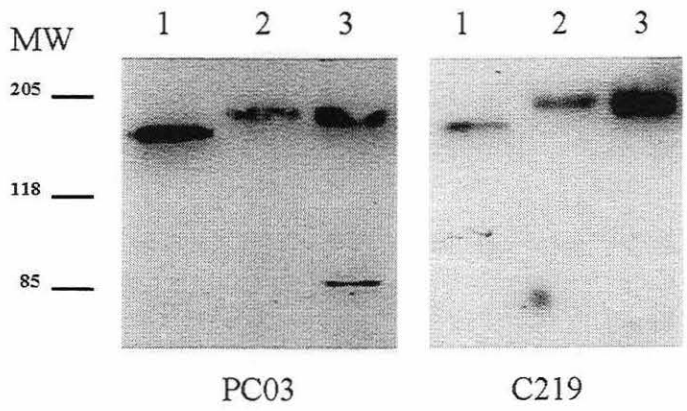

Fig. 4.1. Immunoblot detection of P-glycoprotein(s) in cytoplasmic membrane fractions from rainbow trout liver and cultured hepatocytes. The polyclonal antibody PC03 (left) and the monoclonal antibody C219 (right) were used as primary antibodies. Lane 1: male bovine adrenal (positive control), 2 µg protein /lane; lane 2: trout hepatocytes cultured for 2 days, 10 µg protein/ lane; lane 3: rainbow trout liver, 10 µg protein /lane. MW: position of molecular weight markers of 205; 118 and 85 kDa.

stored at –20°C for later determinations. For Rh123 determinations, cell culture plates were thawed and 2 ml n-butanol added to wells of 24- or 6- well plates to extract Rh123 and to precipitate protein. After 30 min of extraction, the Rh123 concentration in n-butanol was determined fluorometrically (excitation at 517 nm, emission at 532 nm). Doxorubicin determinations were carried out in an analogous way (excitation at 291 nm, emission at 582 nm). Intracellular levels of Rh123 or doxorubicin are reported as $\mu g/10^6$ hepatocytes (viable cell numbers at the time of seeding).

Statistics

Data are given as means ± SE. Means were considered to be significantly different from the corresponding control mean when the probability value (P) was < 0.05 in the appropriate paired T-test or one-way repeated measures (i.e. matching the observations made on cells obtained from the same fish) analyses of variance (ANOVAs) and associated Dunnett's tests.

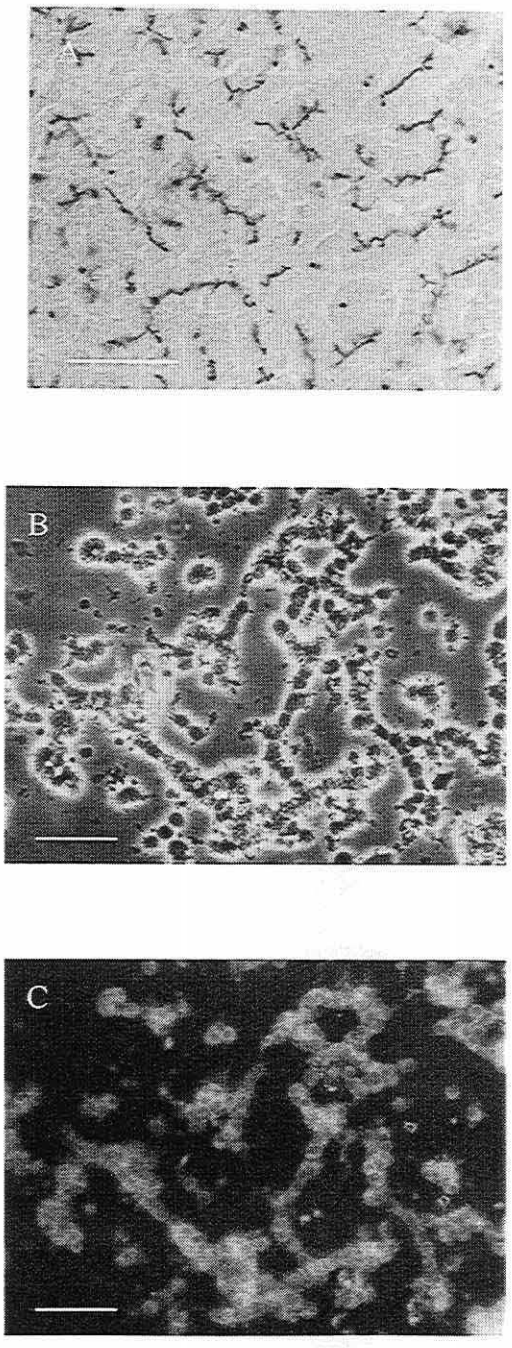

Fig. 4.2. Immunoreactivity of monoclonal antibody C219 against P-glycoprotein(s) in rainbow trout liver and cultured heatocytes. *A*: C219 reacted with bile canalicular structures in rainbow trout liver. Isolated rainbow trout hepatocytes re-aggregated during 24 h of primary culture (*B*: phase contrast microscopy) and their plasma membranes reacted with C219 (*C*: immunofluorescence microscopy). Scale bars = 50 μM. Antibody staining was visualized with avidin-biotin-peroxidase method with diaminobenzidine as chromagen (*A*) or with avidin-biotin-FITC method (*C*).

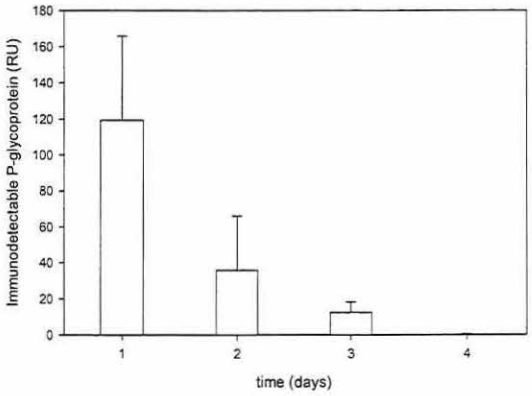

Fig. 4.3. P-glycoprotein levels in rainbow trout hepatocytes during the time course of primary culture. Isolated hepatocytes were cultured as adherent monolayers. After different periods of primary culture, cytoplasmic membrane fractions were prepared from hepatocytes, resolved by SDS-PAGE, and P-glycoprotein determined as described in Materials and Methods. The intensity of the immunoreactive band in trout hepatocyte samples is expressed in relative units (RU) relative to that of a positive control prepared from bovine adrenal which was included on each gel. Values are means ± SE for 3 hepatocyte cultures from different fish.

RESULTS

Immunochemical detection of P-glycoprotein in trout liver and cultured hepatocytes

In immunoblots, the polyclonal antibody PC03 and the monoclonal antibody C219 recognized the same major band of a molecular weight of about 165 kDa in cytoplasmic membrane fractions from trout hepatocytes and trout liver (Fig. 4.1). Both antibodies detected a protein of approximately 158 kDa in cytoplasmic membrane fractions from male bovine adrenal, a tissue showing high levels of P-gp and therefore used as a positive control (Ichikawa-Haraguchi et al. 1993). The 165 kDa immunoreactive protein(s) detected in trout liver and isolated hepatocytes thus most probably represent trout P-gp(s). Depending on species and tissue, the apparent molecular weight of P-gps may vary in the range between 130-180 kDa, reflecting differences in glycosylation (Germann 1996). A minor band of 90 kDa was additionally recognized in trout liver cytoplasmic membrane fractions by the polyclonal antibody PC03 (Fig. 4.1) and, at higher concentrations of sample, by the monoclonal antibody C219 (not shown). The identity of the minor band is not known. It may represent a fragment of trout P-gp or an antigenetically related protein.

In trout liver paraffin sections, immunohistochemical staining with C219 specifically stained bile canalicular structures (Fig. 4.2*A*). The cellular localization of P-gp(s) was also

investigated in trout hepatocyte cultures. When trout hepatocytes are seeded following isolation, they consist of single suspended cells, with very few couplets. Within 24 h of culture, trout hepatocytes partly re-aggregate and form strands of interconnected cells (Fig. 4.2*B*). Immunocytochemistry using the mAB C219 resulted in specific staining of the entire cytoplasmic membrane (Fig. 4.2*C*). The levels of P-gp expression in trout hepatocytes, analyzed in Western blots and expressed relative to the P-gp signal in a preparation from bovine adrenal, were followed over the period of primary culture (Fig. 4.3). P-gp levels in trout hepatocytes strongly decreased with time in primary culture. This did not reflect a general loss in physiological performance, as the liver-specific physiological variables cytochrome P4501A and vitellogenin, respectively, were responsive to appropriate treatment (48 h) with β-naphthoflavone and estradiol (not shown).

Accumulation and efflux studies with fluorescent substrates of mdr1-type P-gps

Because the immunoblot investigations indicated a decrease in P-gp expression during time in primary culture, hepatocytes cultured for 24 h following isolation were used in further

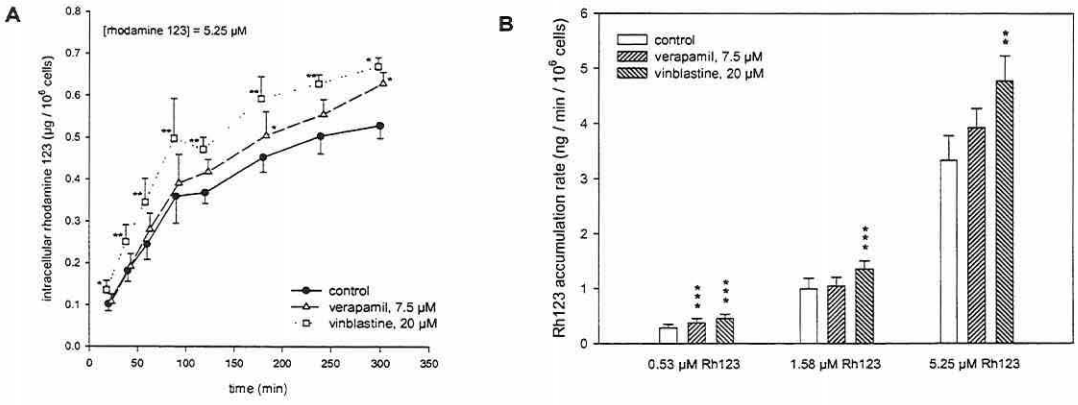

Fig. 3.4. Effects of verapamil and vinblastine on the accumulation of rhodamine 123 by cultured rainbow trout hepatocytes. *A:* Time course of rhodamine 123 accumulation. Trout hepatocytes were incubated with medium containing 5.25 µM rhodamine 123 in the absence (control) or presence of inhibitors of P-glycoprotein (7.5 µM verapamil or 20 µM vinblastine). At the indicated times, incubations were stopped and intracellular rhodamine 123 levels determined as described in Materials and Methods. *B:* Initial accumulation rates of rhodamine 123 by trout hepatocytes. From the experiment shown in *A*, and similar experiments with 0.53 µM and 1.58 µM rhodamine 123, initial rates of rhodamine 123 accumulation were derived, using the measurements after 20 – 90 min of incubation. Values are means ± SE for 5 hepatocyte cultures from different fish. Significantly different from corresponding control values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

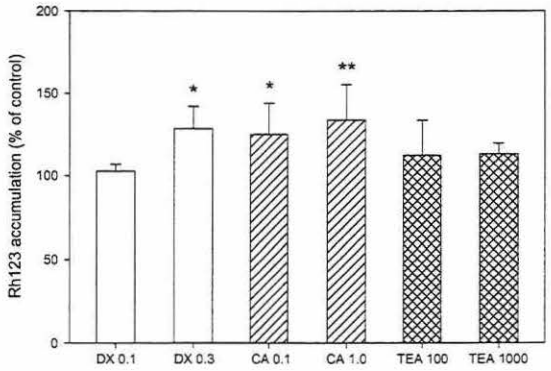

Fig. 4.5. Effects of transport inhibitors on the accumulation of rhodamine 123 by cultured rainbow trout hepatocytes. Trout hepatocytes were incubated with medium containing 5.25 μM rhodamine 123 in the absence (control) or presence of doxorubicin (DX 0.1: 0.1 μM; DX 0.3: 0.3 μM), cyclosporin A (CA 0.1: 0.1 μM; CA1.0: 1.0 μM) or tetraethylammonium (TEA 100: 100 μM; TEA 1000: 1000 μM). After 2 h, incubations were stopped and intracellular rhodamine 123 levels determined as described in Materials and Methods. Values are means ± SE for 3-5 hepatocyte cultures from different fish. Significantly different from corresponding control values: * $P < 0.05$, ** $P < 0.01$.

rhodamine 123 (Rh123), a known substrate of mdr1-type P-gps (Efferth et al. 1989), we investigated whether an mdr1-like efflux mechanism existed in cultured rainbow trout hepatocytes. The calcium channel blocker verapamil and the immunosuppressive peptide cyclosporin A are known as chemosensitizers, i.e. compounds that interfere with P-gp-dependent transport (Ford & Hait 1990). The Vinca alkaloid vinblastine and the anthracyclin doxorubicin are anticancer drugs transported by mdr1-type P-gps (Bellamy 1996; Germann 1996), and therefore compete with other substrates.

The time-course of the accumulation of Rh123 by trout hepatocytes was investigated using different Rh123 concentrations, either in the absence (control) or the presence of verapamil or vinblastine (Fig. 4.4, *A* and *B*). In the presence of 5.25 μM Rh123, the accumulation of Rh123 by trout hepatocytes was linear with time during the initial 90 min of incubation, after which its rate decreased (Fig. 4.4*A*). When compared to controls, the Rh123 accumulation by hepatocytes co-treated with vinblastine was significantly increased at all times of observation, while the Rh123 accumulation by hepatocytes co-treated with verapamil was significantly increased only after 240 min and 300 min (Fig. 4.4*A*). Using linear regression, the initial rates of Rh123 (0-90 min) were derived from the experiment shown in Fig. 4.4*A* and similar experiments using Rh123 concentrations of 0.53 and 1.58 μM (Fig. 4.4*B*). Vinblastine significantly increased the initial rate of Rh123 accumulation with all Rh123 concentrations studied, while verapamil increased Rh123 accumulation significantly

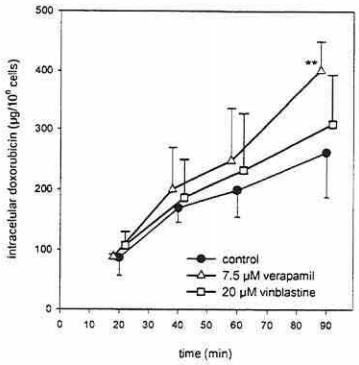

Fig. 4.6. Time course of doxorubicin accumulation by rainbow trout hepatocytes. Hepatocytes were incubated with medium containing 25 µM doxorubicin in the absence (control) or presence of inhibitors of mdr1-type P-glycoproteins (7.5 µM verapamil or 20 µM vinblastine). At the times indicated, intracellular doxorubicin levels were determined as described in MATERIALS AND METHODS. Values are means ± SE for 4 hepatocyte cultures from different fish. Significantly different from corresponding control values: ** $P < 0.01$.

only in experiments with 0.52 µM Rh123, but not with 1.58 µM and 5.25 µM Rh123. The effects of further compounds on the accumulation of Rh123 by trout hepatocytes were investigated using a fixed incubation time of 2 h (Fig. 4.5). Inclusion of doxorubicin or cyclosporin A during the incubation of hepatocytes with 5.25 µM Rh123 significantly increased intracellular Rh123 accumulation (Fig. 4.5). By contrast, the organic cation tetraethylammonium, a substrate for type I sinusoidal organic cation uptake systems and a electroneutral canalicular H^+/ organic cation antiporter (Oude Elferink et al. 1995), had no significant effect on Rh123 accumulation. Moreover, the accumulation of the mdr1 substrate doxorubicin was studied in the absence (control) and the presence of verapamil and vinblastine (Fig. 4.6). Verapamil significantly increased doxorubicin accumulation by trout hepatocytes, while vinblastine caused only slight, non-significant changes.

