Interactive effects of cadmium and benzo(a)pyrene in mummichog (Fundulus heteroclitus).

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INTERACTIVE EFFECTS OF CADMIUM AND BENZO[A]PYRENE IN MUMMICHOG (FUNDULUS HETEROCLOITUS)

A Dissertation Presented to
The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

by

Peter van den Hurk

1998
APPROVAL SHEET

This dissertation is submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Peter van den Hurk

Approved, August 1998

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DEDICATION

This dissertation is dedicated to my father Wilhelmus Jan van den Hurk, and my late grandfather Willem Gerard van den Hurk, who, through their genes, their teachings and their proud and loving support, have laid the basis for what I have achieved with this work. It is a new milestone in multi-generational academic endeavor.
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ABSTRACT

When animals are exposed to mixtures of environmental pollutants, it is generally assumed that the toxic effects of the individual components are additive. However, examples of synergistic and antagonistic effects have been described. To study the mechanisms of interaction between a metal and a polycyclic aromatic hydrocarbon, mummichog (Fundulus heteroclitus) were injected with combinations of cadmium (Cd) and benzo[a]pyrene (BP). Measured effect parameters were: mortality, BP-metabolite production in isolated hepatocytes and microsomes, hepatic induction of CYP1A (the BP metabolizing enzyme) and metallothionein (the Cd binding protein), and biliary excretion of BP-metabolites.

The mortality data demonstrated that both synergistic and antagonistic effects can occur. A Cd dose of 0.32 mg/kg significantly reduced the expected mortality caused by BP. In contrast, a BP dose of 10 mg/kg significantly increased the toxicity of Cd above the expected mortality.

To study the mechanisms of these interactive effects, liver cells (hepatocytes) were isolated from fish that were previously injected with combinations of Cd and BP. These cells were exposed to radiolabeled BP to study the rate of BP metabolism, and the formation of BP-metabolites. Cadmium exposure had an overall inhibiting effect on the metabolism of BP. No effects of Cd were observed on the formation of individual metabolites.

To distinguish between direct interference of Cd with CYP1A at the active site versus indirect interference by inhibiting CYP1A induction, microsomal preparations were evaluated for enzyme activity and enzyme concentration. While there was no direct effect of Cd on enzyme catalytic activity, there was an effect on CYP1A production.

The demonstrated inhibition of BP metabolism by Cd would suggest a reduced excretion of BP-metabolites. However, analysis of bile and water samples after fish were injected with radiolabeled BP demonstrated an enhanced biliary excretion of conjugated BP-metabolites under influence of Cd.

Cadmium exposure caused a significant induction of hepatic metallothionein in the fish. When BP was dosed together with Cd, the induction of MT was inhibited. The hypothesis that reactive BP metabolites would compete with Cd for binding sites on MT could not be confirmed. There was no measurable binding of BP to MT.
INTERACTIVE EFFECTS OF CADMIUM AND BENZO[A]PYRENE IN MUMMICHOG (*FUNDULUS HETEROCLIITUS*)
Chapter 1. Introduction and General Methods

Estuarine and coastal environments are exposed to a variety of anthropogenic pollutants, including nutrients (such as phosphate and nitrate), heavy metals and various organic compounds. As concentrations of these pollutants increase, they are expected to have adverse effects on the natural ecosystems. A well-known case is the nearby Elizabeth River, a small tributary to the Chesapeake Bay, VA, which is heavily contaminated from industrial activities in the area. Wood treatment plants, ship yards and naval activities to name but three have contributed to the environmental degradation of the river (Huggett et al., 1992).

To protect or restore these aquatic communities, environmental managers use different tools. One of them is the application of environmental quality criteria, which are compound specific concentration levels at which some adverse effect is likely to occur (Long et al., 1998). The scientific data for the quality criteria are dose, or concentration-dependent effect studies for individual compounds. To account for the combined effects of more than one chemical, the general, pragmatic approach is to assume additivity of the toxic effects generated by each individual compound in a mixture (Van der Gaag, 1992).

However, it is known from a variety of studies that toxic compounds in mixtures may profoundly influence the toxicity of one another (Marking, 1977; Walker & Johnston, 1989; Broderius, 1991, Haas, 1992). These interactions between compounds in living organisms may enhance the toxicity of individual compounds (synergism), or reduce the toxicity (antagonism). Through study of these interactions, one can gain a better understanding of the underlying mechanisms of interaction. Results of mechanistic studies will ultimately provide a basis for improvement of environmental quality criteria.

Interactive effects for environmental pollutants have been described for combinations of metals (de Nicola, 1992; Naddy et al., 1995), cadmium, PCB and oil (Rhodes et al, 1985), copper and phenanthrene (Moore et al., 1984) and cadmium and benzo[a]pyrene (Fair, 1986; Lemaire-Gony et al., 1992, 1995). The goal of this study was to investigate the mechanisms of interaction between cadmium (Cd), one of the heavy
metals, and benzo[a]pyrene (BP), a polycyclic aromatic hydrocarbon (PAH), in an estuarine fish species (*Fundulus heteroclitus*) that is susceptible to exposure to mixtures of these toxicants in polluted field situations, as in particular, the Elizabeth River.

Benzo[a]pyrene and cadmium were chosen for this study for several reasons:

1) Both compounds are common contaminants in the aquatic environment (Manahan, 1994), and especially in the Elizabeth River.
2) Both are present in relatively high concentrations at sites where cancer epizootics in fish have been reported (Malins et al., 1987; Vogelbein et al., 1990).
3) Though BP as a parent compound is not acutely toxic, some of its metabolites are cytotoxic (Zhu et al., 1995) or carcinogenic in fish (Hawkins et al., 1988). The metabolism of BP is relatively well studied and consequently standards for HPLC-analysis of its metabolites are readily available (Varanasi et al., 1989).
4) The uptake, distribution and toxicity of cadmium are well documented (McLeese et al., 1987)
5) BP and Cd are considered to pose a potential hazard to human health (Casaret et al., 1991).

Mummichog (*Fundulus heteroclitus*) was used for this study because of its ease of collection and maintenance, and because it has been used in a variety of toxicological studies (Vogelbein et al., 1990; Fulton & Scott, 1991; Weis & Weis, 1995). The mummichog is found on the Atlantic coast from Labrador to Florida. It is a euryhaline, schooling species, most abundant in shallow estuarine habitats, like salt marshes, tidal creeks, and barrier beach ponds. The mummichog is extremely resistant to low oxygen levels, sudden salinity changes, and can withstand very foul, polluted or muddy water (Foster, 1967; Hardy, 1978). For instance, populations of mummichog persist in the Elizabeth River, despite the heavy contamination. This makes the species an interesting and suitable subject for the study of physiological responses to environmental contaminants (Van Veld et al., 1991; Van Veld & Westbrook, 1995).

The species shows a clear seasonal migration pattern: in November when water
temperatures drop below 10°C the fish migrate to deeper, more saline waters. In spring (March-April), when water temperatures reach 15°C, they migrate back into the shallower, low salinity areas. The fish spawn in these areas during spring and summer (April until August). Mark-recapture investigations have shown that local populations have narrow summer home ranges (Foster, 1967; Lotrich, 1975; Fritz et al., 1975; Hardy, 1978). This non-migratory life style makes the species suitable for investigating the effects of long term, site specific environmental influences, like the effect of contaminated sediments on physiological processes in the fish. The diet of mummichog consists mainly of small crustaceans and polychaetes, supplemented in summer and fall with plant material, crabs and insects (Kneib & Stiven, 1978).
Polycyclic Aromatic Hydrocarbons and Benzo[a]pyrene

Polycyclic aromatic hydrocarbons (PAH) are compounds with two or more benzene rings in different configurations. The lower molecular weight PAH (less than 4 benzene rings) are relatively water soluble (0.1 - 20 ppm), whereas the higher molecular weight PAH (4 to 6 benzene rings) are hydrophobic (solubility < 0.01 ppm) and generally associate with particulate matter in the aqueous environment (Neff & Anderson, 1981). PAH are formed during incomplete combustion processes of organic material. Although natural sources of PAH exist, most environmental problems are caused by anthropogenic sources (Manahan, 1994).

PAH are easily absorbed by living organisms and can cause an acutely toxic effect. The toxicity increases with increasing molecular weight. High molecular weight PAH (> 225) are generally not acutely toxic. However, because of their lipophilicity these compounds may dissolve in the lipid bilayer of cellular membranes, and thus interfere with membrane-related functions of the cell. Physiological processes are present in most organisms that transform the hydrophobic, difficult-to-excrete parent compound into more hydrophilic, easier to excrete metabolites (Varanasi et al., 1989).

Biotransformation of BP

Benzo[a]pyrene is an example of a high molecular weight (252) PAH, consisting of an arrangement of five benzene rings. BP may enter aquatic organisms through the gills from the aqueous phase, or through the gastrointestinal tract from ingested polluted food or sediment (Lee et al., 1972). Three different metabolic pathways have been described for the primary oxidation of hydrophobic PAH into water soluble compounds that then can be excreted by the cell/organism (Varanasi et al., 1989). Although these pathways are thought to serve primarily for detoxification of xenobiotics or metabolic waste material, some intermediate compounds in the PAH biotransformation have been demonstrated to be carcinogenic, mutagenic or cytotoxic (Gelboin & Tso, 1978). Analysis of bile, urine and faeces show PAH metabolites that are polar derivatives consisting of oxidized and
conjugated parent compound (Neff & Anderson, 1981). Several studies have found that the liver is the most active organ in metabolizing BP (cited in Varanasi et al., 1989), though significant BP metabolism has also been demonstrated in other organs: the intestine (Van Veld et al., 1990), spleen (Ladicz et al., 1992), gills, kidney and heart (Varansi et al., 1989). The occurrence of the early stages of cancer and the actual tumors in fish livers have been related to exposure of sediments polluted with PAH (Malins et al., 1987; Vogelbein et al., 1990). It has also been demonstrated that some BP metabolites have an immunocompromising effect (Ladies et al., 1992).

The three known pathways for BP metabolism are 1) oxidation by cytochrome P450 monooxygenase, 2) oxidation by prostaglandin synthetase, and 3) nonenzymatic cooxidation during lipid peroxidation. These are called the Phase I biotransformation steps. The primary metabolites that are formed during Phase I can be conjugated to other molecules by Phase II conjugating enzymes to facilitate their excretion (see below).

1) Cytochrome-P450 mediated transformation

Oxidation of BP by cytochrome-P450-monoxygenase (CYP1A) is the most studied pathway of BP biotransformation and is considered the most important mechanism in vertebrates. The P450 system is localized on the endoplasmic smooth reticulum inside the cell. P450 activity is measured by fractionating the cells, followed by isolation of the fragmented endoplasmic reticulum parts, called microsomes. Microsomes are then incubated with a suitable substrate (e.g. 7-ethoxyresorufin, BP) and metabolite formation is measured (Varanasi et al., 1989; Schwartzenbach et al., 1993).