The mdr 1 substrates vinblastine and doxorubicin and the chemosensitizers verapamil and cyclosporin A increased the accumulation of Rh123, suggesting the presence of a mdr1-like efflux mechanism in trout hepatocytes. The hypothesis that the observed increases in Rh123 accumulation reflect the inhibition of an active efflux mechanism was further examined in efflux experiments. Hepatocytes were first incubated with 5.25 µM Rh123 for 2 h. After washing with PBS, hepatocytes were further incubated in medium alone, or medium containing different compounds known to interfere with mdr1-dependent transport ('efflux incubation'). Vinblastine significantly delayed the decrease in intracellular Rh123 levels and the increase in extracellular Rh123 concentrations in the medium (Fig. 4.7 *A* and *B*). In similar experiments using a fixed efflux incubation period of 4 h, different chemicals were investigated concerning their effects on Rh123 retention by trout hepatocytes (Table 4.1). Verapamil, vinblastine and cyclosporin A dose-dependently and significantly increased the

intracellular levels of rhodamine 123 retained by hepatocytes after 4 h of incubation in Rh123-free medium (Table 4.1). Doxorubicin effected only slight, non-significant increases in Rh123 retention, while tetraethylammonium had no apparent effect (Table 4.1).

Table 4.1. Effect of transport inhibitors on efflux of rhodamine 123 from rainbow trout hepatocytes

Treatment	N	Intracellular Rhodamine 123
Before Efflux	5	0.295 ± 0.053**
Control	5	0.193 ± 0.049
2.5 μM Verapamil	5	0.221 ± 0.045
7.5 μM Verapamil	5	0.236 ± 0.059*
25 μM Verapamil	5	0.264 ± 0.047**
7.0 μM Vinblastine	5	0.266 ± 0.043**
20 μM Vinblastine	5	0.275 ± 0.042**
60 μM Vinblastine	5	0.277 ± 0.043**
Before Efflux	6	0.215 ± 0.074*
Control	6	0.188 ± 0.071
20 μM Vinblastine	6	0.230 ± 0.084**
0.3 μM Cyclosporin A	6	0.180 ± 0.079
1.0 μM Cyclosporin A	6	0.197 ± 0.079
3.0 μM Cyclosporin A	6	0.214 ± 0.091*
Before Efflux	6	0.168 ± 0.030
Control	6	0.128 ± 0.016
20 μM Vinblastine	6	0.165 ± 0.030**
1.0 μM Doxorubicin	6	0.136 ± 0.023
3.0 μM Doxorubicin	6	0.134 ± 0.027
10.0 μM Doxorubicin	6	0.134 ± 0.029
30.0 μM Doxorubicin	6	0.141 ± 0.029
Before Efflux	5	0.268 ± 0.047**
Control	5	0.218 ± 0.058
20 μM Vinblastine	5	0.268 ± 0.071**
0.1 mM TEA	5	0.225 ± 0.080
1.0 mM TEA	5	0.219 ± 0.074

Values are means ± SE in $\mu g/10^6$ cells; n, no. of hepatocyte cultures from different fish. Hepatocytes were cultured for 24 h after isolation before use in the experiment. Cells were incubated for 2 h in medium containing 5.25 μM rhodamine 123 and washed with PBS. One triplicate cell culture per fish was removed at this stage for determination of intracellular rhodamine 123 levels (before efflux). The remaining cell cultures were incubated for 4 h with medium without (control) and with indicated chemicals in triplicate. After incubations, intracellular levels of rhodamine 123 were determined as described in MATERIALS AND METHODS. TEA, tetraethylammonium. *Significantly different from controls, $P < 0.05$. Significantly different from controls, **$P < 0.01$.

DISCUSSION

Immunochemical evidence for the presence of P-glycoprotein(s) in trout liver

Recent studies in teleost fish have demonstrated the presence of hepatic protein(s) which are immunoreactive to antibodies directed against conserved P-gp epitopes, and which resemble mammalian P-gps with respect to their apparent molecular weight and localization to the canalicular membrane (Cooper et al. 1999; Hemmer et al. 1995). Our immunochemical observations on rainbow trout liver are in line with these reports on other species and, as these studies (Cooper et al. 1999; Hemmer et al. 1995), strongly suggest the hepatic expression of P-gp(s) in teleost fish. However, the results obtained in this and other studies should be interpreted with caution, as the exact specificity of the used P-gp antibodies in fish is not known. The polyclonal antibody PC03 is raised against a conserved 26-amino acid sequence in the C-terminal cytoplasmic P-gp region (see Materials and Methods) which includes the 7-amino acid epitope recognized by C219 (Georges et al. 1990). The epitope recognized by the monclonal antibody C219, a immunochemical probe used in this and other studies (Cooper et al. 1999; Hemmer et al. 1995), is present in all human and rodent P-gp genes known to date, including the *mdr2* genes, the products of which are not related to hydrophobic drug transport. Moreover, C219 also recognizes a canalicular transport protein called

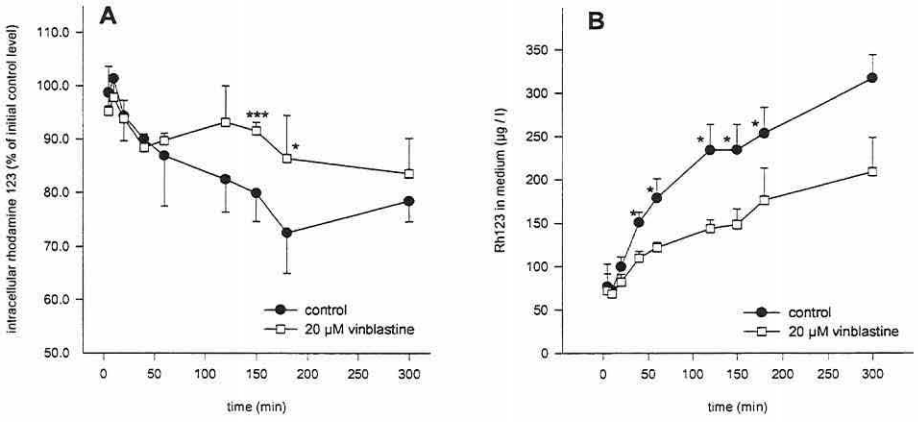

Fig. 4.7. Time course of rhodamine 123 efflux from rainbow trout hepatocytes. After incubation with medium containing 5.25 µM rhodamine 123 for 2 h, hepatocytes were washed and transferred (t= 0) to medium (control) or medium containing 20 µM vinblastine. At the times indicated, rhodamine 123 levels in the cells and in the medium were determined as described in Materials and Methods. *A*: Time course of intracellular rhodamine 123 levels. *B*. Rhodamine 123 concentrations in the medium. Values are means ± SE for 3 hepatocyte cultures from different fish. Significantly different from corresponding control values: * $P < 0.05$, *** $P < 0.001$.

called 'sister of P-gp' (sPgp) (Childs et al. 1995) that mediates the export of bile salts (Gerloff et al. 1998). Partial sequences of both *P-gp* and *sPgp* genes have been identified in teleosts (Chan et al. 1992; Cooper 1999). Hence, the possibility exists that multiple canalicular carrier proteins are detected by the antibodies C219 and PC03 in teleost liver. As long as more specific probes are lacking, antibodies directed against conserved epitopes, such as C219, can yield basic information concerning the occurrence and distribution of P-gps in fish. The molecular cloning of piscine P-gps, currently underway in several laboratories (GenBank. [Online] National Center of Biotechnology Information, Bethesda, MD, USA. http://www.ncbi.nlm.nih.gov/ [2000, Feb 9]), should enable the design of more specific probes that will allow the molecular identification of P-gp gene products in fish tissues.

Expression of P-gp in trout hepatocytes–immunochemical results

Cultured mammalian hepatocytes are well established as an in vitro system for physiological and toxicological studies. Moreover, mammalian hepatocytes have extensively been used in studies on mammalian Pgps (Chieli et al. 1994; Fardel et al. 1993; Hirsch-Ernst et al. 1998; Lee et al. 1993). For this reason, it appeared worthwhile to investigate the expression of P-gp(s) in cultured piscine hepatocytes. Immunohistochemistry with the antibody C219 resulted in specific staining of the entire plasma membrane of cultured trout hepatocytes. This reflects the usual situation in isolated hepatocytes in which, due to the disruption of cell contacts during the isolation procedure, cell polarity is lost and components of the former basolateral (sinusoidal) and apical (canalicular) membrane domains are evenly distributed over the cellular surface (Oude Elferink et al. 1995). During primary culture as adherent monolayers, teleost hepatocytes usually undergo a sequence of morphological changes (Segner 1998). The initially single hepatocytes flatten, form progressively larger clusters of cells, and finally constitute cell strands resembling liver tubuli in vivo (Fig. 4.2*B*). In such cell strands, membrane differentiations may re-appear between neighboring cells which are reminiscent of structures found in vivo, such as junctional complexes, gap junctions and bile canaliculi (Blair et al. 1995; Segner 1998; Vogt & Segner 1997). However, evidence for the formation of functionally integer bile canaliculi in cultured fish hepatocytes is lacking, and also not provided by this study.

The levels of P-gp(s) detected in immunoblots of cultured trout hepatocytes decreased strongly during the time course of culture (Fig. 4.3). By contrast, depending on the species and the culture conditions, mammalian hepatocytes exhibit a stable expression of P-gp(s) or an isoform-specific overexpression of P-gp(s) during primary culture for several days (Fardel et al. 1993; Hirsch-Ernst et al. 1998; Lee et al. 1993). Because of the rapid decrease of the levels

of P-gp expression in trout hepatocytes during the time course of primary culture, hepatocytes cultured for 24 h after isolation were used in accumulation and efflux studies of fluorescent dyes.

Evidence for a mdr1-like efflux mechanism in trout hepatocytes

Drug-resistant tumor cell lines overexpressing *mdr1* genes exhibit a decreased accumulation and increased efflux of Rh123 and other fluorescent dyes when compared to their drug-sensitive parental cell lines lacking mdr1 expression (Bucana et al. 1990; Efferth et al. 1989). The decreased accumulation and increased efflux of fluorescent dyes in these cell lines can partly or completely be reverted by co-administration of compounds that interfere with mdr1-dependent transport (Bucana et al. 1990; Efferth et al. 1989). Accordingly, assays based on the observation of fluorescent dye accumulation/efflux, and the effects of diagnostic compounds hereon, have been used to diagnose mdr1-type P-gp-dependent drug resistance in clinical tumor samples (Efferth et al. 1989). The same approach has been employed to study the function of P-gps in cells from normal tissues, including isolated hepatocytes (Chieli et al. 1994; Hirsch-Ernst et al. 1998).

In the present study, we examined the accumulation and efflux of Rh123 in cultured rainbow trout hepatocytes under the influence of different compounds which interfere with mdr1-type P-gp transport and are themselves substrates (Germann 1996), namely the calcium channel blocker verapamil, the Vinca alkaloid vinblastine, the immunosuppressive peptide cyclosporin A and the anthracyclin doxorubicin. In the mammalian hepatocyte, different transport proteins exist which are thought to contribute to drug transport (Kamisako et al. 1999). Hence, mechanisms of both xenobiotic uptake and secretion may have been underlying processes in our Rh123 accumulation and efflux experiments. The uptake of highly hydrophobic cations, such as Rh123, at the hepatocyte's sinusoidal membrane is assumed to occur mainly passively (Oude Elferink et al. 1995). The uptake of organic cations is further mediated by at least two transport proteins in sinusoidal membrane that preferably transport less hydrophobic cationic compounds (Oude Elferink et al. 1995). If the accumulation of Rh123 by trout hepatocytes had involved active uptake mechanisms, one would expect that other cationic compounds should (partly) inhibit Rh123 accumulation. The accumulation of Rh123 in this study, however, could not be decreased by any of the investigated inhibitors/substrates of organic cation transport systems (Figs. 4.4; 4.5). This argues against the significance of active uptake in Rh123 accumulation. However, all of the investigated compounds known to interfere with mdr1-type P-gp-mediated transport, i.e. verapamil, vinblastine, cyclosporin A and doxorubicin, significantly increased the accumulation of Rh123 by trout hepatocytes (Figs. 4.4; 4.5). This provides evidence for the presence of a mdr1-like efflux mechanism that is sensitive to inhibition and/or competition. Further experiments were carried out concerning the effects of the selected compounds on the efflux

of Rh123 from trout hepatocytes. Verapamil, vinblastine, cyclosporin A and doxorubicin decreased the efflux of Rh123 from hepatocytes (significant for all compounds except doxorubicin, Table 4.1), while TEA had no effect. Moreover, the accumulation of doxorubicin by hepatocytes was increased by vinblastine and verapamil (significant only for verapamil, Fig. 4.6). These observations further support the presence of a mdr1-like mechanism in trout hepatocytes, and suggest that P-gps are involved in the biliary secretion of hydrophobic compounds in trout.

Although our results indicate the presence of a mdr1-like mechanism in the teleost hepatocyte, they do not allow conclusions about the number and exact identity of the transport protein(s) involved, as very little is known on canalicular transport in fish. In the mammalian hepatocyte, the different proteins involved in the active transport of hydrophobic compounds into bile partly overlap in substrate and inhibitor specificity. In addition to mdr1-type P-gp(s), another ABC transport protein expressed in the canalicular membrane is a member of the multidrug resistance-associated protein subfamily (MRP) (Keppler et al. 1997). The canalicular isoform of MRP has been shown to be identical to the functionally defined canalicular multispecific organic anion transporter (cMOAT) (Keppler et al. 1997). It has been speculated that MRP may also transport organic cations (Meijer et al. 1997). Tumor cell lines overexpressing MRP and lacking P-gp expression showed a decreased accumulation of doxorubicin and Rh123 (Barrand et al. 1993; Zaman et al. 1994) that was sensitive to verapamil and cyclosporin A (Barrand et al. 1993), suggesting that these compounds interact with MRP and possibly represent MRP substrates. In conclusion, MRP-like proteins, for the presence of which evidence exists in elasmobranch fish (Miller et al. 1998), could have contributed to the mdr1-like mechanism in trout hepatocytes described in this report. In addition to the ABC proteins discussed above, a H^+/organic cation antiport mechanism exists in the canalicular membrane (Moseley et al. 1992). Of the compounds investigated in this report, TEA is a substrate of the canalicular H^+/organic cation antiport mechanism (Moseley et al. 1992). Because of the lack of effects of TEA on the accumulation and efflux of Rh123 in trout hepatocytes, it appears improbable that the H^+/organic cation antiporter interacted with Rh123 in our experiments.

The mdr1-like Rh123 efflux mechanism in trout hepatocytes characterized in the present report resembles a P-gp-like transport mechanism that has previously been demonstrated in the teleost renal proximal tubule (Miller 1995; Schramm et al. 1995; Sussman-Turner & Renfro 1995). Primary cultures of flounder proximal tubule epithelium mounted in Ussing chambers exhibited an active net secretion of the mdr1-type P-gp substrate daunomycin that was inhibited by verapamil, vinblastine and cyclosporin A (Sussman-Turner & Renfro 1995). The luminal daunomycin accumulation in primary cultured killifish renal tubules was sensitive to verapamil and cyclosporin A (Miller 1995). Similarly, the secretion of a fluorescent cyclosporin analogue in the same in vitro system could be blocked by verapamil, vinblastine, cyclosporin A and other inhibitors/substrates of P-gp (Schramm et al. 1995).

Perspectives

This study demonstrates the presence of a mdr1-like mechanism in cultured rainbow trout hepatocytes and suggests the functional activity of mdr1-type P-gp(s) in teleost liver. The molecular cloning of teleost P-gp genes, presently underway in different laboratories, will facilitate the determination of the molecular identity of the proteins involved in the canalicular secretion of hydrophobic compounds in fish. Together with such molecular studies, functional investigations are needed for a more thorough understanding of this process in fish. Such knowledge will not only significantly contribute to the understanding of liver physiology and its evolution within vertebrates, but appears also highly relevant to predict the effects of potentially toxic environmental contaminants on fish. The role of P-gp-like mechanisms in the biliary excretion may strongly influence xenobiotic body burdens in fish. Subsequent studies should therefore specifically address the question which environmentally relevant xenobiotics interact with piscine P-gp(s). The hepatocyte model used in this report offers one experimental approach for such studies.