The term P450 monoxygenase refers to a large group of related enzymes, all involved in the breakdown of endogenous and exogenous compounds. Each enzyme has a specific substrate, or a defined group of substrates. The CYP1A enzyme catalyzes the epoxidation of PAH and other aromatic compounds (Stegeman & Hahn, 1994). The active site on the cytochrome P450 monoxygenase enzyme consists of an iron porphyrin (a heme group). This heme group is embedded in a protein environment, which is a nonpolar region. This causes nonpolar compounds like BP to be preferentially bound to the active
site of the enzyme as a result of their hydrophobicity. After formation of the substrate:enzyme complex the Fe III in the heme group is reduced to Fe II by NAD(P)H cytochrome P450 reductase. Then oxygen (O$_2$) is bound by the heme group and protonated through the addition of another H$^+$ by again NAD(P)H cytochrome P450 reductase. Then, through an unknown step, the highly reactive electrophilic oxygen that was formed, quickly binds to the BP and a BP-epoxide is formed. This BP-epoxide, which has a less hydrophobic nature than the parent compound, is easily released from the nonpolar active site on the enzyme. Since the active site on the P450 enzyme is not very specific; BP can attach in different configurations to the active site and therefore the oxide can bind at several places on the parent molecule to form BP-1,2, -2,3, -4,5, -7,8, -9,10, or -11,12-epoxide. Of these the -4,5, -7,8, and -9,10 epoxides are the most dominant found in fish (Varanasi et al., 1989).

The BP-epoxides are relatively unstable and will rapidly transform through one of the following 4 major reactions (Varanasi et al., 1989):
1) Spontaneous rearrangement to form phenols (1-, 3-, 7-, or 9-hydroxy-BP)
2) Hydration to form diols (dihydroxydihydro compounds: BP-4,5-diol, BP-7,8-diol, BP-9,10-diol), catalyzed by epoxide hydrolase (EH)
3) Conjugation by Phase II enzymes. In fish, the predominant reaction is the conjugation of metabolites with glutathione (GSH), mediated by glutathione-S-transferase (GST). The conjugates are water soluble and are considered detoxication products that are easily excreted through the bile. The BP-phenols also can be conjugated to either glucuronic acid by UDP-glucuronyl-transferase, or to sulfate by sulfotransferase. These conjugate groups are water soluble as well and are considered detoxification products just as the glutathione conjugates.
4) Covalent binding to cellular macromolecules, e.g. proteins, DNA.

The primary BP metabolites, and especially the diols, can be oxidized a second time by CYP1A. This results in the formation of diol-epoxides. The metabolite BP-7,8-diol-9,10-epoxide (BPDE) is generally seen as the most potent carcinogen. It is highly
reactive and easily binds to cellular macromolecules like proteins, and DNA (Gelboin & Tso, 1978). The formation of DNA adducts on the promotor sites of oncogenes may induce cellular transformation and unrestricted cell proliferation. This will cause neoplastic lesions and may ultimately evolve into actual tumors. Benzo(a)pyrene-diol-epoxides can be further oxidized to form multiple hydroxylated derivatives like triols and tetrols.

A separate group of metabolites, the BP-quinones, can also be formed during the biotransformation of BP. BP can be oxidized by cytochrome-P450 to 6-hydroxy-BP. This 6-OH-BP can then be oxidized to form three different quinone metabolites (BP-1,6-, 3,6-, and 6,12-quinone) (Gelboin, 1980). BP-quinones can be reduced by quinone reductase to an excretable, water soluble conjugate with glucuronic acid or glutathione (Lind et al, 1978; Morgenstern et al., 1981). BP-quinones have been shown to be highly toxic to cultured mammalian cells (Lorentzen et al., 1979; Kawabata and White, 1989). Zhu et al. (1995) exposed primary cultured bone marrow stromal cells from mice to BP, BP-7,8-diol, and 4 of the BP-quinones to investigate the acute toxicity of these compounds. BP and BP-7,8-diol were not toxic to cultured stromal cells. BP-1,6-quinone was the most toxic of the 4 quinones tested; at 20 μM, it caused 30% cell death in 24 h, and up to 90% in 72 h. Mitochondrial function (cellular energy supply) was seriously affected by BP-1,6-quinone, as was shown by the rapid decrease in ATP concentration and structural changes in the mitochondria as seen by electron microscopy. The other BP-quinones also affected ATP levels, though to a lesser extend.

2) Prostaglandin synthetase

Prostaglandin synthetase is known as a potential BP metabolizing enzyme (Marnett et al., 1977; Sivarajah et al., 1978; Kawabata & White, 1989; Smith & Brian, 1991). Arachidonic acid is the normal substrate for prostaglandin synthetase to form prostaglandin, an important hormone. Other compounds with a similar structure, like BP, can slip into this pathway and be co-oxidized. This was tested by Kawabata and White (1989) by incubating splenic microsomes of untreated mice with BP and arachidonic acid, without NADPH to stop any P450 activity (NADPH is an obligate cofactor for P450
activity). Only BP-quinones were formed, in a dose related manner. Phenolic and dihydrodiol metabolites of BP were not detected. The major quinone metabolite formed was 6,12-dione, which, in a separate dosing experiment, was shown to have a direct cytotoxic effect. Prostaglandin synthetase not only mediates the formation of quinones, but also causes BP-7,8-dihydrodiol to be further metabolized to BP-7,8-dihydrodiol-9,10-epoxide (Marrett et al., 1977; Sivarajah et al., 1979).

3) Lipid peroxidation

A third route has been demonstrated for the co-oxidation of BP-7,8-diol to BP-diol-epoxides in the ascorbate- or NADPH-dependent lipid peroxidation pathways in rat liver microsomes (Dix & Marnett, 1983; Colin et al., 1991). Briefly; under favorable conditions unsaturated fatty acid hydroperoxides can be formed by autooxidation. Microsomes can then be triggered to decompose these lipid peroxides by adding metal ion complexes (Fe$^{3+}$-ADP or Fe$^{2+}$-EDTA) and ascorbate or reduced NADPH. When BP-7,8-diol was added to the reaction mixture, it was transformed to BP-diol-epoxides, both in the ascorbate- and NADPH-dependent lipid peroxidation pathway. In addition, Dix & Marnett (1983) were able to separate the contribution of the competing P450-dependent and peroxide-metal-dependent pathways in the formation of BP-diol-epoxides by analyzing the stereo chemistry of the metabolites. With no added metal complexes, no enantiomers from the NADPH-dependent lipid peroxidation pathway were formed, only P450 generated epoxides. Addition of iron complexes dramatically increased the amount of lipid peroxidation and the formation of BP-tetrols. The ratio of anti/syn enantiomers of the BP-diol-epoxides matched the predicted ratio for co-oxidation in lipid peroxidation. These data provide evidence that BP-7,8-diol is epoxidized during NADPH- and ascorbate-dependent lipid peroxidation in rat liver microsomes.
Toxic effects of cadmium

Cadmium is one of the non-essential heavy metals. Because of its close similarity to Zn, which is an essential metal for most organisms, it easily interferes with the Zn homeostasis. Toxic effects have been reported as bone disease, kidney damage, liver toxicity and genotoxicity. Though the symptoms of Cd intoxication are well known, the mechanisms by which Cd exerts toxic action remain unknown (Shopis, 1994, Stohs & Bagghi, 1995). Cd has also been implicated in the formation of DNA-adducts (Frenkel, 1992). In mammals most Cd is stored in the liver during chronic exposure. The liver has a high capacity to synthesize a metal binding protein, named metallothionein (MT), which can trap Cd efficiently, rendering the Cd non-toxic to the liver. Most of the MT-bound Cd is stored in the liver, and only slowly leaks into the bile, or back into the blood stream (Klaassen & Liu, 1997).

Metallothioneins

Metallothioneins (MT) are metal binding proteins that are involved in regulation of essential metals such as Zn and Cu, and in detoxification of nonessential metals such as Cd and Hg. MT was first described in the late 50's from horse kidneys, and have since then been described from other animals, plants, fungi, protists and prokaryotes (Roesijadi, 1992). Only in 1974, metallothioneins were described from marine fish. The structural characterization of MTs has been subject to great difficulties, which has led to the description of considerable differences between species. Reexamination has cleared up some of the reported differences. MTs have the following characteristics: they are low molecular weight (6000 -10,000 D) proteins that are high in metal and cysteine content, have no aromatic acids or histidine, have a unique amino acid sequence and have metallothiolate clusters. Metal-specific MT proteins have been reported for different metals. The specifics of the metal binding process have yet to be reported (Roesijadi, 1992).

Since MT plays a central role in the regulation of essential metals, there is always a pool of MT available in liver cells. When Cd enters a cell it can be bound by this pool of
MT. Because of its close similarity to Zn, and higher affinity for the MT, it is thought that Cd replaces Zn on the Zn-MT complex. Abnormal metal exposure leads to enhanced MT production. Increased metal exposure activates a receptor, this metal-receptor complex binds to a MT gene, mRNA is formed which finally results in increased MT synthesis. MTs are predominantly located in the cytosol. Exposure to a low metal dose has been shown to give future protection against higher doses. Zn in existing Zn-MT can be replaced by Cd when Cd becomes available (Roesijadi, 1992).

**Cadmium and nephrotoxicity**

After formation of a Cd-MT complex, this complex may be slowly released from the liver into the bloodstream and distributed throughout the body. Most of the Cd-MT is taken up by the kidney. Thus the Cd-MT complex plays a crucial role in the transport of Cd to the kidney. Because Cd-MT has a low molecular weight it is easily reabsorbed in the proximal tubuli of the kidney. Chronic exposure to ionic Cd leads to a slow accumulation of Cd in the renal cells and a delay in the onset of toxic effects. Injection of the Cd-MT complex leads to a rapid and selective accumulation of Cd in the kidney, and to a nephrotoxic effect at a much lower dose than when inorganic Cd is administered (Liu et al, 1994; Dorian et al, 1995).

Through lysosomal enzymes the Cd-MT complex is rapidly degraded, and Cd becomes available in the tubular cells. The kidney is able to synthesize MT, but it is thought that this capability is limited so that at a certain level the Cd becomes available as free ions. What causes the actual nephrotoxic effects of Cd is yet unknown. Two hypotheses are currently considered for the occurrence of nephrotoxic effects: A) reabsorption of the Cd-MT complex in the proximal tubule causes the observed membrane damage, B) release of the unbound Cd in the cells causes the nephrotoxic effect (Liu et al, 1994; Dorian et al, 1995).

Dorian et al. (1995) studied the effect of Cd-MT and CdCl₂ injections on renal function in mice to address these questions. Injection of Cd-MT resulted in increased
excretion of glucose and protein, while even 10 times higher doses of CdCl₂ caused no effect. Accumulation of Cd-MT was almost exclusively in the kidney, while inorganic Cd accumulated predominantly in the liver. CdCl₂ was distributed throughout the kidney tissue, while Cd-MT was exclusively found in the cortex and outer medulla, indicating a differentiated bioavailability for both forms.

Within the different segments of the proximal tubule, Cd-MT was found predominantly in two distinct segments. Cd from CdCl₂ was distributed evenly over all the segments. Within the individual cells of the proximal tubulus, the Cd-MT was found mostly in the apical region, indicating an active endocytosis process. Cd from CdCl₂ was distributed more evenly throughout the cell, indicating passive diffusion. Still, it is not clear why Cd-MT is more toxic than Cd from CdCl₂, as it is obviously not a function of the ionic Cd concentration in the cells. Histopathological effects of chronic exposure to low Cd doses in mammals lead to enlarged, pitted and pale kidneys. Tissue damage is reflected in tubular cell necrosis, interstitial fibrosis, and tubular atrophy.