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Chapter 5

Prochloraz and nonylphenol diethoxylate inhibit P-glycoprotein-dependent rhodamine 123 efflux in trout hepatocytes, but do not affect hepatic P-glycoprotein expression in rainbow trout (*Oncorhynchus mykiss*)

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Abstract—*P-glycoproteins (P-gps) encoded by multidrug resistance 1 (*mdr1*) genes are ATP-dependent transporters located in the cytoplasmic membrane which mediate the efflux of a broad spectrum of structurally and functionally unrelated hydrophobic compounds from the cell. Among the mammalian tissues showing a high expression of P-gps are those of the liver and the kidney, suggesting the role of P-gps in excretion. In the present work, P-gp(s) in the liver and in primary cultured hepatocytes of rainbow trout were characterized and their interaction with two model pollutants was studied – the imidazole fungicide prochloraz and the alkylphenolic surfactant nonylphenol diethoxylate (NP2EO). Using a monoclonal antibody (mAB C219) directed against a conserved P-gp epitope, an immunoreactive protein of about 160 kDa was detected in immunoblots of liver extracts from control trout. In sections of control trout livers, immunohistochemistry with the mAB C219 resulted in specific staining of bile canaliculi. In juvenile trout exposed for 7 days to sublethal concentrations of prochloraz (10 µg/L; 100 µg/L) or NP2EO (100 µg/L; 400 µg/L), no changes in levels of immunoreactive hepatic P-gp(s) were found, suggesting that the two chemicals are not able to alter P-gp expression. Isolated hepatocytes from trout cultured as adherent monolayers showed an efflux of the fluorescent mdr1 substrate rhodamine 123 (Rh123) that was partly inhibited by compounds known to interfere with mdr1-dependent transport, such as verapamil and vinblastine. This demonstrates the presence of a mdr1-like mechanism in fish liver most probably mediated by P-gp(s). Non-cytotoxic concentrations of prochloraz and NP2EO were tested for inhibitory effects on the efflux of Rh123 from trout hepatocytes. Prochloraz was a potent inhibitor of the mdr1-like mechanism, being effective at 0.3 µM and above. NP2EO inhibited Rh123 efflux only at the highest concentration tested (31.6 µM). The accumulation and elimination of ^{14}C-prochloraz by cultured trout hepatocytes was not affected by mdr1 substrates (Rh123, vinblastine) and a mdr1 inhibitor (verapamil). This shows that prochloraz is not a substrate of the mdr1-like mechanism in trout liver. The inhibition of Rh123 efflux from trout hepatocytes by prochloraz and NP2EO, however, suggests that water pollutants can interfere with biliary excretion in fish. The use of cultured fish hepatocytes allows the diagnosis of the interaction of xenobiotic with P-gp(s) on a functional level, which is difficult to study in vivo, whereas the in vivo approach is suitable to assess alterations of the P-gp expression level. For this reason, investigations concerning the effects of xenobiotics on hepatic P-gp(s) in fish should be designed as complementary in vitro / in vivo studies.*

Keywords—P-glycoprotein, liver, fish, xenobiotic

INTRODUCTION

Bile is a major route for the excretion of endogenous compounds, such as bile acids and bile pigments, and exogenous compounds (xenobiotics), all of which may either be excreted directly or as conjugates (Arias et al. 1993; Klaasen & Watkins III 1984). The formation of bile takes place at the canalicular pole of the hepatocytes. Hepatocytes are polarized epithelial cells, whose basolateral domain is made up by the sinusoidal membrane and is in contact with blood. The hepatocyte's apical domain — constituting a much smaller proportion of the plasma membrane than the basolateral domain — consists of the canalicular membrane and forms the border of the bile canaliculus, the most proximal part of the biliary tree (Arias et al. 1993; Klaasen & Watkins III 1984). In mammals, hydrophobic compounds with a molecular weight exceeding 400 are predominantly excreted via the hepatobiliary route (Arias et al. 1993). Hydrophobic organic molecules undergo dramatic concentration during bile formation, resulting in concentration gradients of up to 1000 between liver and bile (Groothuis & Meijer 1996). Studies of the canalicular transport of bile acids and other organic anions have suggested that a driving force for their secretion into bile is the negative intracellular membrane potential (Arias et al. 1993; Groothuis & Meijer 1996). However, membrane potential as a driving force could only partly explain the large concentration gradients of organic molecules between liver and bile (Arias et al. 1993; Groothuis & Meijer 1996). Consequently, the involvement of other mechanisms has been postulated. Among other mechanisms, it is well established that diverse transporter proteins located in the canalicular membrane play an essential role in this process (Groothuis & Meijer 1996; Oude Elferink et al. 1995). To date, four distinct canalicular ATP-driven active transport systems have been identified and characterized in mammals (Kamisako et al. 1999).

Among different ATP-dependent transport proteins identified in the canalicular membrane, P-glycoproteins (P-gps) constitute an important group (Meijer et al. 1997; Oude Elferink et al. 1995). P-gps are encoded by the small, highly conserved MDR (multidrug resistance) gene family (Germann 1996), which forms a subfamily of the large superfamily of ATP-binding cassette (ABC) transporters (Bellamy 1996). P-gps consist of about 1200 amino acids and are made up of two homologous halves, each of which includes one nucleotide binding segment and six putative transmembrane domains (Bellamy 1996; Germann 1996). There are two P-gp genes in humans (*MDR1* and *MDR2*; Roninson et al. 1986), whereas three exist in rodents (*mdr1a, mdr1b* and *mdr 2*; Hsu et al. 1989). The overexpression of *mdr1*, but not of *mdr2* genes in human and rodent cell lines confers the resistance towards a broad spectrum of functionally and structurally unrelated drugs (multidrug resistance, MDR) (Germann 1996). MDR mediated by mdr1 P-gps is based on ATP-dependent drug efflux and the resultant decrease of the intracellular accumulation of drugs (Bellamy 1996; Germann

1996). The high level of expression of *mdr1* in certain human tumors is related to their resistance to combination chemotherapy (Bellamy 1996; Germann 1996). By contrast to *mdr1* genes, *mdr2* genes code for phospholipid transporters and are not related to multidrug resistance or decreased drug accumulation (Smit et al. 1993). While P-gps were originally discovered because of their relevance as a determinant of tumor resistance, they are also expressed in normal tissues. A high level of expression of *MDR1* is found in adrenal cortex, renal proximal tubules, the canalicular membrane of hepatocytes, small and large intestinal mucosal cells and pancreatic ductules (Thiebaut et al. 1987). Lower levels of expression of *MDR1* have been found in other tissues, including the capillary endothelial cells of the brain and testis (Bellamy 1996). The patterns of expression among tissues and the substrate specificity of the rodent *mdr1a* and *mdr1b* gene products have suggested that these genes together assume, in a complementary way, a similar physiological role as that subsumed by MDR1 in humans (Germann 1996).

Exposure to xenobiotics, such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-*p*-dioxins or chemotherapeutical agents, effects the induction of *mdr1* genes in rodent liver or hepatocytes from rodents or humans (Chieli et al. 1994; Gant et al. 1991; Schrenk et al. 1996; Schuetz et al. 1995). The inducibility of *mdr1* genes by xenobiotics, and the specificity of the mdr1 P-gps for hydrophobic substrates and their subcellular localization to the apical surface of epithelial cells in tissues involved in excretion, have provoked the hypothesis that one physiological function of mdr1 P-gps is contributing to the organismal defense against xenobiotics (Yeh et al. 1992).

Only little is known about the P-gp(s) of lower vertebrates. The presence of two genes showing homology to mammalian P-gp genes was demonstrated in a teleost (Chan et al. 1992). The tissue distribution of conserved P-gp epitopes in fish resembled that in mammals, with a specific immunohistochemical staining of bile canaliculi in liver (Hemmer et al. 1995). The presence of a mdr1-like transport mechanism was demonstrated in teleost kidney proximal tubules (Schramm et al. 1995; Sussman-Turner & Renfro 1995). As in mammals (Thorgeirsson et al. 1987), an increased level of expression of hepatic P-gp(s) was observed in hepatic carcinomas in fish (Cooper et al. 1999). Elevated levels of P-gp expression were also found in non-neoplastic livers of fish from polluted habitats, suggesting induction by environmental pollutants (Cooper et al. 1999). However, an induction of teleost P-gp(s) by xenobiotics in controlled laboratory experiments has not yet been demonstrated.

The present study characterized the interaction of model compounds, prochloraz and nonylphenol diethoxylate (NP2EO), with hepatic P-gp(s) of rainbow trout, by a combined in vitro / in vivo approach. Prochloraz is a widely used agricultural imidazole fungicide that acts both as an inducer and an inhibitor of cytochrome P450s in mammals (Laignelet et al. 1989) and fish (Bach & Snegaroff 1989; Snegaroff & Bach 1989; Sturm et al. 2000b). NP2EO belongs to the group of alkylphenol polyethoxylates (APnEO; number of ethoxylate units, n = 1-40) which are widely distributed non-ionic surfactants of products in industrial and

domestic use (Servos 1999). Some environmental degradation products of APnEOs, namely alkylphenols and alkylphenol di- and monoethoxylates, show a higher toxicity (Servos 1999) and environmental persistence than the parent compounds (Maguire 1999). In addition, these APnEO degradation products are weak estrogenes, effecting estrogen-dependent responses in fish (Nimrod & Benson 1996; Servos 1999). Both prochloraz and nonylphenol diethoxylate are moderately hydrophobic compounds, which makes them potential substrates of piscine P-gp(s). In fact, certain clinically used imidazole fungicides inhibit and/or induce P-gp in mammalian cell lines (Schuetz et al. 1996; Siegsmund et al. 1994; Takano et al. 1998). Also, it has been demonstrated that several APnEOs are substrates / inhibitors of mammalian P-gp (Charuk et al. 1998; Loo & Clarke 1998).

To evaluate the interaction of the selected model compounds with the level of P-gp expression in trout, we investigated the immunoreactive P-gp(s) in the livers of control animals and in animals after subchronic exposure to sublethal concentrations of prochloraz and NP2EO. At the functional level, the interaction of the model compounds with hepatic P-gp(s) in trout was investigated with cultured trout hepatocytes, using assays with the fluorescent P-gp substrate, rhodamine 123. Mammalian hepatocytes have proven a useful model for the study of the induction of hepatic P-gps by xenobiotics (Gant et al. 1991; Schrenk et al. 1996; Schuetz et al. 1995). During the past ten years, cultured fish hepatocytes have been well established as an in vitro system for the study of liver-specific physiological functions (reviewed by Braunbeck & Segner, in press; Segner 1998). The present study demonstrates that a combined in vivo / in vitro approach offers the opportunity to study the effects of xenobiotics on the expression and function of piscine hepatic P-gp(s).

MATERIALS AND METHODS

Chemicals

Prochloraz (N-propyl-N-[2-(2,4,6-trichlorphenoxy)-ethyl]imidazol-1-carboxamid, 99 % pure) was obtained from Riedel-de-Haën. Prochloraz (95 % pure) was a generous gift from Agrevo UK limited. [Benzene-^{14}C]-prochloraz was purchased from Isotopchim (Ganagobie, France). Its radiochemical purity was at least 98 % as indicated by radio-TLC and radio-HPLC analyses and its specific activity was 2886 MBq/mmol. Technical nonylphenol diethoxylate (NP2EO) was obtained from Aldrich (igepal® CO-210). LC/MS analysis indicated that igepal® CO-210 consisted of approximately 80% NP2EO and 20% nonylphenol monoethoxylate (Cravedi, personal communication). All other chemicals were of analytical grade. Medium 199 (catalogue number M3274), rhodamine 123, vinblastine, verapamil, fetal calf serum, L-glutamine, penicillin-streptomycin and ethyl-4-aminobenzoate were obtained from Sigma. Riedel-de-Haën, Aldrich and Sigma products were purchased from

Sigma-Aldrich, Deisenhofen, Germany. Trypan blue, defatted bovine serum albumin and polyvinylidene difluoride membranes were obtained from Serva, Heidelberg, Germany. Collagenase D was obtained from Roche, Mannheim, Germany. The monoclonal antibody C219 was obtained from Centocor, Malvern, PA, USA. Horseradish-peroxidase- or biotin-coupled secondary antibodies and horseradish peroxidase-coupled streptavidin were obtained from DAKO A/S, Glostrup, Denmark. The ECL system was obtained from Amersham, Braunschweig, Germany.

Hepatocyte Culture and Exposure

Hepatocytes were isolated from female trout (250-350 g) by collagenase perfusion of the liver as described by Mommsen et al. (1994). After isolation, hepatocytes were separated from cell debris by three washing steps with modified Hank's minimal essential medium containing 1% defatted bovine serum albumin (Mommsen et al. 1994), each followed by 3 min of centrifugation at 50 × g and 4°C. After washing, hepatocytes were resuspended in modified medium 199 (M199) containing Hank's salts supplemented with 0.9 g/l HEPES, 0.35 g/l $NaHCO_3$, 0.1 g/l $CaCl_2$, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. During manipulation after isolation and prior to seeding, hepatocytes were kept on ice. Cell viability was assessed by the exclusion of a solution of 0.175% trypan blue in phosphate buffered saline and was at least 80%. Hepatocyte suspensions were brought to a density of 1.5×10^6 viable cells/ml in M199 containing 5% fetal calf serum and seeded into 24 well cell culture plates (Falcon Primaria, Becton and Dickinson). 24 well cell culture plates received 0.4 ml hepatocyte suspension per well. Before use, culture dishes were coated with a commercially available matrix protein (Matrigel, Collaborative Biochemical). Fetal calf serum was added to the medium only during the first 24 h of culture to increase adherence of cells, but was not included in media for later incubations of cells. After seeding, hepatocytes were allowed to recover and form adherent monolayers for at least 12 h. Incubations took place at 15°C and 80% relative atmospheric humidity. To determine the range of concentrations causing acute toxic effects, hepatocytes were exposed to the model pollutants, prochloraz and NP2EO. Dimethyl sulfoxide (DMSO) was used as a solvent carrier (0.1 % final concentration). After 24 h of exposure, the leakage of lactate dehydrogenase (LDH) was determined as a cytotoxicity endpoint as described before (Scholz et al. 1998). Using hepatocytes derived from different fish (n = 3 to 5), LDH determinations were carried out for solvent carrier controls and at least five concentrations per chemical. The lowest observed effect concentrations of prochloraz and nonylphenol diethoxylate, respectively, were 140 µM and 100 µM. The model pollutants were tested for their effects on the Pgp-mediated transport of rhodamine 123 at lower, non-cytotoxic concentrations.

Fish

In *in vivo* experiments, juvenile rainbow trout (*Oncorhynchus mykiss*) of both sexes were used. The fish were obtained from a local hatchery and maintained in the INRA facilities in Rennes, France, for at least 2 weeks before use in experiments. The average body weight of trout was 51 g in experiment 1 (March 1998) and 72 g in experiment 2 (November 1998). The trout were maintained in a flow-through of dechlorinated, aerated city water and fed a commercial trout diet.

In vivo-experiment 1

Experiment 1 was carried out to assess sublethal biochemical effects of the fungicide prochloraz on trout. Fish were exposed for 7-14 days. In addition to a solvent carrier control (0.03% ethanol), experimental treatments included two prochloraz concentrations, 10 µg/l and 100 µg/l, and a mixture treatment of prochloraz (100 µg/l) and NP2EO (33µg/l). Per treatment, three 200 l tanks, housing 20 fish each, were set up. After introduction to tanks, fish were allowed to acclimate for 7 days before begin of exposures. Each of the 200 l tanks received a water flow-through of dechlorinated, aerated city water of 0.14 l / min. At the start of exposures, toxicants were added to the water from 1000-fold concentrated solutions in 30% ethanol (200 ml per tank). To maintain toxicant concentrations during exposures, 0.14 ml/min of 1000-fold toxicant solutions in 30% ethanol were delivered by a peristaltic pump. During the whole experiment, fish were fed a commercial trout diet at 1% body weight per day. Five fish per tank (15 per treatment) were sampled at days 7 and 14 of exposures.

In vivo-experiment 2

Experiment 2 was carried out to assess sublethal biochemical effects of the surfactant NP2EO on trout. Exposure of aquatic animals to toxic contaminants under field conditions is usually to a complex mixture of compounds, and often accompanied by elevated levels of nutrients. To check whether the effects of NP2EO were altered in the presence of other waterborne compounds, both city water and the water of the moderately contaminated river Le Couesnon (area of Rennes, France) were used as the dilution water. The experimental treatments consisted of two concentrations of NP2EO in city water (100 µg/l and 400 µg/l) and one in river water (400 µg/l), and control treatments of fish maintained in either city water or river water. Fish were exposed in 200 l tanks. 50 % of the water/toxicant solution was renewed daily. After introduction, fish were allowed to acclimate for 7 days. During the experiment, fish were fed a commercial trout diet at 0.5% of body weight. Fish sampling took place at day 7 after the beginning of exposures.

Analysis of prochloraz and nonylphenol diethoxylate in water samples

Water samples containing prochloraz or NP2EO were extracted with dichloromethane and analyzed by HPLC as reported by Cravedi et al. (2000) except that the system was equipped with an UV detector set at 296 nm and 260 nm respectively.

Sampling of fish

After removal from the tank, fish were killed by an overdose of ethyl-4-aminobenzoate. The total length and weight was recorded. Fish were opened ventrally and the liver cleared of blood by perfusion with ice-cold Ringer's solution via the portal vein. The liver was removed and weighed. A piece of the liver was fixed in Bouin's fluid. The determination of hepatic P-gp levels of fish from experiment 1 was carried out using crude liver membrane extracts, prepared from 200 mg of liver according to Cooper et al. (1996). With fish from experiment 2, P-gp analyses were performed on cytoplasmic membrane fractions of 300 mg of liver tissue isolated by sucrose gradient centrifugation as described by Simpson et al. (1983). The buffers used for homogenization and centrifugation contained 1 mM phenylmethylsulfonyl fluoride.

In vitro determination of mdr1-like activity in hepatocytes

To measure the effects of prochloraz and NP2EO on the mdr1-like activity in hepatocytes (Sturm et al. 2000a), the efflux of the fluorescent substrate of mammalian mdr1-type P-gps, rhodamine 123 (Rh123) (Efferth et al. 1989), was determined in the presence or absence of these compounds. Two compounds known to interfere with mdr1-dependent transport, verapamil and vinblastine (Germann 1996), were used in positive control treatments in which a decrease of the efflux of Rh123 was expected. After 24 h of primary culture following isolation, hepatocytes were incubated with M199 containing 2 µg/ml Rh123 for 2 h. Then, the medium was removed and the cells were washed with PBS. One set of replicates per experiment was removed and stored at –20°C for later determination of intracellular Rh123 ('before efflux'). The remaining hepatocytes were incubated for 4 h with M199 alone ('control'), or containing verapamil, vinblastine, prochloraz or NP2EO. Rh123 efflux measurements took place at 15°C. After incubations, the medium was removed, and culture dishes containing the cells were stored at –20°C. For Rh123 determinations, cell culture plates were thawed, and Rh123 was extracted by adding 2 ml of n-butanol to the wells of 24-well plates. The Rh123 concentration of the butanol extract was determined fluorometrically (excitation at 517 nm, emission at 532 nm). Intracellular Rh123 levels were normalized, to the cell number introduced to culture dishes upon seeding, or to cellular protein. Only results based on cell number are shown.

In vitro accumulation and elimination of ^{14}C prochloraz by hepatocytes

Trout hepatocytes were cultured for 24 h following isolation before the use in experiments. In accumulation experiments, hepatocytes were incubated in M199 containing ^{14}C-prochloraz, either in the absence or in the presence of mdr1-type P-gp substrates (vinblastine, 20 µM; rhodamine 123 5 µM) or a mdr1-type P-gp inhibitor (verapamil, 25 µM). After different intervals of incubation, the medium and the hepatocytes were collected for later measurement of radioactivity. The hepatocytes were washed with 200 µl of PBS before collection in 200 µl of PBS. In elimination experiments, hepatocytes were incubated in M199 containing ^{14}C-prochloraz for 2 h. After washing with PBS, hepatocytes were incubated in prochloraz-free M199, either in the absence or in the presence of P-gp substrates and inhibitors (as above). After different intervals of incubation, the medium and the hepatocytes were collected pending radioactivity measurements (as above). To monitor the degree of adsorption of prochloraz to the culture dishes, the empty culture dish wells were washed, first with PBS and then with ethanol. In both types of experiments, only a minor fraction of the total radioactivity (< 5%) was recovered from these washes. The measurement of radioactivity was carried out in a Tricarb 2200 CA liquid scintillation counter, using Ultima Gold (all Packard Instruments Co, Downers Grove, IL, USA) as scintillation cocktail.

Immunoblot analysis of P-gp(s)

Aliquots of 30 µg of crude membrane fraction protein, or 10 µg of cytoplasmic membrane protein per lane, determined according to Lowry et al. (1951), were subjected to electrophoresis through 7.5% SDS-polyacrylamide gels (Laemmli 1968). Proteins were then transferred to polyvinyliden fluoride membranes by semidry-blotting (Khyse-Andersen 1984) using a continuous buffer system (48 mM Tris, 39 mM glycine, 0.038% (w/v) SDS and 15% (v/v) methanol, pH 9.0). P-gp was detected by using the primary monoclonal antibody C219 which recognizes a conserved epitope in the C-terminal cytoplasmic region of all known P-gps (Georges et al. 1990). After incubation of membranes with an appropriate secondary horseradish peroxidase-coupled antibody, protein bands were visualized by enhanced chemoluminescence using the ECL system.

Immunohistochemistry of P-gp(s)

Trout livers were fixed in Bouin's fluid for 24 h, paraffin embedded, sectioned and deparaffinized. The following incubations took place in a moist chamber and were at room temperature except where noted otherwise. Rinsing between incubations was four times with PBS/glycine (0.01 M potassium phosphate, 0.123 NaCl, 0.1 M glycine, pH 7.2). Liver sections were incubated for 30 min with 5% non-fat dry milk/PBS (0.01 M potassium

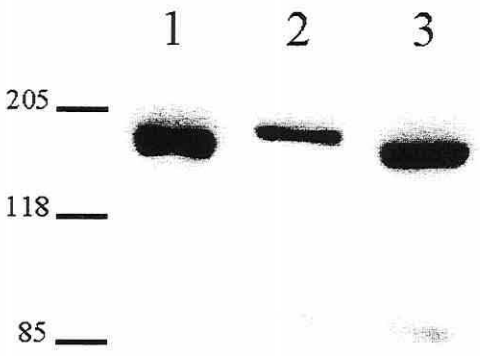

Fig. 5.1. Representative immunoblot of P-glycoprotein. Lane 1, bovine adrenal extract, 5 µg of protein; lanes 2 and 3, rainbow trout liver crude membrane extracts, 30 µg of protein. The bands were visualized by enhanced chemoluminescence, using the monoclonal antibody C219 that recognizes a conserved P-glycoprotein epitope (see Materials and Methods).

phosphate, 0.123 NaCl, pH 7.2), and incubated overnight at 4°C with the monoclonal antibody C219 (2 µg/ml) in 5% non-fat dry milk/PBS. After rinsing, liver sections were incubated for 2 hours at room temperature with biotin-conjugated goat-anti-mouse IGG (1:400) in 5% non-fat dry milk/PBS. Following rinsing, liver sections were incubated for 3 hours with horseradish peroxidase-coupled streptavidin (1:300) in PBS. Liver sections were then rinsed and treated with substrate (diaminobenzidine H_2O_2 mixture) for 10 min, rinsed in tap water and mounted.

Statistics

Except where noted otherwise, the effects of chemical treatments on biochemical markers were tested by one-way analyses of variance (ANOVAs) followed by multiple comparisons using the Student-Newman-Keuls test. In some cases data were log transformed prior to tests to increase homogeneity of variances. With variables observed in hepatocytes, repeated-measures ANOVAs were used, thus matching the observations made on cells obtained from the same fish.

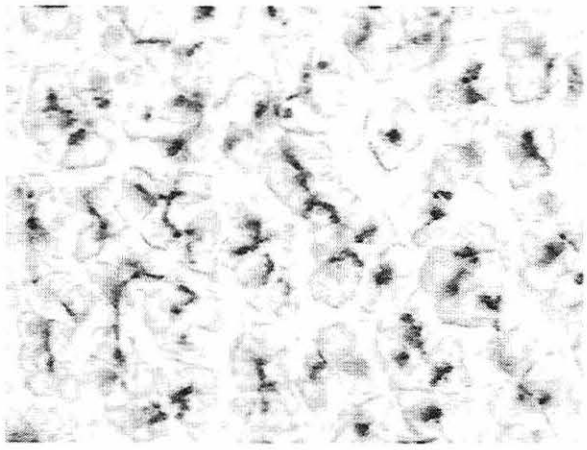

Fig. 5.2. Paraffin section (8 µm) of rainbow trout liver after immunohistochemistry using the monoclonal P-glycoprotein antibody C219. The dark DAB staining product is prepicitated along bile canalicular structures located within hepatic tubuli, which are sectioned sagitally or transversally.

RESULTS

Immunochemical detection of P-gp in control trout

To investigate the expression of P-gp(s) in rainbow trout liver, immunochemical determinations were carried out using the monoclonal antibody (mAB) C219. C219 detects a conserved epitope present in all known P-glycoproteins (Georges et al. 1990). In immunoblots (Fig. 5.1), the mAB C219 recognized a band of approximately 160 kDA in extracts both from bovine adrenal (positive control) and rainbow trout liver. In trout liver, a further minor immunoreactive band of unknown identity occurred at 80 kDa (Fig. 5.1). Immunohistochemistry of sections of fixed paraffin-embedded trout liver with C219 resulted in specific staining of bile canalicular structures (Fig. 5.2).

P-gp levels in trout exposed in vivo

The hepatic expression of P-gp in juvenile trout exposed to sublethal concentrations of prochloraz and/or NP2EO was assessed using immunoblot and immunohistochemical techniques. In immunoblots, the staining intensity of the 160 kDA band detected in trout liver extracts was measured densitometrically and expressed relative to that of a known amount of bovine adrenal extract included on each SDS gel. In immunoblots, no significant effects of the exposure to prochloraz and/or NP2EO on hepatic levels of P-gp were found (Table 5.1). Similarly, immunohistochemical analysis of liver sections of exposed and control animals

Table 5.1. Levels of P-glycoprotein in trout liver after exposure to prochloraz and NP2EO.

Experiment	Treatment[a]	Measured concentration (µg/l)	Dilution water	Exposure	P-glycoprotein[b]
1	untreated control	na[c]	City water	7 days	0.130 ± 0.091 (8)
	Prochloraz, 10 µg/l	8	City water	7 days	0.128 ± 0.053 (8)
	Prochloraz, 100 µg/l	42	City water	7 days	0.118 ± 0.06 (8)
	Prochloraz, 100 µg/l +	43	City water	7 days	0.102 ± 0.071 (8)
	NP2EO, 33 µg/l	nd[d]			
2	untreated control	na	City water	7 days	0.197 ± 0.089 (5)
	untreated control	na	River water	7 days	0.162 ± 0.079 (5)
	NP2EO, 100 µg/l	50	City water	7 days	0.225 ± 0.189 (5)
	NP2EO, 400 µg/l	215	City water	7 days	0.217 ± 0.125 (5)
	NP2EO, 400 µg/l	84	River water	7 days	0.195 ± 0.152 (5)

[a] Nominal concentrations.
[b] The staining intensities of trout P-glycoprotein bands are expressed relative to those of bands obtained with internal standards from bovine adrenal (Fig. 1) at the same protein concentration. Values can not be compared between experiments, as the sample treatment differed (see Materials and Methods). In both experiments, no significant treatment effects were observed on P-glycoprotein expression in trout.
[c] Not applicable.
[d] Not determined.

revealed no differences of staining intensities between control fish and trout treated with prochloraz or NP2EO (not shown).

Rh123 efflux in cultured hepatocytes

The efflux of the fluorescent mdr1-type P-gp substrate rhodamine 123 (Rh123) was studied in rainbow trout hepatocytes. After isolation, hepatocytes had been cultured for 24 h as monolayers on matrigel-coated plastic tissue-culture dishes to allow for recovery from isolation trauma. Then, the hepatocytes were incubated with Rh123 for 2 h, resulting in an accumulation of the fluorescent dye. After washing, hepatocytes were allowed to extrude the intracellular Rh123 (efflux) during 4 h of incubation in rhodamine-free medium, either in the absence or in the presence of chemicals. When hepatocytes were exposed to medium alone during the efflux incubation, they eliminated 32-48% of the Rh123 accumulated during the initial 2 h-loading phase (Table 5.2). In the presence of two compounds known to interfere with mdr1-type P-gp transport, verapamil and vinblastine, hepatocytes eliminated markedly less Rh123 than when incubated in medium alone (decrease of efflux by 50% and 76% respectively, Table 5.2).

Table 5.2. Effects of prochloraz and NP2EO on P-glycoprotein-dependent rhodamine 123 efflux from cultured rainbow trout hepatocytes.

	Treatment[a]	Rhodamine 123 (μg / 10^6 cells)[b]	
experiment 1	before efflux	0.223 ± 0.067	**
	no inhibitor	0.117 ± 0.053	
	Verapamil, 7.5 μM	0.170 ± 0.068	
	Prochloraz, 0.1 μM	0.143 ± 0.061	
	Prochloraz, 0.3 μM	0.189 ± 0.079	*
	Prochloraz, 1.0 μM	0.198 ± 0.084	**
	Prochloraz, 3.1 μM	0.207 ± 0.080	**
	Prochloraz, 10.0 μM	0.219 ± 0.088	**
experiment 2	before efflux	0.304 ± 0.049	
	no inhibitor	0.208 ± 0.041	
	Vinblastine, 20 μM	0.279 ± 0.043	**
	NP2EO, 3.1 μM	0.216 ± 0.042	
	NP2EO, 10.0 μM	0.242 ± 0.038	
	NP2EO, 31.6 μM	0.253 ± 0.035	*

[a] 'Before efflux' values represent levels of rhodamine 123 (Rh123) accumulated by hepatocytes during 2 h of incubation with medium containing 2 μg/ml Rh123. In the other treatments, values are Rh123 levels retained in hepatocytes after further 4h of incubation with medium containing indicated chemicals.

[b] Values are mean and S.E.M. of 3-5 observations on cells derived from different fish. Asterisks indicate whether values differ from the treatment 'no inhibitor'. * $P < 0.05$, ** $P < 0.01$.

In vitro inhibition of Pgp-dependent Rh123 efflux

The influence of the model compounds prochloraz and NP2EO on an mdr1-like activity in trout hepatocytes was estimated as the effect of these compounds on the efflux of Rh123 from hepatocytes. First, hepatocytes were loaded for 2 h with Rh123, as described above, and then the efflux of Rh123 from the cells was measured either in the absence, or the presence of either prochloraz or NP2EO. When prochloraz or NP2EO were included in the culture medium during the efflux incubation, the efflux of Rh123 from trout hepatocytes was significantly lower than in controls (Table 5.2). The concentrations of prochloraz and NP2EO causing Rh123 efflux inhibition were not cytotoxic, as was confirmed by the assay of lactate dehydrogenase leakage (not shown). As an inhibitor of Rh123 efflux, prochloraz was more effective than vinblastine. The inhibitory effects of prochloraz and NP2EO on the Rh123 efflux from hepatocytes suggest that these chemicals might represent substrates of the mdr1-like mechanism.