Accumulation and toxicity of Cd in fish

In fish, substantial accumulation of Cd has only been found in liver and kidney: 10-300 mg/kg tissue (McLeese et al, 1987). Though Cd is generally seen as a highly hazardous compound, acute LC₅₀s show a range from 13 to 60 mg/L for juvenile and adult marine fish. These concentrations are never measured in field situations, not even on the worst polluted sites. Enzyme activities are also only affected at very high concentrations. At more environmentally realistic concentrations (0.05 - 0.5 mg/L) it was shown that Cd has a marked effect on the ion balance in blood plasma of flounder. Na and Cl levels did not change, indicating that osmoregulation was unaffected (McLeese et al, 1987). However, phosphate and Mg levels were elevated, and K and Ca levels were decreased. In mammals this would indicate that renal regulation of blood ion composition was impaired by Cd. For fish, it may be a sign of the impairment of mechanisms responsible for Ca influx. The same blood ion imbalance was observed in Cd exposed rainbow trout. Both flounder and trout showed hypersensitivity, erratic swimming and tetanic contractions.
(McLeese et al, 1987). Given the role of Ca in regulating neuromuscular function, it is assumed that the observed hypocalcemia was contributing to the hyperexcitability.

A histopathological study of Cd effects on kidney tissue in fish was done by Forlin et al. (1986). They found tissue damage in Cd exposed rainbow trout, comparable to mammalian renal damage. Cd exposure resulted in the following changes in the proximal tubules: intracellular granules, inclusion bodies, dilated lumen, deformed mitochondria, apical vacuoles, and intensified lysosomal activity. The phenomena are interpreted as early stages of cell necrosis. The authors argue that this may support the hypothesis that the observed hypocalcemia in Cd exposed fish results from reduced tubular reabsorption of Ca due to kidney damage.

Apart from kidney histopathology, Forlin et al. (1986) also studied the morphological effects of Cd exposure on hepatocytes. They observed hypertrophied Golgi complexes, a deformed endoplasmic reticulum, affected mitochondria, glycogen depletion, inclusion bodies, and increased fibrosis. These phenomena are indicative for accelerated glycolysis, resulting in glycogen depletion, which was also observed in other studies with Cd exposed fish (Larsson & Haux, 1982).

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Interactions between Cd and BP

As is shown in the previous sections a substantial amount of information is available on the toxicity of BP and Cd. In contrast, relatively little is known about interactive effects of the compounds. The study of the interaction of Cd on BP metabolism started in the medical field, because P450 enzymes were initially described as “drug metabolizing enzymes”. This triggered a series of investigations into potential inhibitors of these drug metabolizing enzymes in rats and mice.

**Mammals**

The first studies dealing with interactions of cadmium and BP relate to the effects of Cd on drug metabolizing enzymes in mice and rats (Unger & Clausen, 1973; Ando, 1979). Ichikawa & Yamano (1967) were the first to demonstrate that Cd caused a shift in the spectrophotometric absorbance maximum of cytochrome P450 from 450 nm to 420 nm in rat liver microsomes. Ando (1982) mentions the same change of P450 to P420 at a dose of 2.4 mg Cd/kg in rats.

Schnell et al. (1979) report a threshold dose of 0.84 mg/kg i.p. injected Cd in rats for exhibiting significant decreases in hepatic microsomal metabolism of P450 substrates. The inhibiting effect they found lasted for up to 28 days. It was concluded that Cd reduced the total P450 amount, because the NADPH-cytochrome-C-reductase activity was not affected. Interestingly they found that when the Cd was dosed chronically through the drinking water there was no inhibitory effect. Also, pretreatment (orally and i.p.) with sub-threshold level doses of Cd did not cause inhibition. They concluded that induced metallothioneins are the underlying basis for the tolerance.

One cause for the reduction of the total amount of P450 after Cd exposure may be the induction of heme oxygenase activity, resulting in a decrease in heme available for insertion into the P450 apoenzyme to form the active holoenzyme, as seen in rats (Maines & Kappas, 1977; Means et al., 1979).

Another hypothesis for the inhibition of P450 is that Cd binds directly to
nucleophilic sites on the P450, thus inhibiting the enzyme activity (Jefcoate & Gaylor, 1969). It has been suggested that this nucleophilic site would be a sulfhydryl group on cysteine in the P450 enzyme, though Means et al. (1979) argue that it is unlikely that in vivo there is direct binding of Cd to the P450. They agree that in studies where Cd is added to isolated microsomes, there is a direct, Cd dose-related, inhibition of the P450. But when Cd is dosed in vivo to rats, its maximum concentration in the liver is already achieved after one hour, whereas impairment of the P450 activity takes at least 9 h. It was also found that the concentration needed to inhibit P450 on isolated rat microsomes was 100 times larger than the Cd concentration in microsomes that showed inhibition after in vivo administration. Taken together, these observations imply an indirect effect of Cd on P450 when mammals are exposed in vivo.

Fish

Fair (1986) described BP-Cd interaction experiments with Black Sea Bass (Centropristis striata). Fish were injected i.p. for two days with 0.075, 0.75, or 7.5 mg/kg BP alone or with 1.4 mg/kg Cd. In addition there were two treatments with a low (0.42 mg/kg) and high (2.5 mg/kg) Cd pretreatment 3-4 days before the BP dose. Livers were sampled on day 3 for analysis of BP hydroxylase and GST activity. Cd had an inhibitory effect on BP hydroxylase activity at 0.75 mg/kg, but not at the higher or lower doses. At the lower BP doses, co-administration of Cd also had an inhibiting effect on GST activity, while at the highest BP dose, this effect was not demonstrable. Pretreatment of the fish with Cd did not change the hydroxylase and GST activities for the highest Cd pretreatment dose. For the lower Cd dose, the hydroxylase activity was significantly reduced in the combined BP-Cd follow-up dosage, while GST activity was significantly increased in this treatment. The observations were explained by assuming that when BP and Cd are administered together the Cd inhibits the hydroxylase activity, but stimulates the GST activity. When the fish were pretreated with Cd, the Cd binding metallothioneins were induced. Subsequent combined dosage of BP and Cd resulted in sequestering the Cd by MT, which did not result is a difference in the BP transforming and conjugating enzyme.
activities when compared to the BP alone treatment.

In a subsequent study, Fair & Fortner (1987) dosed Black Sea Bass with 0.2 \( \mu g/g \) BP and/or 10 \( \mu g/g \) Cd on a daily basis for 10 days through the food. Tissue accumulation of BP and "BP hydroxylase" activity were not affected by the presence of Cd. On the other hand there was significantly more Cd accumulation in the combined treatment than in the Cd alone treatment. That Cd, unexpectedly, did not inhibit accumulation and hydroxylase activity is attributed by the authors to the low Cd dose.

George & Young (1986) studied the time related interactive effects of 3-methylcholanthrene (3MC, an artificial P450 inducer) and Cd in plaice (Pleuronectes platessa). The compounds were administered by i.p. injections of 10 mg/kg 3MC and/or 1 mg/kg Cd. In the 3MC alone treatment, EROD activity doubled in 24 h, and reached a maximum of 15x control value, 10 days after the injections. EROD activity dropped back to double the control value 14 days post-injection. Cd abolished EROD activity immediately, to 10% of control at day 2 in combined 3MC-Cd. MT induction was delayed from 4 days in the Cd alone treatment to 10 days in combined treatment. This suggests that not only has Cd an inhibitory effect on P450, but vice versa, an activated P450 system has an inhibiting effect on MT induction.

Cadmium dose-dependency for P450 inhibition was demonstrated by George (1989). He showed that EROD activity in flounder was reduced in a dose-dependent manner upon Cd injection (0.01 - 1 mg/kg). Immunoblot analysis showed dose-dependent reduction of total P450 content. In contrast to the study of Fair (1986) there was no effect at all on GST activity at any Cd dose.

Forlin et al. (1986) exposed rainbow trout to cadmium using i.p. injections (0.5 mg/kg of CdCl\(_2\)-2.5 H\(_2\)O) and aqueous exposure at 10 and 100 \( \mu g \) Cd/l for 4 weeks. After four days, liver and kidney of the injected fish showed reduced P450 activity on ECOD, EROD and EMND, three substrates that cover a broad range of monooxygenase activities representing different P450 isozymes (resp. CYP2B, CYP1A and CYP3B). There was a trend, though not statistically significant, towards lower total amount of P450 in the Cd treated fish, compared to the control. When Cd was added in micromolar amounts to the
in vitro enzyme incubations, it strongly inhibited both Phase I and Phase II reactions. The Phase I activity was 100 times more sensitive than the Phase II. The authors suggest that Cd binds directly to nucleophilic sites on the P450, possibly sulphhydryl (SH) groups, thus inhibiting the enzyme activity. After 4 weeks of aqueous exposure the total amount of P450 was significantly increased in liver microsomes, but ECOD activity was unchanged (though slightly increased in the kidney). UDPGT activity was decreased, while GST activity was increased.

Lemaire-Gony & Lemaire (1992) studied Cd and BP interactive effects in European eel (Anguilla anguilla). Fish were held in seawater with 5µg Cd/l for 25 days. Half the fish received a BP i.p. injection of 20 mg/kg after the Cd exposure, and were sampled 24 hours later. Hepatic tissue structure showed complete disorganization and nuclear degeneration of the hepatocytes in all Cd exposed fish. Total P450 content was not increased in the Cd alone and BP alone treatments, but doubled in the combined treatment. EROD activity was not increased in the Cd alone treatments, but more than 10 fold increased in the BP treatment, and almost 20 fold increased in the combined exposure. GST activity was not influenced by Cd alone, but slightly decreased in the combined treatment.

Lemaire-Gony et al. (1995) did a comparable study with European sea bass (Dicentrarchus labrax). Fish were exposed to Cd through seawater (40 µg Cd/l) for 15 days, and were then injected i.p. with BP (20 mg/kg). After 14 h the following assays were performed: macrophage phagocytosis assay, respiratory burst assay, gill Na/K-ATPase assay and EROD assay. The immunotoxicity assays showed a synergistic effect in the combined treatment, the Na/K-ATPase assay showed no effect in any of the treatments, and EROD activity was significantly increased in all treatments, but most in the combined treatment.
Synthesis, conclusions and preliminary hypotheses.

From the studies described above it is clear that Cd can interfere with the biotransformation of BP. Various endpoints have been measured by different researchers to show the inhibition of the metabolic pathway. The extent of the interaction is dependent on a variety of parameters: species, dose, exposure time, pre-exposure history, and mode of exposure (i.p., dietary, aqueous). Though most of these parameters were different in the cited studies, a few conclusions emerge that would support the following model for the interactive effects: In most studies Cd was administered at the same time as BP in a single dose. At low Cd doses, all the Cd is bound by the available pool of MT and other Cd binding agents. When the metal binding capacity of the cell/organism is exceeded, the Cd starts to have a negative effect on BP metabolism. This effect may be a direct effect, involving a direct intervention with the catalytic activity of CYP1A. A direct effect can be caused by binding to the active site, disruption of the heme group on the active site, or binding on other places on the enzyme, disturbing the tertiary structure of the enzyme and thus influencing the affinity for the substrate.