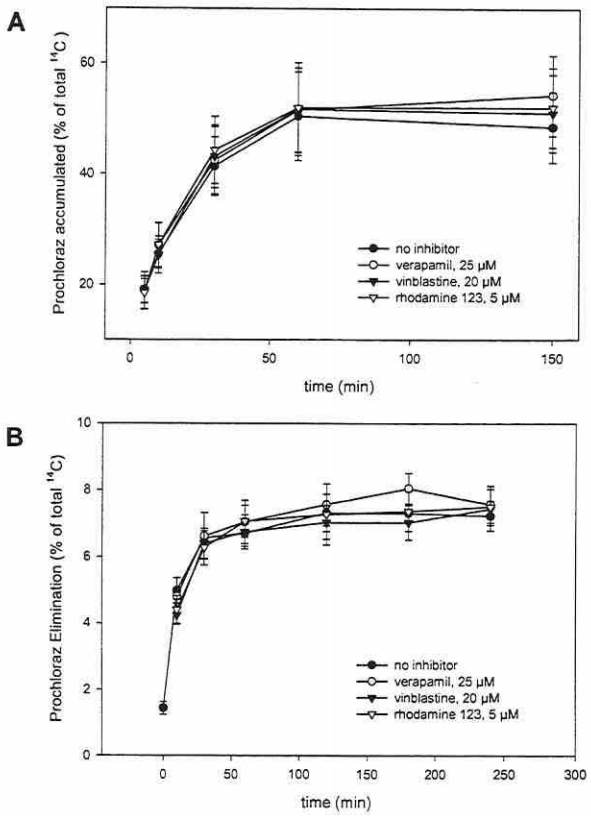

Fig. 5.3. Kinetics of accumulation and elimination of prochloraz by cultured rainbow trout hepatocytes in the presence or absence of inhibitors and substrates of P-glycoprotein. (A) Hepatocytes were incubated with medium containing 4 µM of ^{14}C-prochloraz and indicated chemicals. After different incubation periods, levels of ^{14}C-prochloraz accumulated by cells were determined. (B) Hepatocytes were incubated with medium containing 4 µM of ^{14}C-prochloraz for 2 h and, after washing with PBS, incubated with medium containing the indicated chemicals. After different incubation periods, levels of ^{14}C-prochloraz in medium were determined. Values are mean and S.E.M. of 6 (A) or 3 (B) determinations on cells derived from different fish. In both experiments, the inhibitors and substrates of P-glycoprotein did not exert any significant effects ($P < 0.05$).

Inhibitors and substrates of Pgp lack effects on ^{14}C-prochloraz accumulation and efflux

To test the hypothesis that prochloraz was a substrate of the mdr1-like mechanism in trout hepatocytes, the kinetics of the accumulation and elimination of ^{14}C-prochloraz by hepatocytes was recorded in the absence and presence of either Rh123, verapamil or vinblastine. Rh123 and vinblastine are known substrates of mdr1-P-gps, while verapamil is a known chemosensitizer, i.e. a drug known to interfere with the transport mediated by mdr1-type P-gps (Germann 1996). A substrate of mdr1-type P-gp would be expected to show an increased accumulation by hepatocytes, but decreased efflux from these cells, in the presence of the above compounds. However, none of the compounds tested had a significant effect on

the accumulation or efflux of total ^{14}C-prochloraz radioactivity (Fig. 5.3). This suggests that prochloraz inhibits the mdr1-type mechanism in trout hepatocytes, but is not one of its substrates.

DISCUSSION

Immunochemical evidence for the hepatic expression of P-gp in trout

Using the monoclonal antibody C219, a main immunoreactive protein of 160 kDA was found in rainbow trout liver in immunoblots (Fig. 5.1), while immunohistochemistry of liver sections resulted in specific staining of bile canaliculi (Fig. 5.2). These findings strongly suggest the hepatic expression of P-gp(s) in rainbow trout and confirm results reported for other teleost fish (Cooper et al. 1999; Hemmer et al. 1995). However, it must be kept in mind that different proteins may be detected by C219. The epitope recognized by C219 is present in all known Pgps (Georges et al. 1990), including the mammalian *mdr1* and *mdr2* gene products, both of which are expressed in the canalicular membrane (Meijer et al. 1997; Oude Elferink et al. 1995). In addition, C219 also detects a related transport protein called 'sister of P-glycoprotein' (sPgp) (Childs et al. 1995), which is mainly expressed in liver (Török et al. 1999). *Mdr1* genes confer drug resistance in cell lines and encode proteins that transport hydrophobic xenobiotics (Bellamy 1996), suggesting that one of their roles in liver is the biliary secretion of hydrophobic xenobiotics. By contrast, the *mdr2* and *sPgp* genes do not confer drug resistance (Bellamy 1996; Török et al. 1999). The physiological role of the mdr2 proteins is the biliary secretion of phospholipids (Frijters et al. 1997; Oude Elferink et al. 1995), while sPgp is probably identical with the functionally characterized canalicular bile acid transporter (cBAT) (Kamisako et al. 1999; Török et al. 1999). Mdr2 and sPgp are not directly involved in the biliary secretion of xenobiotics. However, a recent hypothetical model postulates an active role for mdr2 and cBAT in the formation of biliary vesicles, and suggests that this may contribute to biliary drug transport (Frijters et al. 1997). Little is known on *P-gp* genes in fish. Two genes showing homology to *P-gp* genes were partly sequenced from winter flounder and termed flounder P-gpA and flounder P-gpB (*fPgpA; fPgpB*) (Chan et al. 1992). However, recent data suggest that *fPgpA* represents a piscine *sPgp* gene, and only *fPgpB* encodes a true P-gp (Cooper et al. 1999). As long as more specific probes are lacking, the mAB C219 may provide initial information on the interaction of xenobiotics with the expression of hepatic P-gp(s) in fish.

Levels of P-gp expression in trout exposed to prochloraz or NP2EO

Following the exposure of juvenile rainbow trout to sublethal concentrations of prochloraz and NP2EO, no effects were observed on hepatic P-gp levels, as apparent from immunoblots (Table 5.1) and immunohistochemistry with the mAB C219. This lack of response does not reflect a lack of xenobiotic accumulation, as a number of other hepatic biochemical parameters responded to the chemical treatments in the same experiment. For instance, prochloraz and NP2EO effected alterations in biotransformation enzymes (Sturm et al. 2000b), while NP2EO caused vitellogenin induction (F. LeGac, INRA Rennes, personal communication). The responsiveness of piscine P-gp(s) to xenobiotic inducers is not understood to date. Elevated hepatic levels of P-gp(s) were reported in mummichog (*Fundulus heteroclitus*) from polluted environments when compared to fish from pristine waters (Cooper et al. 1999). However, P-gp expression in mummichog remained unchanged after laboratory exposure to the polycyclic aromatic hydrocarbon, 3-methylcholanthrene (Cooper et al. 1999). In mammals, polycyclic aromatic hydrocarbons and polychlorinated dibenzo-*p*-dioxins were reported as inducing P-gp (Gant et al. 1991; Schuetz et al. 1995). However, the responsiveness of mammalian P-gps to such xenobiotics has also been doubted (Russell et al. 1994; Teeter et al. 1991). The inducibility of P-gps by xenobiotics appears to vary strongly between species (Lecureur et al. 1996; Schrenk et al. 1996), and between individuals in human (Schuetz et al. 1995). Kurelec proposed that P-gps represent a mechanism of pollution-tolerance in aquatic animals, and experimentally demonstrated the induction of P-gp(s) expression in aquatic invertebrates by pollutant mixtures, such as diesel oil or contaminated sediments (Kurelec 1997). The immunochemical detection of P-gps, however, reflected pollutant exposure less reliably and sensitively than assays based on the accumulation or efflux of P-gp substrates (Kurelec et al. 1996). This illustrates that the functional activity of P-gp is not necessarily related to P-gp protein amount (Chieli et al. 1994).

Xenobiotic effects on P-gp function

As a consequence, it appears important to analyze P-gp function, in addition to the determination of P-gp expression. Since transport processes mediated by mdr1-type P-gps are difficult to determine in vivo, in vitro systems are preferable. In fish, mdr1-like transport mechanisms have been identified in primary cultured kidney tubules or kidney tubule epithelium (Schramm et al.. 1995, Sussman-Turner and Renfro 1995). In this study, primary monolayer cultures of trout hepatocytes were used to assess the effects of the xenobiotics on a hepatic mdr1-like mechanism. That two compounds known to interfere with mdr1-type transport, verapamil and vinblastine, markedly decreased the efflux of the mdr1-type substrate Rh123 from cultured rainbow trout hepatocytes (Table 5.2) suggests that the efflux of Rh123 from trout hepatocytes is at least partly due to the activity of transport protein(s), most

probably P-gp(s). A more in-depth characterization of this mdr1-like mechanism in trout hepatocytes is presented elsewhere (Sturm et al. 2000a, chapter 4 of this work). Concerning the functional interaction of the selected xenobiotics with trout liver P-gp(s), the imidazole fungicide prochloraz and the alkylphenolic compound NP2EO inhibited the efflux of Rh123 from cultured trout hepatocytes, with prochloraz being a very effective inhibitor (Table 5.2). Further experiments showed that prochloraz was not a substrate of the mdr1-like mechanism in trout hepatocytes (Fig. 5.3). These findings are in accordance with studies using mammalian systems. Nonylphenol ethoxylates, but not nonylphenol, have been shown to be substrates of human MDR1 P-gp and inhibit the transport of other substrates (Charuk et al. 1998; Loo & Clarke 1998). The clinically used imidazole fungicide ketoconazole has been reported to be a potent inhibitor of mdr1 P-gps (Siegsmund et al. 1994), but not a substrate itself (Takano et al. 1998).

Extensive research has aimed to identify inhibitors of P-gp in order to find antagonists of P-gp-mediated multidrug resistance in cancer (Ford & Hait 1990). Compounds that reverse drug resistance, and/or the decreased accumulation of cytoctoxic drugs accomplished by mdr1 proteins, are called chemosensitizers or resistance modifiers (Ford & Hait 1990). Many, but not all chemosensitizers are substrates of the P-gp pump (Ford & Hait 1990). Compared to the knowledge on potential therapeutics, much less is known about the ability of water pollutants to interact with P-gp. Water from polluted environments can increase the accumulation and decrease the efflux of fluorescent P-gp-substrates in aquatic invertebrates (Kurelec 1997; Smital & Kurelec 1997), suggesting unidentified water pollutants as substrates and/or inhibitors of P-gp. Several moderately hydrophobic pesticides, but not highly hydrophobic compounds such as polychlorinated biphenyls or DDT, act as inhibitors of mussel P-gp(s) (Cornwall et al. 1995). The inhibition of a P-gp like mechanism in trout hepatocytes by the model pollutants prochloraz and NP2EO demonstrated in this study suggests that the exposure of fish to water pollutants may result in a decreased biliary secretion of endogenous and exogenous compounds. The threshold medium concentrations for the inhibition of P-gp in rainbow trout hepatocytes by NP2EO were in a similar range as concentrations effecting vitellogenin production in trout hepatocytes in vitro (Petit et al. 1997). With prochloraz, an inhibition of P-gp occurred at concentrations lower than those required for EROD induction in trout hepatocytes in vitro (Sturm et al. 2000b, chapter 3 of this work). Following sublethal exposures of juvenile rainbow trout in vivo to NP2EO, an induction of vitellogenin production was observed, while in vivo exposure to prochloraz resulted in EROD induction (F. LeGac, INRA Rennes, personal communication; Sturm et al. 2000b). This suggests that in the rainbow trout exposed in vivo to NP2EO and prochloraz, the activity of hepatic P-gp(s) was probably inhibited.

The toxicological relevance of an inhibition of P-gp(s), however, is difficult to judge, as the function of P-gp(s) remains unclear. Several lines of evidence argue for a role for mdr1-P-gp(s) in the defense of mammals against toxic xenobiotics. Firstly, the *mdr1* gene

products are expressed at high levels in tissues involved in excretion, such as liver and kidney, or representing blood-tissue barriers, such as capillary endothelia of brain and testis (Bellamy 1996; Thiebaut et al. 1987). In these tissues, P-gp is localized to the apical poles of epithelial cells, which supports the hypothetical protective role. Secondly, the administration of inhibitors of Pgp increases access to brain and neurotoxicity of certain toxicants (Marques-Santos et al. 1999), and decreases their biliary excretion (Schrenk et al. 1993). Thirdly, an increased toxicity of certain toxicants compared to that normal mice is observed in *mdr1a*-, *mdr1b*- and *mdr1a/mdr1b*-knockout mice, who also show an increased accumulation of toxicants in tissues normally expressing *mdr1* gene products (Borst & Schinkel 1996; Schinkel et al. 1997). Apart from a role in the organismal defense against xenobiotics, however, different other physiological roles of P-gp(s) have been suggested, including the transport of steroid hormones, the extrusion of (poly-)peptides and other compounds involved in cellular signaling, and the intravesicular transport of cholesterol (Borst & Schinkel 1996; Sharma et al. 1992; Ueda et al. 1992). Arguing against such roles of P-gp; however, no overt signs of disease or pathology are observed in *mdr1a/mdr1b* double knockout mice.

More research is needed to elucidate the relevance of P-gp(s) as a protective mechanism against environmental toxicants in fish, and the inducibility of fish P-gp(s) by environmental pollutants. Such studies will be significantly facilitated by the identification of piscine P-gp genes and the development of selective molecular probes. Moreover, it appears important to study piscine P-gp(s) on a functional level also, in order to reveal which environmentally relevant chemicals interact with P-gp function and/or are excreted by mechanisms involving P-gp. Studies with cultured fish hepatocytes have the potential to contribute significantly to such research, because they represent a system that, firstly, expresses a P-gp-like extrusion mechanisms and secondly, exhibits a similar biotransformation capacity as liver in vivo.

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General Discussion

INTRODUCTION

This work investigated selected biochemical mechanisms of the organismal defense against xenobiotics in fish. The first thematic line is concerned with butyrylcholinesterase (BChE) which has been proposed to function as a scavenger of certain xenobiotics. Little is known on butyrylcholinesterase in fish. Therefore, this work provides a characterization of some basic properties of BChE in a teleost species. Then, the interaction of BChE with organophosphate insecticides is studied. The investigations presented in this volume focus primarily on the use of BChE as a biomarker of organophosphate exposure. However, the results presented here further suggest that BChE deserves attention as a potential biochemical defense mechanism in fish.

The second thematic line of this work deals with the suitability of cultured hepatocytes as a cellular in vitro system to investigate the effects of xenobiotics on hepatic biotransformation enzymes in fish. Because of the prominent role of these enzymes in xenobiotic detoxification and/or activation, and their relevance as biochemical targets of xenobiotic toxicity, there is a need for cellular systems that allow the study of hepatic biotransformation enzymes in aquatic toxicology. The effects of two model water pollutants on biotransformation variables were investigated in a combined in vitro / in vivo approach, using cultured hepatocytes and juvenile trout, respectively. This study thus contributes to the validation of the cultured trout hepatocyte system as a tool in aquatic toxicology.

The third thematic block of the present work is concerned with P-glycoprotein (P-gp) in the liver of teleost fish. P-gp is a tranporter expressed, among other organs, in the vertebrate liver and kidney, and is probably involved in the excretion of lipophilic organic compounds. This work investigates of the basic properties of P-gp in the teleost liver and its interaction with xenobiotics. Cultured hepatocytes from rainbow trout have been used to characterize hepatic P-gp in trout on a functional level, and the potential of xenobiotics to interfere with P-gp function.

BUTYRYLCHOLINESTERASE

Chapters 1 and 2 of this work showed that in the fish three-spined stickleback (*Gasterosteus aculeatus*), cholinesterase activity is due to AChE exclusively in the brain, due to BChE exclusively in the liver, and due to both AChE (75-80%) and BChE (20-25%) in the skeletal muscle. The BChE of stickleback showed some features unusual for a BChE and was much (>1000-fold) more sensitive to the inhibition by OPs in vitro than AChE. In small agricultural streams in the area of Braunschweig, Germany, the BChE in the skeletal muscle of stickleback was strongly (>80%) decreased, compared to two pristine reference streams, in streams which showed low levels of the OP parathion (0.2 to 0.3 µg/l) during runoff events. That the short-term exposure to low levels of parathion leads to the inhibition of BChE in stickleback was confirmed in laboratory tests. By contrast to BChE, AChE remained unchanged in stickleback following both environmental and laboratory exposures to the low levels of OPs typically found in the field situation of agricultural streams.