Cadmium may also cause indirect effects on BP metabolism, in the form of a disturbance of the induction or production of new enzyme. This would involve an effect on one or more of the steps in the CYP1A induction cycle: affinity of the Ah receptor, DNA-mRNA transcription, P450 protein synthesis, and the basic cellular energy supply, as located in the mitochondria. Inhibition of BP biotransformation occurs predominantly on the phase I enzymes; the phase II conjugating enzymes do not seem to be affected. However, even though the conjugating enzyme activity may not be affected, there will be competition for glutathione by the BP metabolites and Cd. Glutathione is a general anti-oxidizing compound, that also has affinity for Cd (Viarengo, 1989). Depletion of glutathione may lead to toxic effects from either Cd, or the reactive BP metabolites.

It appears that the induction of MT may take several days, while induction of P450 can be measured on a time scale of hours (George & Young, 1986). This would explain why Cd effects are more moderate in combined Cd-BP treatments than in single Cd
treatments: as long as MT is not fully induced, induced P450 may act as a temporary (or permanent) Cd scavenger. Exposure of fish to Cd, by either injection or aqueous exposure, will induce MT production, which will subsequently bind and immobilize Cd. Pre-exposed animals or cells show no, or reduced inhibiting effects of Cd on BP metabolism. Interestingly, the results of Lemaire-Gony et al. (1992, 1995) show that prolonged exposure to Cd results in synergistic effects on BP metabolism. This would mean that the acute effect of Cd exposure is inhibition, but the chronic effect is stimulation of BP metabolism. A possibility could be that the Cd-MT complex, which is formed in chronic exposure, stimulates the AH receptor and induces P450 production. Another aspect of the combined dosage of Cd and BP may be that the highly reactive BP metabolites that are released from the microsomes into the cytosol may bind to the induced MT, thus rendering this MT incapable of binding Cd.
Goal and objectives

The overall goal of this study was to determine the influence of Cd on BP metabolism, and the influence of BP exposure on Cd toxicity in mummichog. The study was subdivided in three parts:

1) Interactive effects of Cd and BP on acute lethal toxicity. To study sublethal cellular and molecular effects, appropriate dose levels had to be established for both compounds. Investigating dose combinations up to lethal levels also gave the opportunity to study interactive effects with mortality as an endpoint.

2) Interactive effects of Cd on BP metabolism. The influence of Cd on BP metabolism was studied on three levels of organization: in the entire organism, in isolated liver cells (hepatocytes) and in microsomes. The hierarchical approach gave the opportunity to separate different processes in the total biotransformation pathway of BP. The influence of Cd on CYP1A concentrations and activity was measured in liver microsomes (the cell fraction containing the CYP1A enzyme). Measurement of BP metabolism in isolated hepatocytes was performed to collect information on the relative importance of non-P450 metabolic pathways. In the entire fish the influence of Cd on the total rate of excretion and the metabolite spectrum was studied by analyzing bile samples. For all levels of organization the influence of time after a single dosage was part of the investigation.

3) The role of metallothioneins. The induction of MT was measured in Cd exposed fish, together with the influence of simultaneous BP dosage on MT induction. In addition the potential binding of BP metabolites to MT, which may inhibit detoxification of Cd, was studied.
General Methods & Materials.

Some aspects of the experiments described in the following chapters were kept constant across all experiments. These aspects are described here and are not repeated elsewhere.

Fish and exposure system

For all experiments mummichog were selected in the size range of 65 to 85 mm (4 - 8 g wet weight). Fish at this size are in their second growing season (Kneib & Stiven, 1978). To avoid potential interference of processes involved with female oocyte production on BP metabolism, only males were used for the experiments in which BP metabolism was measured. The animals were collected from the bridge connecting Carmine’s Island (York River, VA.) with the main land. Sediments collected from this site have been analyzed for PAHs, and were shown not to have elevated contaminant levels (unpublished results). Prior to the experiments, the fish were acclimated to the laboratory environment for at least two weeks. During this period they were treated against exoparasites with a 1 h. fresh water dip upon arrival in the laboratory, followed by a 1 h. diluted formaldehyde treatment (200 mg/l), which was repeated one week later if necessary. Speare & MacNair (1996) have demonstrated that twice weekly exposure of juvenile rainbow trout to 200 mg/l formalin in a static bath did not affect growth rate, appetite, feed conversion or body condition index. This exposure is recommended as disease prophylaxis.

On day 0 of the experiment the fish were injected intraperitoneally, just posteroventrally of the pectoral fin. The fish were anaesthetized in 200 mg/l tricaine methanesulfonate (MS-222) before injection. Standard length, total length and wet weight were recorded for each fish. The injection site was cleaned with 70% ethanol just before injection. Injections consisted of 2 x 25µl containing a selected dose of Cd and/or BP or the solvent (as controls). Cd was dissolved in a saline solution (teleost Ringer's), BP was dissolved in corn oil. For the experiments in which metabolism in entire fish was studied,
animals were injected with $^3$H-BP. Radiolabeled metabolites were analyzed in bile samples. Both “cold” and “hot” BP were purified before use, either by cleanup over solid phase extraction columns, or by HPLC.

Animals were generally kept in 200 l tanks, one tank per treatment, under flow through conditions (> 0.5 l/min). One experiment was done in a closed, recirculation system because fish were injected with radiolabeled BP, and the excreted metabolites could not be recovered before disposal of the effluent. Water temperature was maintained at 20 (±2)°C. Water quality parameters (temperature, salinity, dissolved oxygen and pH) and flow rate were measured on a daily basis. The fish were fed daily with TetraMarin at a rate of 4 % of the body dry weight (Fisher, 1985).

Experimental set-up

The experiments in which hepatocytes and/or microsomes were harvested from injected fish, typically consisted of the following treatments: saline+oil injected control fish, BP alone, Cd alone, BP plus Cd, Cd 4 days before BP. Results from one of the first experiments showed that there was no difference in P450 amount and activity between saline control, oil control and saline+oil control. For that reason only a saline+oil control was used for the following experiments. Fish were generally sampled over a time period of 14 days. For microsomal studies samples were taken on days 2, 3, 4 and 7 post-injection, and for other experiments samples were taken 3, 7 and 14 days post-injection. Efforts were made to sample 4 or 5 replicate fish per treatment per sample day, but for some experiments, unexpected mortality reduced the number of fish available, forcing reduction of sample size to 3, or only 2 in one case. The methods used for measuring BP metabolism by isolated hepatocytes and microsomes are described in the respective chapters.

Extraction and analysis of BP metabolites and MT

Non-polar (non-conjugated) metabolites were extracted from cell cultures and microsomal incubations with ethyl acetate. BHT (0.01M) was added to the organic solvent as an anti-oxidant to prevent auto-oxidation of the BP metabolites. The aqueous
fraction was kept for analysis of polar (conjugated) metabolites. The organic fraction was blown to dryness and reconstituted in methanol for HPLC analysis (Kawabata & White, 1989). Bile samples were not extracted, but analyzed directly after dissolution in methanol (James et al., 1991). Non-conjugated BP metabolites were analyzed by reversed phase HPLC with a UV detector, set at 254 nm, and a flow cell radio detector. Total conjugated metabolites were quantified by scintillation counter. Metallothioneins were analyzed by gel filtration HPLC after heat denaturation of hepatic cytosol (Jin et al, 1993).

**Statistical analysis**

The mortality experiments described in Chapter 2 required a special approach to analyze for statistically significant differences between treatments, which is described there. All other experiments consisted of a set of treatments (e.g. control, BP, Cd, BP+Cd), sampled at intervals over a certain time period. Endpoints consisted of BP breakdown, BP metabolite production, and MT production. Significant differences for the endpoints were analyzed between treatments on a certain day. The time factor was used as a blocking factor. Differences between treatments on a given day were analyzed with ANOVA, followed by a multiple comparisons test (Tukey’s HSD). Homogeneity of variance was tested with Bartlett’s test, and when necessary log transformation was applied before using ANOVA (Zar, 1984).

Practical limitations did not allow sampling from a fully randomized, fully independent time series for the experiments. Therefore treatments were kept in separated tanks, but the time series was sampled from the same tank each time. This may trigger the question whether there was true independence between different sampling times within a treatment. However, the actual treatment (the injected dose) was given before the fish were put into the tanks, which means that the individual fish is the statistical unit for consideration in these experiments, not the population of fish in each tank. Pseudoreplication was therefore not an issue in these experiments (Hurlbert, 1984).
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Chapter 2. Interactive Effects of Cd and BP on Acute Mortality^1

Introduction

As a model to study the mechanisms underlying toxicant interactions, a metal (cadmium) and a polycyclic aromatic hydrocarbon (benzo[a]pyrene) pair was selected to challenge a common estuarine fish species (*Fundulus heteroclitus*). Benzo[a]pyrene (BP) and cadmium (Cd) are two potentially harmful pollutants that often co-occur in contaminated aquatic environments. Both compounds are known to induce physiological changes in fishes and other organisms (Varanasi et al., 1989; Roesijadi, 1992). BP induces the production of a metabolizing enzyme (CYP1A), Cd exposure enhances the production of metal-immobilizing proteins (glutathione, metallothioneins). Though the reactions of organisms to exposure to the individual compounds has been well studied, effects of exposure to a combination of BP and Cd is less well-known. Interactive effects have been demonstrated in fish at cellular and sub-cellular levels, showing effects of Cd on BP metabolism (Fair, 1986; Lemaire-Gony et al., 1995; Bruschweiler et al., 1996; Sandvik et al., 1997). Effects of co-administration on mortality have not been reported.

The overall goal of this study was to investigate the mechanisms of interactive effects of Cd and BP at cellular and sub-cellular levels over a time frame of several weeks. To study these effects, fish would have to be dosed with amounts low enough to keep them alive over this period, and high enough to see any effects. Because no information was available on the acute lethal levels of Cd and BP after i.p. injection, these effect doses had to be established. Challenging the fish with different dose combinations would also

give the opportunity to select combinations that would result in clear interactive effects.

The objectives for the experiments described in this chapter were first to establish dose-effect relationships, with mortality as endpoint, for individual doses of BP and Cd in mummichog (*Fundulus heteroclitus*). Secondly, to investigate at which doses interactive mortality effects of co-exposure of BP and Cd would occur in the sublethal- lethal dose range. The results of the mortality experiments would be used to select those dose combinations that showed interactive effects for subsequent studies investigating interactive effects of Cd on hepatocytic and microsomal BP metabolism.
Material and Methods

Experimental design

Four experiments were performed for this study, following a sequence of range finding and definitive tests (Parrish, 1985). The first two range finding tests were done to find the concentration range in which the 14-day LD50 for Cd and BP alone would lie. The third experiment was a range finding test for interactive effects when Cd and BP were dosed together in different combinations, and the fourth, final experiment was designed to determine definitively the actual dose combinations at which interactive effects can be seen.

Test animals

One year old mummichog were collected with baited traps from Carmine's Island, York River, VA. The fish were selected in the size range of 65-85 mm, these fish had an average weight of 6.2 ±1.3 g. The fish were acclimated to the laboratory environment and treated for ectoparasites two weeks before the start of the experiment, as described previously.

Experimental set up

The fish were injected intraperitoneally (i.p.) after being anaesthetized in 200 mg/l MS-222. In the first two experiments the test compounds were dosed in 50 µl aliquots. For the combined Cd and BP treatments in the last two experiments the compounds were dosed in two separate injections of 25 µl each per fish. Cd was dissolved in teleost Ringer's saline solution, BP was dissolved in corn oil. Ten fish were exposed per treatment for all experiments. Fish were kept in 45 l seawater tanks, one tank per treatment, under flow through conditions (0.5 l/min). The fish were fed daily with dried fish food at a rate of 4 % of body dry weight. Water quality parameters and mortality were monitored on a daily basis for 14 days.
Data handling and statistics

LD$_{50}$ values with 95% Confidence Intervals (C.I.) were calculated for Cd and BP using the trimmed Spearman Karber method, which is judged as a better method than the traditional logit and probit methods (Hamilton et al., 1977). Based on the LD$_{50}$ values, doses of the last experiment were expressed in Toxic Units (=dose/LD$_{50}$) for each compound. This generates a unitless variable for a toxicant that expresses the contribution of the dose to the observed effect. For each combination treatment, contributing Toxic Units for both compounds were added to obtain Combined Toxic Units (Marking, 1977). If linear additivity would exists for both compounds, then the mortality would be 50% when the Combined Toxic Units would equal one. Combined Toxic Units lower than one would result in lower than 50% mortality, Combined Toxic Units higher than one would produce more than 50% mortality. The relation between Combined Toxic Units and mortality was modeled by probit analysis, which is basically a transformation followed by a nonlinear regression. Probit analysis was used for this data set because the calculating routine in SAS also generates a 95% confidence interval around the regression sigmoid. Treatments that fell outside this 95% confidence interval were considered significantly different from the linear additivity model, and represent synergistic or antagonistic effects.
Results

Mortality

The mortality data for the LD$_{50}$ range finding experiment for Cd show that Cd doses of 240 and 2400 mg/kg were lethal instantaneously: the injected fish did not even survive the anaesthesia (Table 2-1). At 24 mg/kg a 100% mortality was achieved within 24 h post-injection. For BP it took 6 days to obtain 50% mortality in the highest dose of 177 mg/kg (Table 2-2). The 14 day LD$_{50}$ for Cd was calculated to be 0.23 mg/kg (95% C.I.: 0.14 - 0.37) and for BP 14.58 mg/kg (95% C.I. 8.52 - 24.95).

The calculated LD$_{50}$ s for Cd and BP were used to design the third experiment, in which fish were dosed with combinations of both compounds. The dose ranges for this experiment were supposed to bracket the calculated LD$_{50}$ s. The results of this experiment showed that the selected dose ranges were too low to produce 100% mortality in the highest dose combinations (Table 2-3), which is one of the criteria for an acceptable dose-response test (Parrish, 1985).

For the final interaction experiment, higher doses were used for both compounds in an effort to meet the 100% mortality criterium for the highest doses. The results show that for this experiment the criterion was met (Table 2-4). The calculated 14 day LD$_{50}$ value for BP was 35.7 mg/kg (95% C.I.: 29.3 - 43.6), the 14 day LD$_{50}$ value for Cd was 6.5 mg/kg (95% C.I.: 4.4 - 9.5). The data from this experiment show that there are bimodal response trends for several dose ranges. For BP doses of 18, 32 and 56 mg/kg the pattern shows that a concomitant dose of Cd causes a decrease in toxicity after the initial high mortality. Though less pronounced, the same pattern shows for Cd doses below 10 mg/kg when combined with BP.
Table 2-1. Cumulative mortality for cadmium over 14 day period. N=10 fish per treatment, Cd concentration in mg/kg.

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37
Table 2-2. Cumulative mortality for benzo[a]pyrene over 14 day period. N=10 fish per treatment, BP concentration in mg/kg.

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<tr>
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Table 2-3. Mortality (%) of mummichog 14 days after i.p. injection of Cd, BP or combinations of both (N = 10 per treatment, dose in mg/kg).

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<th>Cd- BP†</th>
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Table 2-4. Mortality (%) of mummichog 14 days after i.p. injection of Cd, BP or combinations of both (n = 10 per treatment, dose in mg/kg). Light shade indicates lower mortality than expected, dark shade is higher mortality than expected.

<table>
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<tr>
<th>Cd- BP†</th>
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<th>0.32</th>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>60</td>
<td>60</td>
<td>90</td>
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</table>

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A scatterplot of mortality as a function of Combined Toxic Units suggests that the probit model might be used to analyze the data (Figure 2-1). Probit analysis was performed to describe the expected dose-response curve for linear additivity of both compounds, together with its 95% C.I. Data points outside this 95% C.I. were identified as having higher mortality than expected (synergistic effect), or lower mortality than expected (antagonistic effect). The dose combinations that had a lower or a higher mortality than expected, turn out to be grouped together (Table 2-4). It appears that for higher BP doses a simultaneous low Cd dose had an antagonistic effect: co-administration of Cd reduced the toxicity of BP below expected. On the other hand, at higher Cd doses a simultaneous dose of BP caused a synergistic effect: the combination was more toxic than expected.

Behavioral and pathologic observations

Fish dosed with high concentrations of BP showed clear signs of distress after several days. They became lethargic, refused food, and did not respond to outside stimulation. Other pathologic signs were dark coloration, swelling of the peritoneal cavity, anal excretion of white mucous-like “feces”, and internal hemorrhaging around the snout. Some fish recovered from these signs, but for most it was indicative for later mortality. For the Cd dosed fish no specific clinical signs were observed.
Figure 2-1. Dose-response model for Combined Toxic Units versus percent Mortality, based on probit analysis. Dotted lines indicate 95% confidence interval.
Discussion

BP toxicity

Acute toxicity data for BP have to be treated with caution. It is generally thought that toxicity is not caused by the parent compound itself, but by the metabolites which are formed during biotransformation (Gelboin & Tso, 1978). Toxicity of the parent compound will thus be dependent on the activity of the metabolizing pathways. Acute BP toxicity might also be caused by phototoxicity under the influence of UV light (Arfsten et al, 1996). However, limited UV radiation in the laboratory environment makes it unlikely that phototoxicity contributed substantially to the observed mortality (Di Giulio, pers. com.).

The observed behavioral and morphological changes in the BP dosed fish may give an indication for the mode of action of BP in mummichog. Drummond & Russom (1990) exposed juvenile fathead minnows to a large number of toxicants from a variety of chemical classes, and described behavioral changes in the fish. The observed behavioral changes were classified into three stress syndromes: hypoactivity syndrome (Hypo-AS), hyperactivity syndrome (Hyper-AS), and physical deformity syndrome (Physical-DS). The Hypo-AS is indicative of exposure to narcosis, or narcosis-like producing chemicals that depress central and peripheral nervous system activity, as opposed to the Hyper-AS, which is induced by chemicals that disrupt metabolic activity or function. Physical-DS is indicative of neurotoxic chemicals. For all syndromes there is mortality within 24-96 h.

The observations on mummichog match most with the Physical-DS (depressed locomotor activity, increase in rate and amplitude of opercular movements, darker body coloration, hemorrhage), though the severe abdominal edema is typical of Hyper-AS. These signs are remarkable because the physical characteristics of BP (relatively large, very lipophilic) would suggest a nonpolar narcosis-like mode of action. Schultz (1989) describes nonpolar narcosis as a physical change owing to the migration of the agent into the cellular membranes. Narcosis is therefore correlated with lipophilicity, and the relative effect depends primarily on the quantity of agent absorbed. Narcotics do not produce a chemical change in the membranes, and the phenomenon is rapidly and completely
reversible. Tricaine methanesulfonate (MS-222) is one of the model compounds for narcosis. Another argument against Physical-DS may be that BP exposure did not cause changes in neurotransmitter levels in channel catfish (Fingerman & Short, 1983). Combining the observations on BP injected mummichog with the information from the references cited above, it is unlikely that BP caused an acute narcosis effect at the doses used for this study. The behavioral effects are more likely caused by the metabolites that are formed during BP metabolism, and do not clearly match one of the described stress syndromes.

Cd toxicity

The EPA-Aquire data set contains about 20 references with toxicity data for Cd in mummichog. However, all derived toxicity values were based on aqueous exposure, which hampers comparison with the LD<sub>50</sub> values derived from i.p. injections in this study. Intraperitoneal injection of Cd is an often used technique to study induction of metal binding proteins (metallothioneins) and other physiological responses to heavy metal exposures (Hamilton et al., 1987).

Toxicity and tissue distribution of Cd as a function of exposure method have been described for sticklebacks and carp. Sticklebacks dosed i.p. with 5 mg/kg Cd retained about 60% of the dose till 60 h post-injection. The primary sites of retention were the liver, gall bladder, kidney and gut. For aqueous exposure, most Cd was retained in the gills and gut (Woodworth & Pascoe, 1983). When Cd was injected i.p. in carp, the LD<sub>50</sub> was 9.5 mg/kg (±0.7), while oral dose LD<sub>50</sub> was 650 mg/kg (±75) (Yarzhombek et al., 1992). The results from these studies indicate that the method of exposure is an important consideration for interpretation of toxicity data. For oral or aqueous exposure much higher doses are required to reach an effect level than i.p. injected cadmium. The gill and gut appear to be buffers against Cd toxicity in other organs.

Interaction

The results presented here show that both synergistic and antagonistic effects can
occur when BP and Cd are dosed together, depending on the dose range. At sublethal doses of Cd, lower mortality was observed in BP+Cd dosed fish than would be expected from BP dosage alone. Assuming that the toxic effects of BP are caused by metabolites that are formed during biotransformation, and not by a direct narcotic effect as described above, the Cd effect would be an inhibition of the biotransformation of BP. This effect has been described before (Fair, 1986; Lemaire-Gony et al., 1995; Bruschweiler et al., 1996; Sandvik et al., 1997), and results of new experiments investigating this phenomenon will be described in the following chapters.

At sublethal doses of BP there was higher mortality in BP+Cd dosed fish than would be expected for fish treated only with Cd. Most organisms have a certain capacity to bind metals through glutathione (GSH) (Singhal et al., 1987) or metallothionein (MT) (Roesjiadi, 1992). Binding of Cd to MT reduces the toxic effect of Cd. A higher mortality than expected could be caused by an inhibition of the binding of Cd to GSH or MT. In the combined BP+Cd treatments, the BP metabolites that are formed may interfere with the availability of Cd scavenging proteins, and thus increase the toxic effects of Cd. Experiments to investigate a possible interaction of BP metabolites with MT are described in Chapter 6.

The combination of both synergistic and antagonistic effects for certain dose combinations of Cd and BP poses serious difficulties for modeling the effects. Broderius (1991) and Haas et al. (1996) describe joint toxicity models based on isobolic graphs. For each dose of one compound, an LD_{50} for the other compound is calculated, and vice versa. The LD_{50} values are then plotted and compared with the line for linear additivity. This method could not be used for the BP-Cd data set because of the bimodal character of the effect curve at most of the dose levels. This would not allow the calculation of a reliable LD_{50} for that specific dose level, and would thus make the use of the isobolic graphs inappropriate.
References


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Chapter 3. Effect of Cd on BP biotransformation in isolated hepatocytes.

Introduction

The results described in the previous chapter demonstrated antagonistic effects of Cd on the toxicity of BP. Because BP itself is not considered to have a direct toxic effect, as discussed above, toxicity, resulting in mortality, is most likely caused by BP metabolites. Biotransformation of BP is found in a number of organs, but most actively in the liver.