Atypical features of BChE in stickleback

The atypical features of stickleback BChE found in this work (chapter 1) are not surprising, as BChE often shows unusual properties in fish (Leibel 1988b; Toutant et al. 1985). Some investigators have been puzzled by atypical cholinesterases occurring together with AChE in fish and hesitated to attribute these enzymes to either AChE or BChE (Lundin 1968; Stieger et al. 1989). However, the prevailing view is that these cholinesterases represent BChEs with somewhat unorthodox features (Leibel 1988a; Massoulié et al. 1993; Toutant et al. 1985), and that the duality of AChE and BChE is found in all of the gnathostome vertebrates, including teleost and elasmobranch fish (Leibel 1988b; Massoulié et al. 1993; Toutant et al. 1985), but not in the agnathan vertebrates which possess only AChE (Sanders et al. 1996). The properties of BChE in fish do not only deviate from those in mammals, but also show a high variability among (Leibel 1988b; Magnotti et al. 1994). In particular, it appears noteworthy from a practical point of view, that selective inhibitors of both BChE (e.g., iso-OMPA) and AChE (e.g., BW284C51) are active against BChE in some, but not all fish species (this study; Leibel, 1988c; Lundin, 1968; Stieger et al., 1989; Sturm et al., 1999a, 1999b). Similarly, fish BChEs may show high activities on substrates normally selective for AChE (e.g. AcβMeSCh; this study; Sturm et al. 1999a), or, as observed in other cases, be practically inactive on substrates usually selective for BChE (e.g. BSCh; Leibel, 1988c). Hence, general recommendations for selective substrates or inhibitors of fish BChE cannot be made. A careful characterization of cholinesterases present in the tissue under question should precede any investigation of fish AChE or BChE.

BChE as a potential biomarker in fish

The much higher sensitivity of BChE than AChE to the inhibition by OPs in stickleback, shown in this report by in vitro and in vivo experiments and a field study (chapters 1 and 2), suggests the usefulness of BChE as a biomarker of OP exposure, particularly under scenarios where AChE is not sufficiently sensitive. An example of such a situation is provided by the runoff-borne contamination of agricultural streams by OPs (chapter 1). Insecticide contamination in these streams typically occurs at water concentrations in the low µg/l range and is present only for short time intervals (hours). Despite the low level and brief presence of OP insecticides in stream water, a single contamination event can have dramatic, long-lasting (up to 6 months) adverse effects on benthic invertebrate communities (Liess & Schulz 1999; Schulz & Liess 1999). Because the inhibition of fish cholinesterases by OPs remains detectable for weeks (Carr et al. 1997; Straus & Chambers 1995; Weiss 1959), the measurement of BChE in feral fish can be used to cost-effectively identify OP contamination events in retrospective manner, as it was demonstrated in a field investigation in this work (chapter 1). However, prior to an application of BChE as a biomarker of OP exposure in routine monitoring, different aspects require investigation. In the present investigation, insecticidal carbamates were not considered because they were of minor importance in the area of investigation. For the general application of BChE as biomarker, however, it is necessary to characterize the sensitivity of BChE to these compounds. Moreover, the time course of the recovery of BChE after the inhibition by OPs and insecticidal carbamates should be studied. It appears further necessary to examine factors other than OP and carbamate exposure for their potential influence on BChE activities. The most important natural factors affecting AChE activitiy in fish are body size (Weiss 1959) and habitat temperature (Baslow & Nigrelli 1964). A sex difference in AChE activities is mostly not observed in fish, but may occur with certain species (Bocquené & Galgani 1995). Certain cyanobacteria produce toxins that are cholinesterase inhibitors and that may occur at significant amounts in freshwaters during algal blooms (Henriksen et al. 1997; Hyde & Carmichael 1991). Apart from natural factors, water pollutants other than anti-cholinesterase insecticides, such as other pesticides and heavy metals (Davies & Cook 1993; Gill et al. 1990a, 1990b), have been reported to decrease AChE activities, albeit such chemicals are effective only at relatively high water concentrations.

Toxicological relevance of BChE in fish

When the higher sensitivity of BChE compared to AChE is interpreted from toxicological, rather than from an environmental monitoring perspective, two questions arise: Firstly, has an inhibition of BChE in fish toxicological consequences; or is BChE dispensable for normal life functions in fish, as it is the case in mammals? Secondly, to which extend can the presence of high activities of a BChE in the skeletal muscle in certain fish provide a

protection of AChE during the exposure to OPs and insecticidal carbamates? It was beyond the scope of the present investigation to address these questions, which should be the subject of further studies. Both issues are linked; BChE can obviously only be considered as a protective mechanism in the toxicity of OPs and carbamates if its inhibition does not itself result in adverse effects. The physiological role of BChE, as that of AChE occurring outside of synapses and neuromuscular junctions, is not known (Chatonnet & Lockridge 1989; Massoulié et al. 1993). Different roles have been suggested for extra-synaptic cholinesterases: 1. the hydrolysis of acetylcholine at nonsynaptic locations, 2. the hydrolysis of other choline esters originating from food (particularly suggested for BChE in the liver and the plasma), 3. the scavenging of exogenous toxic compounds (particularly suggested for BChE), 4. a role during the development and morphogenesis of the nerve system, 5. non-cholinesterasic calalytical functions, such as arylacylamidase and peptidase activities (Massoulié et al. 1993).

The occurrence of BChE in the skeletal muscle of certain fishes appears less unusual when it is taken into account that BChE is quite common in the nerve and muscle tissues of lower vertebrates and/or of early developmental stages of vertebrates (Leibel 1988a; Massoulié et al. 1993). In histochemical studies, (Lundin 1962; Lundin & Hellstrom 1968) showed that the BChE in the body muscle of the pleuronectid flatfish plaice (*Pleuronectes platessa*) is extrajunctional and localized to the sarcolemma, which forms the surface of muscle fibers. This suggests that the BChE in the skeletal muscle in fish is not involved in cholinergic neurotransmission. The presence of high levels of BChE in the skeletal muscle in stickleback (a species also occurring in coastal marine systems; Brunken & Fricke, 1985) and some other marine and brackish fish (Magnotti et al. 1994; Sturm et al. 1999a) could point to a role of BChE as a scavenger of natural toxins occurring in the marine environment. Many marine organisms produce toxins (Aune 1997; Trevino 1998). Certain toxins produced by cyanobacteria are inhibitors of cholinesterases; however, these cyanobacteria toxins have only been reported for freshwater, but not marine environments (Henriksen et al. 1997; Hyde & Carmichael 1991).

B-esterases are esterases irreversibly inhibited by OPs and include cholinesterases and carboxylesterases (Aldridge 1953). B-esterases other than AChE can provide protection against OP toxicity if they are present at sufficient levels, and if they are at least as sensitive as AChE to the inhibition by OPs. Although BChE is more sensitive than AChE to many OPs in mammals (Herzsprung et al. 1989), it is considered of minor importance as a protective mechanisms because its whole body level is orders of magnitude lower than that of CaE (Maxwell et al. 1987). However, a quite different situation appears to be present in fish. First, the levels of CaE in fish are generally low, i.e. orders of magnitude smaller than those found in mammals (Barron et al. 1999; Carr et al. 1997; Maxwell et al. 1987; Straus & Chambers 1995). Second, different fish species showing high BChE activities in the skeletal muscle. In stickleback, the contribution of BChE to the cholinesterase activity in the skeletal muscle was 20 to 25% while it can be greater than 80% in certain marine fishes (Leibel 1988b; Stieger et al. 1989). The high contribution of skeletal muscle to the body mass in fish (e.g., 40% in

crucian carp; Smith et al. 1996) suggests that the BChE in skeletal muscle of fishes with significant BChE should be relevant as a scavenger of OPs on a whole body level. The role of the BChE in skeletal muscle as a protective mechanism is further supported by the extreme (>1000-fold) difference in sensitivity to OPs of AChE and BChE in the fish studied in this respect (stickleback, chapter 1; dab, flounder and sea bass, Sturm et al., 1999a).

Conclusions

This study showed that BChE is much more sensitive to OPs than AChE in three-spined stickleback, and should therefore be considered as a potential biomarker of OP exposure in environmental monitoring. Studies of AChE and/or BChE in fish have to take into account the unusual characteristics of BChE in fish. The findings of this work further suggest that BChE deserves further attention as a potential protective factor in the toxicology of OPs in fish.

HEPATIC BIOTRANSFORMATION ENZYMES

In chapter 3 of the present work, it was examined whether xenobiotics cause the same type of effects on hepatic biotransformation enzymes in cultured hepatocytes from rainbow trout (*Oncorhynchus mykiss*) when compared to the response of these variables in juvenile rainbow trout. Such information is needed to validate the usefulness of the trout hepatocyte system in the assessment of xenobiotic effects on hepatic biotransformation in fish. With cytochrome P450-dependent variables, the same types of response were found in vitro and in vivo, while with the phase II enzyme UDP-glucuronyltransferase the response differed between in vitro and in vivo investigations. The imidazole fungicide prochloraz effected the induction of cytochrome P4501A (CYP1A) levels and of the CYP1A-dependent catalytic activity EROD, while it inhibited testosterone hydroxylation rates. The surfactant nonylphenol diethoxylate (NP2EO) also caused an inhibition of testosterone hydroxylation, but remained without effects on CYP1A levels and EROD.

CYP1A-dependent variables

In this work, prochloraz induced CYP1A levels and EROD activity both in vitro (hepatocytes) and in vivo (juvenile trout). At higher concentrations of prochloraz in vitro, however, an apparent induction was observed only for CYP1A levels, but not for EROD activity. This can be interpreted as an inhibition of the catalytic activity of CYP1A at higher concentrations of the inducer. Such a bimodal way of action is found with many environmental inducers of CYP1A, including polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) (Behrens et al. 2000; Gooch et al. 1989; Hahn et al. 1996). Prochloraz is a potent inducer of CYP1A in mammals (Laignelet et al. 1989), birds (Rivière et al. 1985) and fish (Bach & Snegaroff 1989). However, as other imidazole

fungicides (Rodrigues et al. 1987; Wilkinson & Hetnarski 1974), prochloraz may also inhibit P450-dependent enzyme activities (Monod et al. 1993; Snegaroff & Bach 1989).

In the present work, NP2EO had no inductive effects on CYP1A-dependent variable, which is in accordance with the present view of the toxicology of alkylphenolic compounds (Servos 1999). Nonylphenol has been reported to decrease EROD activity and suppress CYP1A-expression in mammals and fish (Arukwe et al. 1997; Lee et al. 1996a), which could be related to the weakly estrogenic properties of nonylphenol (see also below). In this study, the weakly estrogenic xenobiotic NP2EO did not cause such effects in juvenile trout. However, the levels of EROD activity and CYP1A in juvenile trout are low. Consequently, decreases caused by chemical treatments, if any, are difficult to resolve statistically.

The ability of CYP1A inducers and other xenobiotics to act as inhibitors of CYP1A has important implications for the use of CYP1A in environmental monitoring (Fent et al. 1998; Goksøyr & Förlin 1992; Stegeman & Hahn 1994). During the application of CYP1A as a biomarker, the use of immunochemical techniques, such as ELISA or Western blotting, is recommended in addition to the measurement of CYP1A-dependent EROD activity to avoid false negatives.

Effects on the hepatic testosterone hydroxylation

With both model xenobiotics, prochloraz and NP2EO, an inhibition of testosterone hydroxylation rates was observed in vitro and in vivo. The 6β- and 16β-hydroxylations of testosterone in the liver of rainbow trout are mainly due to the constitutive trout P450s LMC5 and, to a somewhat lesser extend, LMC2 (Miranda et al. 1989). LMC5 most probably corresponds to the gene *CYP3A27*, while LMC2 is encoded by *CYP2K1* (Buhler & Wang-Buhler 1998; Buhler et al. 1995). The levels of LMC2 are substantially higher in mature male when compared to female and immature trout (Buhler et al. 1995). The levels of steroid hydroxylation markedly differ between pre-spawning male and female salmonids (Andersson & Förlin 1992; Hansson & Gustafsson 1981). In maturing female salmonids, the levels of putative CYP3A protein(s) and the rates of the 6β-hydroxylation of steroids are decreased compared to male or immature fish (Hansson & Gustafsson 1981; Pajor et al. 1990). The strong inhibition of hepatic steroid hydroxylation activities observed in this study suggests that exposure of fish to prochloraz and NP2EO could potentially result in adverse effects on reproductive function.

Different mechanisms could have been behind these effects. On the one hand, the decreased steroid hydroxylation caused by prochloraz and NP2EO could reflect the direct inhibition of the cytochrome P450 system. On the other hand, the decrease of steroid hydroxylation rates could reflect the down regulation of the respective CYP isoforms. With prochloraz, it appears probable that a direct inhibition of steroid-hydroxylating CYP forms occurred, as ample evidence exists for the ability of prochloraz to inhibit P450-dependent catalytic activities in mammals, birds, insects and fish (Laignelet et al. 1989; Monod et al.

1993; Pilling et al. 1995; Rivière et al. 1985; Snegaroff & Bach 1989). Alkylphenolic compounds, including nonylphenol, octylphenol and octylphenol diethoxylate, have also been shown capable to inhibit different P450-dependent catalytic activities, including steroid hydroxylation rates (Hanioka et al. 1999; Lee et al. 1996a; Lee et al. 1996b). However, the effects of NP2EO on steroid hydroxylation in this study could also be related to the weakly estrogen-like properties of this and other alkylphenolic compounds in mammals and fish (Nimrod & Benson 1996; Servos 1999). Alkylphenol mono- and diethoxylates can cause different estrogen-dependent responses in trout, such as the induction of vitellogenin and zona radiata (eggshell) proteins (Arukwe et al. 1999; Jobling & Sumpter 1993). Estrogens are involved in the regulation of P450 in fish. Experiments with juvenile and hypophysectomized fish suggest that the down regulation of different P450 isoforms and P450-dependent catalytic activities in maturing females is mediated by estradiol (Andersson & Förlin 1992; Pajor et al. 1990; Vodicnik & Lech 1983). Recent studies indicate that also xenobiotics with estrogen-like properties (xenoestrogens), including nonyl- and octylphenol, can suppress the expression of P450s in fish (Arukwe et al. 1997; Buhler & Wang-Buhler 1998). Whether the effects on steroid hydroxylation observed in this study were due to decreased levels of expression of constitutive P450 such as CYP3A27 and CYP2K1, rather than due to the direct inhibition of catalytic activity, could not be addressed because of the lack of appropriate immunochemical probes.

Further studies are required to elucidate the interaction with the endocrine system of xenobiotic compounds that affect the cytochrome P450 system, and the interaction with the P450 system of endocrine-disrupting compounds. The results of the present study show that cultured fish hepatocytes, which also have successfully been used to study the estrogenic effects of xenobiotics (Anderson et al. 1996; Jobling & Sumpter 1993), provide a promising cellular in vitro system for such research (see also Navas & Segner 2000a, 2000b).

Conclusions

The comparative in vivo / in vitro investigation of the hepatic biochemical effects of prochloraz and NP2EO contributes to the validation of the cultured trout hepatocyte system as a tool in the study of hepatic biotransformation in fish. Up to now, few studies exist that have examined the relation between responses in vivo and in hepatocytes. This work demonstrates the suitability of rainbow trout hepatocytes to identify the types of responses of cytochrome P450-dependent variables, while it shows that the response of glucuronidation can differ between in vivo and in vitro investigations.