The liver is one of the most important organs for the clearance of xenobiotics from the animal body. Both Phase I and Phase II enzymes for the oxidation and conjugation of PAHs, such as benzo[a]pyrene, are found abundantly in the liver (Varanasi et al., 1989). To study the induction and catalytic activity of these enzymes, they are usually isolated from homogenized liver tissue. Phase I enzymes are located on endoplasmatic reticulum (ER), and can be studied in the cell fraction that contains parts of this ER in the form of microsomes. Phase II enzymes are located on ER (UDP-glucuronidase) or in the cytosol (glutathione-S-transferase), and can be studied in the appropriate cell fractions. However, separating out the different cell fractions, and studying individual steps of PAH metabolism may give an impression of underlying mechanisms, but does not provide insight in how the complete process proceeds in vivo.

To study BP metabolism in vivo in whole fish can be a challenging endeavor. The use of isolated fish hepatocytes allows one to study the complete metabolic system in a small, living entity. Some benefits of using in vitro studies for toxicological research are:

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1) Simple control of environmental conditions, 2) reduced variability between experiments, 3) possibilities for simultaneous or repeated sampling over time, 4) small amounts of test materials are needed, which reduces the amount of waste, 5) cheaper and quicker than whole animal studies (Baksi & Frazier, 1990). Because of these benefits of using cell cultures for testing environmental toxicants, cell culture techniques are advocated as environmental bioassay techniques (Marion & Denizeau, 1983; Baksi & Frazier, 1988). Immortal fish hepatoma cell lines have been established, and are used for toxicological research (Hahn et al., 1993; Bruschweiler et al., 1996), but may not fully represent the metabolic processes that occur in healthy liver cells. Primary cultures of liver cells are most closely related to intact liver functions.

Several techniques have been developed for isolating and culturing hepatocytes. For most larger fish species the livers are perfused in situ with collagenase to digest the connective tissues (Moon et al., 1985; Braunbeck & Storch, 1992). For smaller fish, like mummichog, it is more appropriate to excise the entire liver, and digest it in trypsin after mincing the liver tissue into small pieces (Moerland & Sidell, 1981; Faisal et al., 1995).

The use of primary hepatocyte cultures not only allows one to study the effects of pretreatment of the fish, but can also be used to expose liver cells to a variety of conditions. Cells can be incubated with toxicants in the media (Kelly & Maddock, 1985; Bruschweiler et al., 1996), and other variables can be studied, like temperature effects on biotransformation rates (Gill & Walsh, 1990).

Metabolism of BP can occur through several different pathways. Most dominant is the oxidation by P450 monooxygenases which is inducible upon exposure to PAHs and other aromatic compounds (Varanasi et al., 1989). However, other pathways have been reported, like cooxidation during lipid peroxidation (Colin et al., 1991) and oxidation by prostaglandin synthetase (Sivarajah et al., 1978). Each pathway results in a different spectrum of metabolites, compared to the P450 pathway. Cd has been reported to have a direct inhibiting effect on P450 enzymes (Forlin et al., 1986). If there is inhibition of the P450 pathway by Cd, but not of the other pathways, this should become obvious from the comparison of the metabolite spectra produced by hepatocytes from fish that have been...
treated with or without Cd.

Exposure of fish to BP induces the production of P450, resulting in a faster metabolism of the toxicant. Coexposure to Cd may have both direct and indirect effects. Direct effects would involve interference with the catalytic activity of the P450 enzyme. Indirect effects would be the inhibition of the production of new enzyme, or accelerated breakdown of the enzyme. Direct effects can be studied by incubating induced hepatocytes in a media with Cd.

The objective for the experiments described in this chapter were to examine a series of questions:

• When fish are dosed with Cd and BP, do the hepatocytes from these fish show an effect of Cd on the BP metabolizing capacity? Is this effect time dependent?
• Are there differences in the spectra of metabolites formed by hepatocytes from fish that received different treatments?
• What are lethal media concentrations of Cd for hepatocytes?
• Is there an acute effect of sublethal Cd concentrations on BP metabolizing capacity of hepatocytes?
• Are there differences over time in metabolites formed by hepatocytes from fish receiving different pretreatments?
Material and Methods

Experimental design

Three types of experiments were designed. In the first type, hepatocytes from different fish treatments were incubated with 3H-BP to investigate the effect of fish treatment on the ability of hepatocytes to metabolize 3H-BP (experiment I). In the second type, hepatocytes from BP induced fish were incubated in media with 3H-BP and Cd to investigate if Cd has a direct effect on BP metabolism in Cd exposed cells (experiment V). To do this experiment with effective Cd doses, Cd dose-response curves were first generated (experiment II, III, IV). Experiment II was planned to establish a dose-response relationship for Cd after a 4 h incubation period. Unfortunately, a complete power outage forced postponement of sampling to 16 h. Therefore the experiment was repeated to get the actual 4 h exposure time (experiment III).

Exposure of hepatocytes to media with Cd may cause specific problems with regard to the complexation of Cd (Marion & Denizeau, 1983). The RPMI media used for experiments II and III contains a suite of amino acids and other vital compounds for optimal cell growth. These compounds may bind Cd, and make it unavailable. Therefore, Minimal Essential Media, have been suggested for short term exposures of hepatocytes (Denizeau & Marion, 1990). In experiment IV Hanks balanced salt solution was used as a media, to study if this media would yield different results for Cd toxicity than the RPMI media.

In the third type of experiments, production of various BP-metabolites by hepatocytes over time was determined (experiment VI).

Experimental set up

One year old mummichog were collected from Carmine’s Island, York River, VA. Males only were used, 60 to 85 mm long, with a body weight of 4.0 - 9.0 g. The animals were acclimated to the laboratory conditions over two weeks, and treated for ectoparasites by a one hour fresh water dip, and a 1-h 200 ml/l formalin treatment (Speare
The fish were injected intraperitoneally (i.p.) after being anaesthetized in 200 mg/l MS-222. The treatments consisted of: solvent control, Cd alone, BP alone, BP simultaneous with Cd, and Cd four days before BP. Cd and BP were dosed in separate injections of 25μl. Cd was dissolved in teleost Ringer’s saline solution, BP was dissolved in corn oil. Fish were kept in 200 l seawater tanks, one tank per treatment, under flow through conditions (1.2 l/min). The fish were fed daily with dried fish food at a rate of 4 % of the body dry weight.

For experiment I doses of 0.32 mg/kg Cd and 10 mg/kg BP were applied, and 4 replicate fish were sampled on days 3, 7 and 14 post-injection, from each treatment. For experiment VI a BP dose of 26.5 mg/kg, and Cd doses of 0.32 and 3.2 mg/kg were used. Fish were sampled 6 days post-injection, with 2 replicates per treatment.

Cell cultures, incubation and metabolite analysis

For all experiments the following procedure was used for preparation of primary hepatocyte cultures, incubation with radiolabeled BP, and extraction and analysis of metabolites. Fish were anaesthetized in MS-222 (200 mg/l), and rinsed with 70 % ethanol. Livers were dissected aseptically, and washed in Hanks BBS. With a scissor the livers were minced in small pieces, and transferred into a beaker with 2 ml trypsin (0.25%). Trypsinization was performed with a magnet bar on a stir plate at the lowest speed for 20 min. Digested tissue was filtered over sterile gauze, followed by addition of 8 ml RPMI with 10 % FCS to stop the digestion. The cell suspension was centrifuged immediately at 1200 rpm for 5 min, followed by a second wash with RPMI. Red blood cells in the cell suspension, which may have BP metabolizing activity, were then lysed by hypertonic shock with ammonium sulfate in Tris buffer. (An initial attempt to separate cells by density gradient centrifugation with Histopaque resulted in unacceptable large loss of hepatocytes in some samples, obviously due to density differences between hepatocyte batches.) Final cell suspension was brought to 0.25 x 10⁶ cells/ml in salinity adjusted RPMI media. Cell viability was measured by trypan blue exclusion, and was generally more than 95%.

To measure BP metabolism by hepatocytes, one ml of each cell suspension was
exposed to 5μCi 3H-BP, and incubated in 24 well plates on a rotary shaker at 27° C in a 5% CO2 atmosphere. Exposed cells were lysed after 4 h incubation by adding an equal volume of deionized water to each well. BP metabolism was stopped by adding 1 ml ice cold acetone. Non-conjugated metabolites were extracted in ethyl acetate with 0.01 M BHT as an anti-oxidant. The organic fraction was blown to dryness and reconstituted in methanol for HPLC analysis. The aqueous fraction was subsampled, and total radioactivity was established on a scintillation counter as a measure for the total conjugated metabolite fraction. BP metabolites and residual parent compound in the organic fraction were analyzed on a HPLC configuration with two Waters 510 pumps, a Waters 717plus autosampler, Waters 486 absorbance detector, and a Radiomatic Flo-one\Beta, type A-100 radio detector with a 500 μl flow cell. The column used was a reversed-phase 4.6 x 25 mm Partisil 10 ODS-2 kept at room temperature. Compounds were separated using a linear water-methanol gradient at a flow rate of 0.8 ml/min, starting with 40% water, changing to 92% methanol over 15 min, changing to 100% methanol over the next 15 min, followed by 30 min of 100% methanol to remove any parent compound.

Statistics

Data sets were analyzed for statistically significant differences between treatments on a given day as described in Chapter 1.
Results

Effect of Cd dosed in fish on BP metabolism of hepatocytes

Isolated hepatocytes from mummichog demonstrated a clear capacity to metabolize BP (Table 3-1). In the 4 h exposure period, 70 to 93 % of the radiolabeled BP dose was transformed to non-conjugated and conjugated metabolites. The conjugation of primary metabolites was rapid, with only 2 - 7 % of the total recovered BP present as non-conjugated metabolites (Figure 3-1). On day 3, BP metabolism was significantly increased in the hepatocytes from BP-treated fish (Figure 3-2). The induced metabolizing activity of the BP-treated fish remained high on day 7, but started dropping on day 14. When fish were dosed simultaneously with Cd and BP, induction of BP metabolism was significantly inhibited on day 3. However, after day 3, the metabolizing activity of the hepatocytes increased significantly on day 14 to a level comparable to that of BP alone on day 3. This suggests that Cd initially had an inhibiting effect on BP metabolism, but after 3 days this inhibiting effect was diminished. When fish were dosed with Cd 4 days before BP, the induction on day 3 induction was apparent, though significantly reduced. On days 7 and 14 post-injection an effect of Cd was not detectable. In the Cd alone treatment the resident BP-metabolizing activity of the hepatocytes was not affected.