P-GLYCOPROTEIN

Using immunochemical methods, the present work demonstrated the hepatic expression of P-glycoprotein (P-gp)-like protein(s) in the liver of rainbow trout. In cultured trout hepatocytes, a P-gp-like mechanism was investigated using the fluorescent P-gp substrate rhodamine 123 and characterized with respect to its specificity towards specific inhibitors of P-gp. To the author's best knowledge, this work is the first demonstration of a hepatic P-gp-like mechanism in teleosts. The P-gp-like mechanism in trout hepatocytes was inhibited by the model xenobiotics prochloraz and nonylphenol diethoxylate (NP2EO). One week of exposure of juvenile trout to sublethal levels of these compounds, however, did not alter the levels of expression of P-gp-like protein in the liver of the exposed animals.

Immunochemical evidence for the expression of P-gp(s) in trout liver

The evidence for the hepatic expression of P-gp(s) in rainbow trout, presented by immunoblotting and immunohistochemistry in this report, is in line with studies on other fish species that have demonstrated the presence of P-gp-like proteins in teleost liver by similar techniques (Cooper et al. 1999; Hemmer et al. 1995). In a teleost, two genes showing a high degree of homology to mammalian P-gps have been identified (Chan et al. 1992). However, newer data have revealed that only one of these genes likely encodes a true P-gp, whereas the other resembles the mammalian 'sister of P-gp' gene (*sPgp*) (Cooper et al. 1999; Cooper 1999), which encodes a hepatic bile salt transporter (Childs et al. 1995; Gerloff et al. 1998). The antibodies used in this study are directed against epitopes conserved among P-gps and sPgps (Childs et al. 1995; Georges et al. 1990). Hence, the immunochemical data of this and other extant studies on teleosts using the same antibodies (Cooper et al. 1999; Hemmer et al. 1995) should be interpreted with caution. The molecular cloning of piscine *P-gp* genes, currently being undertaken in several laboratories (GenBank. [Online] National Center of Biotechnology Information, Bethesda, MD, USA. http://www.ncbi.nlm.nih.gov/ [2000, Feb 9]), will enable the design of more specific probes, and the further characterization of the expression of *P-gp* genes in fish tissues.

Functional evidence for the expression of P-gp(s) in trout liver

In this study, the accumulation and efflux of the mdr1-type substrate rhodamine 123 was studied in the presence and the absence of compounds known to interfere with transport mediated by mdr1-type P-gps (verapamil, vinblastine and cyclosporin A). Generally, the latter chemicals increased the accumulation, but delayed the efflux of rhodamine 123. This demonstrates the presence of an mdr1-type mechanism in trout liver and suggests the presence of mdr1-type P-gp(s) in the teleost liver. However, it should be borne in mind that different

transporters of overlapping specificities are present in the vertebrate liver (Kamisako et al. 1999; Meijer et al. 1997; Oude Elferink et al. 1995). A group of transporters existing in mammals which display a substrate and inhibitor specificity overlapping with that of mdr1-type P-gps are the multidrug resistance associated proteins (MRPs) (Loe et al. 1996; Twentyman et al. 1994). Evidence exists for the presence of a MRP-like protein in the rectal gland of elasmobranch fish (Miller et al. 1998), suggesting the conservation of MRPs among vertebrates, and consequently their presence in teleosts. Hence, MRP(s) could also have contributed to the mdr1-like mechanism observed in trout hepatocytes in this study. Although the responsible protein species have not fully been identified, the data in this report provide functional evidence for a P-gp-like mechanism in the teleost liver and suggest its function in the biliary secretion of hydrophobic endogenous and xenobiotic compounds.

Interference of xenobiotics a hepatic P-gp-like mechanism in trout

In the present study, both prochloraz and NP2EO inhibited the mdr1-like efflux of rhodamine 123 from cultured hepatocytes, suggesting these model water pollutants as substrates of the P-gp pump. Further investigations with prochloraz showed, however, that this imidazole compounds is, despite its high potency as an inhibitor, not a substrate of the trout mdr1-like mechanism. This is in line with studies which have shown that other imidazole fungicides are potent inhibitors, but not substrates of the human MDR1 P-gp (Siegsmund et al. 1994; Takano et al. 1998). Although not demonstrated in this work it appears likely that NP2EO is a substrate of the mdr1-like mechanism in trout hepatocytes, as nonylphenol ethoxylates have been shown to represent substrates of the human MDR1 protein (Charuk et al. 1998; Loo & Clarke 1998).

Extensive research has aimed to identify inhibitors of P-gp in order to find antagonists of the P-gp-mediated multidrug resistance in cancer (Ford & Hait 1990). Compounds that reverse drug resistance, and/or the decreased accumulation of cytoctoxic drugs accomplished by mdr1 proteins, are called chemosensitizers or resistance modifiers. Many, but not all chemosensitizers are substrates of the P-gp pump (Ford & Hait 1990). From an ecotoxicological perspective, it is important to know which types of environmentally relevant compounds interact with P-gp in aquatic animals. Compounds that inhibit P-gp could either be substrates, i.e. chemicals the excretion of which may involve P-gp, or inhibitors but not substrates. Xenobiotics of the latter category may interfere with the excretion of other xenobiotics or endogenous compounds. However, little is known about the potential of environmental pollutants to inhibit P-gp. It has been shown in mammalian systems that different pesticides and the PAH benzo-a-pyrene interact with P-gp (Lanning et al. 1996; Lanning et al. 1995; Yeh et al. 1992). (Kurelec & Pivcevic 1991; Kurelec & Pivcevic 1992) identified a P-gp-like mechanism in aquatic invertebrates for which they coined the term multixenobiotic resistance (MXR). The presence of inhibitors and/or substrates of MXR in the aquatic environment has been demonstrated (Kurelec 1997; Smital & Kurelec 1997; Toomey

& Epel 1993); however, little is known concerning their chemical identity. Different moderately hydrophobic pesticides have been shown to be inhibitors and/or substrates of the MXR mechanism in a marine bivalve (Cornwall et al. 1995).

The identification of environmental xenobiotic substrates of piscine P-gp could also contribute to elucidate the function of hepatic P-gp in fish. Different lines of evidence exist that the primary role of mdr1-type P-gps in mammals is the defense against xenobiotics. These include the preferable expression of mdr1-type P-gps in organs involved in excretion or epithelia representing tissue boundaries (Fojo et al. 1987; Thiebaut et al. 1987; Thiebaut et al. 1989), the responsiveness of mdr1-type P-gps to xenobiotic induction (Burt & Thorgeirsson 1988; Fardel et al. 1996; Schrenk et al. 1994), and the fact that mdr1 knock-out mice are phenotypically normal except that they show an increased susceptibility to certain toxicants (Borst & Schinkel 1996; Schinkel et al. 1997). Similarly, the association of high levels of expression and functional activity of the MXR mechanism in mollusk populations from heavily polluted sites with their resistance toward environmental toxins suggests for MXR a role in the biochemical defense against xenobiotics (Kurelec 1997; Kurelec et al. 1996; Waldmann et al. 1995). The resemblance in substrate and inhibitor specificity of the P-gp-like mechanism in fish to mammalian mdr1-type P-gps, shown in the present and other investigations (Miller 1995; Schramm et al. 1995; Sussman-Turner & Renfro 1995), argues for the role of piscine P-gps as in the defense against xenobiotics. However, more research is required to substantiate or refute this hypothesis.

Lack of effects of xenobiotics on the hepatic levels of expression of P-gp-like protein(s) in trout

Following sublethal in vivo exposures of juvenile trout to prochloraz and nonylphenol diethoxylate, the levels of the hepatic expression of P-gp-like protein(s) remained unchanged compared to controls (chapter 5). Xenobiotic inducers of hepatic P-gps in mammals include polycyclic aromatic hydrocarbons, dibenzo-p-dioxins, cytostatic agents and pesticides (Burt & Thorgeirsson 1988; Lanning et al. 1996; Schrenk et al. 1996; Schuetz et al. 1995). These compounds do not present common structural or toxicological features; and it has been shown that the induction of P-gp in mammals is independent of that of CYP1A (Gant et al. 1991). (Cooper et al. 1999) found increased levels of hepatic P-gp in the fish mummichog from a heavily polluted environment when compared to reference populations. However, attempts to induce P-gp in mummichog through laboratory exposure to 3-methylcholanthrene, a PAH and known inducer of CYP1A, were unsuccessful (Cooper et al. 1999). The elevated levels of P-gps in mummichog populations from polluted environments (Cooper et al. 1999) could also reflect a genetically fixed increase of basal levels of P-gp expression. Hence, the responsiveness of P-gps in fish to xenobiotic induction remains to be demonstrated.

Conclusions

This work demonstrates the presence of P-gp and of a P-gp-like mechanism in the rainbow trout liver, suggesting the role of P-gp(s) in the biliary secretion of hydrophobic compounds in teleost fish. Moreover, the capability of environmentally relevant xenobiotics to interfere with the function of the P-gp-like mechanism is shown. The exposure of juvenile rainbow trout to prochloraz and nonylphenol remained without effect on the hepatic levels of expression of P-gp-like proteins, so it remains unclear whether xenobiotics are able to affect of the expression of P-gp-like proteins in fish.

GENERAL CONCLUSION

The present work characterized two potential biochemical defense mechanisms in fish, butyrylcholinesterase and P-glycoprotein, about which there is little existing information. It is demonstrated here that both butyrylcholinesterase and P-glycoprotein interact with certain environmentally relevant xenobiotics, which suggests their role in the organism's defense against toxicants. However, more research is required to judge the toxicological relevance of butyrylcholinesterase and P-glycoprotein in the toxicology of different classes of environmental pollutants.

A further subject of this study was the validation of cultured rainbow trout hepatocytes in the assessment of xenobiotic effects on hepatic biotransformation enzymes. The responses of different cytochrome P450-dependent variables to xenobiotics in cultured trout hepatocytes was similar as those observed in vivo, which demonstrates the general suitability of the trout hepatocyte system for such studies. Research into the potential of xenobiotics to interfere with endogenous metabolic roles of cytochrome P450 in fish is clearly needed, and or which cultured hepatocytes appear to offer a promising system for this.

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Summary

In the present work, selected biochemical mechanisms which potentially provide a defense against the toxicity of organic environmental chemicals, were investigated in fish. The enzyme butyrylcholinesterase, dealt with in chapters 1 and 2, can bind organophosphate insecticides irreversibly and thus act as a defense mechanism. The aim of the present investigation was firstly, to characterize the basic properties of butyrylcholinesterase in a teleost fish, the three-spined stickleback (*Gasterosteus aculatus*), and secondly, to study the interaction of stickleback butyrylcholinesterase with organophosphate insecticides. Chapter 3 examines the extend to which the cellular in vitro system of primary cultured rainbow hepatocytes is suitable for predicting the effects of environmental chemicals on hepatic biotransformation enzymes. The subject of chapters 4 and 5 is the tranporter P-glycoprotein which is probably involved in the renal and hepatobiliary excretion of lipophilic chemicals. The present investigation characterized some basic properties of P-glycoprotein in the liver of rainbow trout were characterized and studied the interactions of P-glycoprotein with two model chemicals.

Butyrylcholinesterase

Three-spined stickleback (*Gasterosteus aculeatus*) was selected as a teleost model species to study butyrylcholinesterase. Stickleback appear suitable as a bioindicator in the monitoring of water contamination by organophosphate insectides (OPs), because they occur as a dominant species in small streams heavily influenced by agriculture, and are found in wide parts of central and Western Europe. In stickleback, the cholinesterase of the brain is exclusively made up of acetylcholinesterase (AChE), while that of the liver consists exclusively of butyrylcholinesterase (BChE), and that of the skeletal muscle is composed of both AChE and BChE. Stickleback BChE shows atypical properties. With median inhibition concentrations in the nanomolar range, stickleback BChE is more than three orders of magnitude more sensitive to the OPs paraoxon and dichlorvos in vitro than AChE. In a field study, the contamination of eight small headwater streams by OPs was compared to the activities of AChE and BChE in stickleback populations from the streams. In three streams, the OP parathion was found at low water concentrations (0.05 to 0.3 µg/l) during precipitation events associated with surface runoff. Compared to populations from two pristine reference streams, the BChE activities in the skeletal muscle were significantly decreased (>85%) in stickleback from the three OP-contaminated streams. By contrast, no significant differences of brain and body muscle AChE activities were found between populations from reference and contaminated streams. Strongly decreased BChE activities, however, were also found at one stream at which no insecticides were detected. In experiments under controlled laboratory conditions, the BChE activity was decreased by 60% in the liver and by 30% in the gills and

the skeletal muscle after stickleback had been exposed to 1 µg/l parathion for one hour and had been allowed to recover for 48 h. By contrast, no effects of parathion on AChE activities were observed in this experiment.

The present investigations show that in stickleback, BChE is more sensitive toward the inhibition by OPs than AChE. Consequently, fish BChE is suggested as a biomarker of OP exposure which allows a much more sensitive detection of contamination than AChE. The results further suggest that BChE may function in fish as a protective mechanism in the toxicology of OPs. This hypothesis, however, requires further experimental examination.

Biotransformation

Chapter 3 examines whether the in vivo effects of environmental chemicals on hepatic biotransformation enzymes can be predicted from in vitro investigations with primary cultured hepatocytes from rainbow trout. The imidazol fungicide prochloraz and the surfactant nonylphenol diethoxylate were selected as model chemicals. Prochloraz is an inducer of the cytochrome P450 subfamily CYP1A, but may also inhibit cytochrome P450-dependent catalytic activities. Nonylphenol diethoxylate is a know xenoestrogen, i.e. a compound with estrogen-like properties in biological systems. Generally, a good correspondence of the patterns of the responses of biotransformation enzymes was found between exposed hepatocytes (in vitro) and juvenile trout (in vivo). As expected, prochloraz effected the induction of the CYP1A-dependent enzyme EROD and of CYP1A levels, both in vitro and in vivo. By contrast, nonylphenol diethoxylate had no effects on EROD and CYP1A levels in both biological systems. Both prochloraz and nonylphenol diethoxylate inhibited the rates of the 6β und 16β-hydroxylation of testosterone, both in vitro and in vivo. In fish, there are marked differences in the activities of different cytochrome P450-dependent regioselective steroid hydroxylation reactions between adult males and females, particularly during the (pre)spawning period. The decreases in testosterone hydroxylation rates by prochloraz and nonylphenol diethoxylate found here suggest that an exposure of fish to these compounds could result in adverse effects on reproduction. The inhibition of testosterone hydroxylation could, on the one hand, have been due to a direct inhibition of the enzyme. On the other hand, the decreased steroid hydroxylation activities could also reflect a suppression of the expression of the responsible cytochrome P450 isoforms.

The present work confirms the suitability of primary cultures of rainbow trout hepatocytes in the assessment of xenobiotic effects on cytochrome P450-dependent biotransformation at a qualitative level. However, differences were observed in the response of testosterone glucuronidation to the investigated chemicals between in vitro and in vivo experiments. This could be because of methodological differences between the in vitro and in vivo measurements, or could have been due to the different periods of exposure used in vitro and in vivo.

P-Glycoprotein

The present work examined firstly whether P-glycoproteins exist in the liver of the rainbow trout (*Oncorhynchus mykiss*). With antibodies recognizing conserved epitopes of P-glycoprotein, a protein band of 165 kDA was detected in immunoblots of liver extracts, while in immunohistochemical investigations bile canaliculi showed a strongly positive reaction. These findings suggest the presence of P-glycoprotein in the liver of teleosts. Secondly, the work investigated the kinetics of the accumulation and the efflux of rhodamine 123, a substrate of P-glycoprotein, in trout hepatocytes in order to elucidate the function of hepatic P-glycoprotein in trout. Specific inhibitors of P-Glycoprotein increased the accumulation of rhodamine 123 by hepatocytes, but delayed the efflux of rhodamine 123 from hepatocytes. This demonstrates the presence of a mdr1-P-glycoprotein-like transport mechanism in the liver of rainbow trout. A further question was whether environmentally relevant xenobiotics interact with P-glycoprotein in the liver of rainbow trout. The imidazole fungicide prochloraz and the surfactant nonylphenol diethoxylate inhibited the P-glycoprotein-like mechanism in trout hepatocytes. Despite the high potency of prochloraz as an inhibitor, this fungicide is not a substrate of the P-glycoprotein-like mechanism. Juvenile rainbow trout showed no changes in the hepatic levels of P-glycoprotein after sublethal exposures to prochloraz and nonylphenol diethoxylate.