Chromatographic analysis of the non-conjugated metabolites showed that BP-9,10-diol, BP-4,5-diol, BP-7,8-diol, and 3-hydroxy-BP were the predominant metabolites (Figure 3-3). For these metabolites, a lower amount was present in almost all of the BP treatments, with or without Cd, than the solvent control and the Cd only treatment (Table 3-1). This indicates that conjugation in hepatocytes from BP dosed fish is faster than in the control treatments. Also, at the Cd levels used for this experiment, no change in the spectrum of primary BP metabolites was observed as a result of Cd coadministration.
Table 3-1. Unmetabolized parent compound and metabolites in hepatocyte cultures after 4 h incubation with 5μCi $^3$H-BP. Mean of 4 independent samples, expressed in pmol/10$^6$ cells, ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>Parent</th>
<th>Nonconjug. total</th>
<th>Conjugated total</th>
<th>9,10-diol</th>
<th>7,8-diol</th>
<th>4,5-diol</th>
<th>9-OH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 0</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24.46 ±7.88</td>
<td>9.82 ±1.69</td>
<td>113.28 ±13.86</td>
<td>4.30 ±5.50</td>
<td>0.05 ±0.11</td>
<td>0.16 ±0.19</td>
<td>1.44 ±1.52</td>
</tr>
<tr>
<td><strong>Day 3 sal &amp; oil</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cd</td>
<td>27.64 ±3.97</td>
<td>7.06 ±1.86</td>
<td>110.30 ±5.28</td>
<td>2.59 ±3.89</td>
<td>0.09 ±0.23</td>
<td>0.12 ±0.16</td>
<td>0.98 ±1.29</td>
</tr>
<tr>
<td>BP</td>
<td>27.08 ±8.92</td>
<td>7.35 ±2.78</td>
<td>104.51 ±17.33</td>
<td>2.29 ±2.91</td>
<td>0.07 ±0.22</td>
<td>0.08 ±0.10</td>
<td>1.09 ±1.42</td>
</tr>
<tr>
<td>BP &amp; Cd</td>
<td>11.04 ±5.09</td>
<td>2.90 ±0.57</td>
<td>145.32 ±10.08</td>
<td>0.20 ±0.33</td>
<td>0.00 ±0.00</td>
<td>0.05 ±0.09</td>
<td>0.39 ±0.50</td>
</tr>
<tr>
<td>Cd bef. BP</td>
<td>27.02 ±7.83</td>
<td>3.55 ±0.56</td>
<td>118.30 ±11.69</td>
<td>0.26 ±0.43</td>
<td>0.00 ±0.00</td>
<td>0.04 ±0.07</td>
<td>0.51 ±0.74</td>
</tr>
<tr>
<td></td>
<td>22.21 ±5.74</td>
<td>4.46 ±1.00</td>
<td>119.01 ±11.60</td>
<td>0.66 ±1.17</td>
<td>0.08 ±0.14</td>
<td>0.04 ±0.08</td>
<td>0.45 ±0.55</td>
</tr>
<tr>
<td><strong>Day 7 sal &amp; oil</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cd</td>
<td>27.18 ±6.65</td>
<td>5.88 ±0.83</td>
<td>105.68 ±12.86</td>
<td>1.47 ±2.00</td>
<td>0.14 ±0.18</td>
<td>0.08 ±0.13</td>
<td>0.67 ±1.11</td>
</tr>
<tr>
<td>BP</td>
<td>33.76 ±16.42</td>
<td>5.01 ±1.99</td>
<td>128.31 ±16.12</td>
<td>1.40 ±2.14</td>
<td>0.13 ±0.17</td>
<td>0.05 ±0.10</td>
<td>0.97 ±1.33</td>
</tr>
<tr>
<td>BP &amp; Cd</td>
<td>11.35 ±5.51</td>
<td>5.04 ±1.87</td>
<td>113.43 ±26.61</td>
<td>0.98 ±1.55</td>
<td>0.11 ±0.16</td>
<td>0.04 ±0.08</td>
<td>0.99 ±1.46</td>
</tr>
<tr>
<td>Cd bef. BP</td>
<td>15.31 ±4.80</td>
<td>4.59 ±0.72</td>
<td>141.21 ±20.68</td>
<td>0.55 ±0.93</td>
<td>0.13 ±0.20</td>
<td>0.06 ±0.10</td>
<td>0.55 ±0.78</td>
</tr>
<tr>
<td></td>
<td>15.98 ±5.16</td>
<td>3.91 ±1.09</td>
<td>118.17 ±22.15</td>
<td>0.50 ±0.69</td>
<td>0.10 ±0.13</td>
<td>0.06 ±0.10</td>
<td>0.40 ±0.49</td>
</tr>
<tr>
<td><strong>Day 14 sal &amp; oil</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cd</td>
<td>39.07 ±13.22</td>
<td>8.56 ±2.84</td>
<td>78.52 ±14.75</td>
<td>4.05 ±6.39</td>
<td>0.06 ±0.09</td>
<td>0.11 ±0.17</td>
<td>1.19 ±1.70</td>
</tr>
<tr>
<td>BP</td>
<td>34.42 ±21.39</td>
<td>4.56 ±0.71</td>
<td>100.00 ±22.82</td>
<td>0.91 ±1.02</td>
<td>0.04 ±0.05</td>
<td>0.14 ±0.20</td>
<td>0.71 ±1.04</td>
</tr>
<tr>
<td>BP &amp; Cd</td>
<td>19.77 ±12.82</td>
<td>3.29 ±0.89</td>
<td>112.06 ±25.07</td>
<td>0.38 ±0.65</td>
<td>0.03 ±0.05</td>
<td>0.11 ±0.19</td>
<td>0.46 ±0.71</td>
</tr>
<tr>
<td>Cd bef. BP</td>
<td>13.19 ±6.63</td>
<td>6.20 ±0.73</td>
<td>132.76 ±5.01</td>
<td>0.16 ±0.27</td>
<td>0.10 ±0.13</td>
<td>0.16 ±0.24</td>
<td>1.07 ±1.48</td>
</tr>
<tr>
<td></td>
<td>30.98 ±5.18</td>
<td>5.52 ±0.48</td>
<td>104.06 ±9.50</td>
<td>0.77 ±0.93</td>
<td>0.08 ±0.13</td>
<td>0.13 ±0.18</td>
<td>0.57 ±0.75</td>
</tr>
</tbody>
</table>
Figure 3-1. Mass balance for $^3$H-BP metabolism by hepatocytes, isolated from fish 3 days after different injections.
Figure 3-2. BP metabolism by isolated hepatocytes from fish with different treatment history, expressed as percentage of total recovered radiolabel on days post-injection. Each data point represents average of 4 fish.
Cd dose-mortality relationship for hepatocytes

The calculated LC<sub>50</sub> values for Cd were significantly different for all three treatments: 22.3 μM (95% C.I.: 17.2-29.1) for 16 h RPMI, 72.0 μM (50.5-102.7) for 4 h RPMI, and 126.4 μM (61.5 - 259.7) for 4 h Hank’s. As could be expected, the longer incubation time lowered the effect concentration for the RPMI media. The effect concentration in Hanks balanced salt solution is significantly higher, for unknown reasons. The dose-response curve for the Hanks treatment shows a higher mortality in the low concentration range, and a lower mortality in the higher concentrations, when compared to the RPMI treatments (Figure 3-4). A possible explanation is that exposure in a salt solution affects the viability of the cells, and thus disturbs the dose-response curve for a toxicant. Based on these results Hanks was excluded as a suitable exposure media. For the RPMI treatments significant effects occurred at 14 μM and higher. Therefore 4 and 14 μM were chosen as sublethal exposure concentrations for experiment V.

Effect of Cd in media on hepatocytes

Incubation of hepatocytes in media with sublethal concentrations of Cd does not seem to influence the BP metabolizing capacity of those hepatocytes under the conditions used in this experiment (Table 3-2). There were no significant differences in the amount of BP metabolized between Cd exposed hepatocytes and the control, and there were no differences in the amount of major metabolites formed during the exposure. These results indicate that there is no acute inhibiting effect of Cd on BP metabolizing enzymes.
Figure 3-3. Chromatogram of radiolabeled BP-metabolites produced by isolated hepatocytes. Identified peaks were: peak A: tetrols; peak B: BP-9,10-diol, peak C: BP-4,5-diol; peak D: BP-7,8-diol; peak E: 9-hydroxy-BP; peak F: 3-hydroxy-BP; peak G: unmetabolized BP.
Figure 3-4. Cd dose-response relations for isolated hepatocytes in 3 different media treatments. Each data point represents average of 3 replicates, except 2 replicates for RPMI 16 h data points.
Table 3-2. Amount of $^3$H-BP parent compound and metabolites in Cd spiked RPMI media after incubation with hepatocytes from untreated fish. Mean values (n=4) with standard deviation. Conditions: $0.25\times10^6$ cells per replicate, 4 h. incubation time with 5 $\mu$Ci $^3$H-BP.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BP left over</th>
<th>9,10-diol</th>
<th>4,5-diol</th>
<th>7,8-diol</th>
<th>9-OH</th>
<th>3-OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.402 (0.127)</td>
<td>0.061 (0.042)</td>
<td>0.004 (0.003)</td>
<td>0.006 (0.003)</td>
<td>0.025 (0.005)</td>
<td>0.083 (0.122)</td>
</tr>
<tr>
<td>4 $\mu$M Cd</td>
<td>0.415 (0.085)</td>
<td>0.062 (0.041)</td>
<td>0.009 (0.006)</td>
<td>0.006 (0.002)</td>
<td>0.026 (0.006)</td>
<td>0.025 (0.002)</td>
</tr>
<tr>
<td>14 $\mu$M Cd</td>
<td>0.378 (0.102)</td>
<td>0.060 (0.040)</td>
<td>0.006 (0.005)</td>
<td>0.004 (0.001)</td>
<td>0.025 (0.004)</td>
<td>0.023 (0.002)</td>
</tr>
</tbody>
</table>
Metabolite production over time

The results of experiment VI showed that there was a rapid metabolism of parent compound in hepatocytes from fish that were treated with BP and Cd, six days after the dosage (Figure 3-5). At the first time interval the BP metabolism was significantly faster in both combined BP +Cd fish treatments than in the control and BP alone treatment. Hepatocytes from fish treated with BP and a high Cd dose (3.2 mg/kg) metabolized almost 90% of the radiolabeled BP in the first 45 min. After this first time interval the differences were not significant anymore. The results from this experiment confirm those of the first experiment in that injection of fish with BP causes a rapid induction of BP metabolism over the first few days, followed by a return to background levels. Under the conditions used in this experiment there was no significant difference between the BP treatment and the control after 6 days. Combined Cd+BP treatment of the fish delays the induction of BP metabolism in the first few days, and induction peaks after about a week, as shown in this experiment.