The investigations in rainbow trout confirm, using immunochemical techniques, that P-glycoprotein exists in the liver of teleosts. Moreover, they demonstrate for the first time the presence of a P-glycoprotein-like mechanism in the teleost liver. An assumed role of mdr1-type P-glycoproteins in the mammalian liver is the secretion of lipophilic xenobiotics into bile. It appears probable that the P-glycoprotein-like mechanism in the liver of rainbow trout also assumes such a role. More research is required, however, to resolve the function of P-glycoprotein in the piscine liver. The model compounds selected in this study did not alter hepatic levels of P-glycoprotein in trout. Hence, the question whether xenobiotics are capable to induce the hepatic expression of P-glycoprotein in fish remains open. Because of the presence of different transporters in the vertebrate liver, it seems possible that the immunochemical and functional measurements reflect the presence and activity of different transporters. Further studies are necessary to determine the number isoforms of P-glycoprotein in fish and their function. The molecular characterization of P-glycoproteins and further hepatic transporters, currently being undertaken by different laboratories, will considerably facilitate investigations into the expression, function and regulation of piscine P-glycoproteins.

Zusammenfassung

In der vorliegenden Arbeit wurden biochemische Mechanismen in Fischen untersucht, die potentiell eine Schutzwirkung gegen die toxische Wirkung organischer Umweltchemikalien darstellen. Die in den ersten beiden Kapiteln behandelte Butyrylcholinesterase kann Organophosphorinsektizide irreversibel binden und dadurch eine Schutzwirkung ausüben. Ziel der vorliegenden Untersuchung war es, in einem Teleosteer, dem Dreistachligen Stichling (*Gasterosteus aculeatus*) die Butyrylcholinesterase bezüglich ihrer grundlegenden Eigenschaften und ihrer Interaktion mit Organophosphorinsektiziden zu charakterisieren. Im dritten Kapitel wurde untersucht, inwiefern das zelluläre in vitro-System der Primärkultur von Regenbogenforellen-Hepatozyten zur Vorhersage der in-vivo-Effekte von Umweltchemikalien auf Biotransformationsenzyme geeignet ist. Gegenstand der Kapitel 4 und 5 war der Transporter P-Glykoprotein, der wahrscheinlich an der renalen und hepatobiliären Exkretion von lipophilen Chemikalien beteiligt ist. In der vorliegenden Untersuchungen wurden einige grundlegende Charakteristika von P-Glykoprotein in der Regenbogenforelle bestimmt und seine Interaktion mit zwei Modell-Umweltchemikalien charakterisiert.

Butyrylcholinesterase

Für die Untersuchung der Butyrylcholinesterase wurde als Modellspezies der Dreistachlige Stichling (*Gasterosteus aculeatus*) ausgewählt. Der Stichling ist als Indikatorart im Biomonitoring von Organophosphorinsektiziden (OPs) geeignet, da er auch in kleinen, landwirtschaftlich stark belasteten Fließgewässern als dominante Art vorkommt und über weite Teile Mittel- und Westeuropas verbreitet ist. Im Stichling besteht die Cholinesterase des Gehirns ausschließlich aus Acetylcholinesterase (AChE), die der Leber ausschließlich aus Butyrylcholinestterase (BChE) und die des Skelettmuskels sowohl aus AChE als aus BChE. Die BChE des Stichlings zeichnet sich durch atypische Eigenschaften aus. Mit medianen Inhibitionskonzentrationen (IC50s) im nanomolaren Bereich ist die BChE des Stichlings gegenüber den OPs Paraoxon und Dichlorvos um mehr als drei Größenordnungen empfindlicher als seine AChE. In einer Freilanduntersuchung wurde die Belastung von acht kleinen Fliessgewässer (im folgenden Bäche) durch OPs mit den AChE- und BChE-Aktivitäten der Stichlings-Populationen dieser Bäche verglichen. In drei Bächen wurde das OP Parathion in geringen Konzentrationen (0.05 bis 0.3 µg/l) während Regenereignissen nachgewiesen, die zu Oberflächenabfluss führten. Bei den Stichlings-Populationen dieser OP-belasteten Bächen waren im Vergleich zu Populationen aus zwei unbelasteten Referenzgewässern die BChE-Aktivitäten im Skelettmuskel signifikant erniedrigt (>85%), während keine signifikanten Unterschiede der AChE-Aktivitäten im Gehirn und im

Skelettmuskel vorlagen. Stark erniedrigte BChE-Aktivitäten wurden allerdings auch an einem Bach gefunden, in dem keine Insektizide nachgewiesen wurden. Dies könnte auf Kontaminationen zurückgehen, die mit der verwendeten Methodik nicht nachgewiesen wurden. In Experimenten unter kontrollierten Laborbedingungen war nach einer einstündigen Exposition von Stichlingen an 1.0 µg/l Parathion und einer Erholungs- und Metabolisierungsphase von 48 h war die BChE-Aktivität im Skelettmuskel und in den Kiemen um 30% und in der Leber um 60% gegenüber den Kontrollen erniedrigt, während keine Effekte auf AChE festgestellt wurden.

Die vorliegenden Untersuchungen zeigen, dass BChE im Stichling empfindlicher als AChE gegenüber der Inhibition durch OPs ist. BChE in Fischen wird daher als Expositionsbiomarker für OPs vorgeschlagen, der einen wesentlich empfindlicheren Nachweis von Kontaminationen erlaubt als die Acetylcholinesterase. Die Ergebnisse sprechen ferner dafür, dass BChE in der Toxikologie von OPs in Fischen als potentieller Schutzmechanismus fungieren könnte. Diese Hypothese erfordert jedoch weitere experimentelle Prüfung.

Biotransformation

In Kapitel 3 der vorliegenden Arbeit wurde untersucht, ob sich die in vivo Effekte von Umweltchemikalien auf die Biotransformations-Enzyme der Leber in Fischen aus in vitro-Untersuchungen an Primärkulturen von Forellenhepatozyten vorhersagen lassen. Als Modell-Umweltchemikalien wurden das Imidazol-Fungizid Prochloraz und die oberflächenaktive Substanz verwendete Verbindung Nonylphenol-Diethoxylat ausgewählt. Prochloraz ist ein Induktor der Cytochrom P450-Unterfamilie CYP1A, übt aber daneben auch inhibierende Wirkungen auf Cytochrome P450-abhängige Enzymaktivitäten aus. Nonylphenol-Diethoxylat ist als Xenoestrogen bekannt, d.h. bewirkt Estrogen-abhängige Wirkungen in biologischen Systemen. In den Mustern der Antworten der hepatischen Biotransformations-Enzyme auf die Exposition an die Modellchemikalien zeigte sich eine im allgemeinen gute Übereinstimmung zwischen exponierten Hepatozyten (in vivo) und juvenilen Forellen (in vivo). Prochloraz bewirkte, wie erwartet, sowohl in vitro als auch in vivo eine Induktion der CYP1A-abhängigen Enzymaktivität EROD und der Konzentrationen des CYP1A Proteins. Nonylphenol-Diethoxylat zeigte dagegen in beiden biologischen Systemen keine Wirkung auf CYP1A und EROD. Sowohl Prochloraz als auch Nonylphenol-Diethoxylat inhibierten die Raten der Testosteron-Hydroxylierung an den Positionen 6β und 16β, sowohl in vitro als auch in vivo. Die Aktivitäten der P450-abhängigen, regioselektiven Hydroxylierung von Steroiden

differieren stark zwischen adulten männlichen und weiblichen Fischen, besonders zur Fortpflanzungszeit. Die in dieser Arbeit festgestellte Herabsetzung der Testosteron-Hydroxylierungs-Raten durch Prochloraz und Nonylphenol-Diethoxylat legen nahe, dass eine Exposition von Fischen an diese Stoffe negative Wirkungen auf die Reproduktionfunktionen haben könnte. Die Inhibierung der Testosteron-Hydroxylierung könnte einerseits auf einer direkten Enzym-Inhibition beruhen. Auf der anderen Seite könnten die herabgesetzten Aktivitäten der Testeron-Hydroxylierung auch eine Supprimierung der Expression der beteiligten Cytochrom P450-Isoformen anzeigen.

Die vorliegende Arbeit bestätigt die Eignung von Primärkulturen von Forellenhepatozyten, die in vivo Effekte von Umweltchemikalien auf P450-abhängige Enzyme der Biotransformation qualitativ zu bewerten. Dagegen ergaben sich bei der Glucuronidierung von Testosteron Unterschiede zwischen den in vitro und den in vivo Versuchen in der Wirkung der untersuchten Chemikalien. Die Unterschiede könnten zum einen methodische Unterschiede zwischen den Messungen in vitro und in vivo widerspiegeln, und zum anderen auf den in vitro und in vivo unterschiedlichen Expositionsdauern beruhen.

P-Glykoprotein

In der vorliegenden Arbeit wurde zunächst mit immunchemischen Methoden geprüft, ob P-Glykoproteine in der Leber der Regenbogenforelle (*Oncorhynchus mykiss*) vorhanden sind. Mit Antikörpern, die konservierte Epitope von P-Glykoprotein erkennen, lässt sich in Immunblots von Leberextrakten eine Proteinbande von 165 kDa nachweisen, während in immunhistochemischen Untersuchungen die Gallenkanalikuli eine stark positive Reaktivität zeigen. Diese Befunde sprechen für das Vorhandensein von P-Glykoproteinen in der Leber der Knochenfische. Des weiteren wurden in der vorliegenden Arbeit für P-Glykoprotein die Kinetik der Akkumulation und des Effluxes von Rhodamine 123, einem Substrat von P-Glykoprotein, in Primärkulturen von Hepatozyten der Regenbogenforelle untersucht, um die Funktion von P-Glykoprotein in der Leber der Forelle zu beleuchten. Spezifische Inhibitoren von P-Glykoprotein erhöhten die Aufnahme von Rhodamin 123 durch die Hepatocyten, während diese Inhibitoren die Abgabe von Rhodamin 123 durch die Hepatozyten verlangsamten. Dies zeigt, dass ein mdr1-P-Glykoprotein-ähnlicher Transportmechanismus in der Leber der Regenbogenforelle vorhanden ist. Es stellte sich die weitere Frage, ob umweltrelevante Xenobiotika mit P-Glykoprotein in der Leber der Regenbogenforelle interagieren. Das Imidazol-Fungizid Prochloraz und die oberflächenaktive Substanz Nonylphenol-Diethoxylat hemmten den P-Glykoprotein-ähnlichen Mechanismus in

Forellen-Hepatozyten. Trotz hohen Wirksamkeit von Prochloraz als Inhibitor stellt dieses Fungizid kein Substrat des P-Glykoprotein-ähnlichen Mechanismus dar. Juvenile Regenbogenforellen zeigten nach subletalen Expositionen an Prochloraz und Nonylphenol-Diethoxylat keine Veränderungen der hepatischen P-Glykoprotein-Konzentrationen.

Die Untersuchungen an der Regenbogenforelle bestätigen mit immunchemischen Methoden, dass in der Leber der Teleosteern P-Glykoprotein vorliegt. Ferner weist die vorliegende Arbeit zum ersten Mal die Präsenz eines mdr1-ähnlichen Mechanismus in der Teleosteer-Leber nach. Eine vermutete Rolle der mdr1-P-Glykoproteine der Säugerleber ist die Sekretion lipophiler Xenobiotika in die Gallenflüssigkeit. Es ist wahrscheinlich, dass der P-Glykoprotein-ähnliche Mechanismus in der Leber der Regenbogenforelle ebenfalls eine solche Rolle einnimmt. Um die Funktion von P-Glykoprotein in der Fischleber aufzuklären sind jedoch noch weitere Untersuchungen nötig. Die Frage, ob Xenobiotika die hepatische Expression von P-Glykoprotein in Fischen induzieren können, bleibt nach der vorliegenden Untersuchung weiterhin offen. Wegen der Existenz verschiedener ähnlicher Transporter in der Vertebratenleber erscheint es möglich, dass die immunchemischen und funktionellen Messungen in dieser Arbeit die Präsenz und Aktivität verschiedener Transporter widerspiegeln. Weitere Untersuchungen sind notwendig, um die Anzahl von P-Glykoprotein-Isoformen in Fischen und ihre Funktion zu bestimmen. Die molekulare Charakterisierung von P-Glykoproteinen und weiteren hepatischen Transportern in Fischen, die momentan in verschiedenen Laboratorien stattfindet, wird die Untersuchung der Expression, Funktion und Regulation der P-Glykoproteine in Fischen erheblich erleichtern.

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List of Abbreviations

mAB	Monoclonal antibody
AChE	Acetylcholinesterase (EC 3.1.1.7)
AcβMeSCh	Acetyl-β-(methyl)thiocholine
AcSCh	Acetylthiocholine
AhR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
anti-ChE	Anti-cholinesterase insecticide
APnEO	Alkylphenol ethoxylate
cBAT	Canalicular bile acid transporter
BChE	Butyrylcholinesterase (EC 3.1.1.8)
BW284C51	1,5-Bis(4-allyldimethylammoniumphenyl)pentan-3-one-dibromide
BuSCh	Butyrylthiocholine
BSA	Bovine serum albumine
CaE	Carboxylesterase (EC 3.1.1.1)
ChE	Cholinesterase
CYP	Cytochrome P450
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
EROD	7-Ethoxyresorufin-*O*-deethylase
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
IC50	Median inhibition concentration
IgG	Immunoglobulin G
LDH	Lactate dehydrogenase (EC 1.1.1.27)
M199	Medium 199
MDR	Multidrug resistance
MRP	Multidrug resistance-associated protein
NADPH	Nicotinamide-adenin-dinucleotide phosphate, reduced form
NP2EO	Nonylphenol diethoxylate
iso-OMPA	Tetraisopropyl pyrophosphoramide
OP	Organophosphate insecticide
P450	Cytochrome P450
PAGE	Polyacrylamide gel electrophoresis
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate-buffered saline
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo-p-dioxin
PCDF	Polychlorinated dibenzofuran
P-gp	P-glycoprotein
sPgp	Sister of P-glycoprotein
PrSCh	Propionylthiocholine
Rh123	Rhodamine 123
SDS	Sodium dodecyl sulfate

List of Publications

Manuscripts independent from this thesis

Steinberg, C. E. W., Sturm, A., Kelbel, J., Lee, S. K., Hertkorn, N., Freitag, D., Kettrup, A.A. 1992. Changes of acute toxicity of organic chemicals to *Daphnia magna* in the presence of dissolved humic material (DHM). *Acta hydrochim. hydrobiol.* 20:326-332

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Manuscripts arising from this thesis

Sturm, A., Wogram, J., Segner, H., Liess, M. 2000. Different sensitivity to organophosphates of acetylcholinesterase and butyrylcholinesterase from three-spined stickleback (*Gasterosteus aculeatus*): Application in biomonitoring. *Environ. Toxicol. Chem*, 19(6):1607-1615.

J. Wogram, J., Sturm, A., Segner, H., Liess, L. Effects of parathion on acetylchlinesterase, butyrylcholinesterase and carboxylesterase in three-spined stickleback (*Gasterosteus aculeatus*) following short-term exposure. Submitted to: *Environ. Toxicol. Chem.*

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Sturm, A., Ziemann, C. Hirsch-Ernst, K.I., Segner, H. Expression and functional activity of P-glycoprotein in cultured hepatocytes from rainbow trout (*Oncorhynchus mykiss*). Submitted to: *Am. J. Physiol.*

Sturm, A., Cravedi, J.-P., Segner, H. Prochloraz and nonylphenol diethoxylate inhibit P-glycoprotein-dependent rhodamine 123 efflux in trout hepatocytes, but dot affect hepatic P-glycoprotein expression in rainbow trout (*Oncorhynchus mykiss*). In press in: *Aquat. Toxicol.*

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Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die den benutzten Werken inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Ich habe mich zu früherer Zeit noch nicht um den Doktorgrad beworben.

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