Production and conjugation of the predominant primary metabolites show a comparable trend. There is a rapid production of primary metabolites in the first 45 min (Figures 3-6, 3-7, 3-8). The metabolism of $^3$H-BP in the BP+CdHigh treatment is so rapid that the production of 9,10-diol is significantly higher than in the other treatments. After the rapid production of metabolites in the first 45 min. there was a gradual decline of the primary metabolites. This decline is attributed to conjugation, causing the metabolites to disappear from the ethyl acetate extractable fraction into the aqueous fraction. Conjugation of 7,8-diol appears to be the fastest in general; after 3 h. there is almost nothing left, while for 9,10-diol and 3-hydroxy-BP there were still measurable amounts available. The 3-hydroxy-BP metabolite is the most persistent, and in the control treatment the amount even exceeds significantly over the other treatments after 12 h.
Figure 3-5. Amount of BP in primary hepatocyte cultures from fish with different treatment history. Hepatocytes were isolated 6 days post injection, and were incubated with 5 μCi ³H-BP for 24 h. Each data point represents average of 2 independent cell incubations, sampled at increasing intervals during the incubation. Data are presented as percent metabolized parent compound.
Figure 3-6. Amount of BP-9,10-diol over time in hepatocyte cultures from fish with different treatment history. Hepatocytes were isolated 6 days post injection, and were incubated with 5 μCi ³H-BP for 24 h. Each datapoint represents average of 2 independent cell incubations, sampled at increasing intervals during the incubation.
Figure 3-7. Amount of BP-7,8-diol over time in hepatocyte cultures from fish with different treatment history. Hepatocytes were isolated 6 days post injection, and were incubated with 5 μCi $^3$H-BP for 24 h. Each data point represents average of 2 independent cell incubations, sampled at increasing intervals during the incubation.
Figure 3-8. Amount of 3-hydroxy-BP over time in hepatocyte cultures from fish with different treatment history. Hepatocytes were isolated 6 days post injection, and were incubated with 5 $\mu$Ci $^3$H-BP for 24 h. Each data point represents average of 2 independent cell incubations, sampled at increasing intervals during the incubation.
Discussion

Effect on BP breakdown

The results of the experiments presented here show that Cd delays the induction of BP metabolism in mummichog livers. This confirms previous studies which demonstrated that Cd affects the induction and activity of the CYP1A enzyme system (Fair, 1986; Forlin et al, 1986; George & Young, 1986). The observation that Cd does not have an effect on BP metabolism in non-induced fish is an indication that, upon i.p. injection, Cd more likely interferes with CYP1A induction than with the actual enzymatic activity.

At the dose levels used here, Cd exposure does not seem to have an effect on the spectrum of BP metabolites being produced during biotransformation. A shift in metabolite spectrum would have been expected if other metabolic pathways for BP transformation increased in importance when CYP1A is inhibited by Cd (Sivarajah, 1978; Colin et al., 1991). However, base level BP metabolism by P450 in uninduced control fish was probably high enough to mask these other pathways. And because the Cd alone treatment did not affect the base level of BP metabolism, no change in metabolite spectrum was predicted or observed. In the combined BP+Cd treatments an initial inhibition of BP metabolism was observed, but this also did not result in a change of metabolite spectrum. These results indicate that Cd does not affect the activity of resident P450, but does affect the production of new P450.

Long term Cd exposure

In the experiments described here, fish were only dosed with a single Cd injection. This design was chosen to assure a consistent dosage per animal, which might be more difficult to achieve when fish are dosed through the water column. In three different studies, effects of Cd on P-450 enzymes are reported after exposure to aqueous Cd. Forlin, et al. (1986) exposed rainbow trout to 100 µg Cd/l for 4 weeks. This treatment did increase the liver P-450 content, but not the ECOD activity, which might be explained by direct inhibition of the enzyme: it takes more enzyme to oxidize the same amount of
substrate if the enzyme is partially inactivated. Lemaire-Gony & Lemaire, (1992, 1995) also exposed fish (eel and sea bass) to aqueous Cd (5 and 40 μg Cd/l) for several weeks, before dosage of BP. The cadmium pretreatment caused a higher induction of EROD activity than the BP alone treatment. These studies show that, contrary to the results obtained in single dose studies, long term exposure to Cd has a stimulating effect on the P-450 system. This would mean that several regulating mechanisms are involved in the effect of Cd on P-450 induction and activity.

Cd toxicity to hepatocytes

Various researchers have investigated the toxicity of Cd to fish cell cultures, and the results show a remarkable variation for the toxicity of Cd. Babich et al. (1986) compared effects of Cd on bluegill fry cells (BF-2) to effects on rainbow trout gonadal cells (RTG-2). Effect concentrations for Cd were 0.08 and 0.18 mM respectively (50% reduction in the uptake of neutral red). Cd toxicity for an immortal cell line, originating from a hepatic carcinoma in topminnow (PLHC-1) was 0.096 mM (Ryan & Hightower, 1994) and 0.14 mM (Bruschweiler et al., 1996). The effect concentrations for Cd reported here (0.02 - 0.07 mM) are below the values in the studies mentioned above, i.e. the primary cell cultures used in this study were more sensitive to Cd. This may result from differences in cell types. Denizeau & Marion (1989) compared primary cultures of trout hepatocytes with RTG-2 cells for Cd sensitivity, and found that the primary culture of trout hepatocytes was more sensitive to Cd than the RTG-2 cell line.

In experiments described here, incubation of hepatocytes in a media with sublethal Cd concentrations for 4 h did not affect the BP metabolizing capacity of the cells. These observations differ from results by Bruschweiler et al. (1996), who demonstrated for PLHC-1 cells that exposure of the cell cultures to a variety of heavy metals significantly reduced the amount of CYP1A protein, and consequently on EROD activity, with Cd as the strongest inhibitor. However, these results were obtained after an incubation period for the cells of 3 days in a Cd media. Combining the results of both studies indicates that there is no acute effect on the activity of resident P450, but Cd exposure does inhibit the
production of new P450.

**Metabolite spectra**

The metabolite spectra obtained from the hepatocyte incubations in this study are comparable to those obtained by others (Nishimoto et al., 1992; Gill & Walsh, 1992). The 9,10-diol is the dominant primary metabolite, followed by 7,8-diol, and 3-, or 9-hydroxy-BP. The study of Nishimoto (1992) revealed that glucuronides form the major Phase II BP metabolites in English sole hepatocytes, while very little sulfate and glutathione conjugates were formed. Treatment of these glucuronides with β-glucuronidase showed that 7,8-diol, and 1-, and 3-hydroxy-BP were the major conjugated metabolites. Obviously 9,10-diol does not conjugate very well, which would explain the relatively high abundance of this compound in the ethyl acetate-extractable fraction.

Interpretation of primary BP metabolite spectra is a difficult endeavor. Some metabolites are formed faster than others, and conjugation rates also differ for each metabolite, as was shown in the experiments presented here. This means that sampling an incubated cell culture at a certain moment gives a snap shot of the balance between production and conjugation of primary metabolites for that moment only. If a culture from a different parent is slower, or faster in metabolizing BP, that may result in a different metabolite spectrum for that same specific moment. Considerable individual variation in hepatocyte cultures has been observed in other fish studies (Moon et al., 1985) and in human hepatocyte cultures (Moore & Gould, 1984). For these human hepatocyte cultures, as for the mummichog hepatocytes, 9,10-diol, 7,8-diol and 9-hydroxy-BP were the predominant metabolites. However, the ratios among these metabolites varied widely among the six donors investigated, while they were highly reproducible within cultures from the same donor. The observed variability could not be correlated to cell viability, or overall levels of BP metabolism, and is therefore considered true individual variability.
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Chapter 4. Effects of Cd on Microsomal Biotransformation of BP

Introduction

The mortality experiments presented above show that Cd can have an inhibiting effect on the toxicity of BP. In isolated hepatocytes from fish that had been treated with Cd and BP, a reduced metabolism of BP was observed in the first days after dosage, compared to hepatocytes from fish that received BP only. Reduced mortality and biotransformation of BP under influence of Cd suggest that Cd has a negative effect on the BP-metabolizing enzyme CYP1A.

The first step in the biotransformation of benzo[a]pyrene (BP) is oxidation by CYP1A, one of the cytochrome P-450 monooxygenases, located on the endoplasmic reticulum (ER) of a variety of cells, like hepatocytes (Bruschweiler et al., 1996), intestinal cells (Van Veld et al., 1988), and immunocompetent cells (Ladies et al., 1992). When ER is isolated from homogenized tissue it is broken up in small parts called microsomes, from where the term microsomal biotransformation originates. A detailed description of the metabolic pathway for hydroxylation and conjugation of BP was presented in Chapter 1.

Exposure to a number of environmental toxicants (PAHs, PCBs) has been shown to induce the production of P-450 enzymes in fish (Stegeman et al., 1992). This induction is therefore widely used as a biomarker for exposure to these inducing compounds. Induction of CYP1A is generally measured by the EROD assay. In this assay the catalytic activity of microsomal CYP1A is measured, expressed as the rate of dealkylation of the CYP1A specific substrate 7-ethoxyresorufin (Klotz, 1984).

Effects of Cd exposure on BP metabolism have been documented for laboratory studies with mammals (Schnell et al., 1979; Means et al., 1979) and fish (Fair, 1986; George & Young, 1986; Lemaire-Gony & Lemaire, 1992, 1995). Not only laboratory studies, but also two field studies suggest actual interaction of heavy metals and CYP1A
in fish. Addison & Edwards (1988) found an effect of copper exposure on EROD activity in flounder from polluted sites in Norway. And Romeo et al. (1994) measured a high metal content combined with a low EROD activity in two Mediterranean fish species from waste water disposal locations.

A number of explanations has been proposed for the inhibitive effect of Cd on BP metabolism (Unger & Clausen, 1973; Means et al., 1979; George & Young, 1986; Forlin et al., 1986). Cd may have a direct or an indirect effect on the catalytic activity of the CYP1A enzyme. A direct effect would be caused by binding of Cd to thiol groups near the active site of CYP1A, causing competitive inhibition for BP. Alternatively, Cd could interfere with the binding characteristics of the heme group on the active site of the enzyme, thus destroying the integrity of the enzyme. Indirect effects could consist of the inhibition of CYP1A production, or interference with the CYP1A reducing enzyme: NADPH cytochrome P-450 reductase. Also, accelerated breakdown of CYP1A by heme oxygenase, which is induced by Cd exposure, has been suggested (Kutty et al., 1988).

The purpose of the experiments presented in this chapter was to investigate the effects of Cd on BP metabolism at microsomal level, in liver microsomes from fish that had been dosed with a combination of BP and Cd. The following questions were addressed:

• Does Cd affect the activity of microsomal CYP1A in preexposed fish, as measured by EROD activity and by BP metabolism?
• Does Cd affect the total amount of CYP1A?
• Does Cd affect the metabolite spectrum?
• Does Cd have a direct inhibiting effect on CYP1A, or does Cd have an indirect effect by affecting enzyme production?
Materials and methods

Experimental design

Six experiments were conducted using microsomes from preexposed fish. Experiments I and II were designed to measure EROD activity and CYP1A amount in microsomal protein from preexposed fish. Experiment III was a pilot experiment to determine a useful incubation time for a low amount of microsomal protein with $^{3}H$-BP. Experiments IV, V and VI were used to measure the breakdown of $^{3}H$-BP by microsomes from preexposed fish, and to analyze the spectrum of primary metabolites formed.

Experimental set-up

Fish were injected and maintained in flow-through tanks as described before. For all experiments the following treatments were used: saline/oil control, BP alone, Cd alone, BP together with Cd, and Cd four days before BP. For experiment I the BP dose was 18 mg/kg, and the Cd dose was 0.32 mg/kg, and fish were sampled on days 3 and 7. For experiments II and VI the BP dose was increased to 26.5 mg/kg to obtain a more pronounced CYP1A induction in the first 2 days post-exposure, and 3 Cd doses were used: 0.32, 1 and 3.2 mg/kg to study the effect of different Cd doses. Samples were taken on day 2 and 4 to obtain a better picture of the effects in the first few days post injection. For experiments III, IV and V 10 mg/kg BP and 0.32 mg/kg Cd were used. A lower BP dose than in the previous experiment was used to avoid mortality towards the end of the two week exposure period. Livers were harvested on day 3, 7 and 14, frozen immediately in liquid nitrogen, and stored at -80° C until further use.

Microsome preparation and protein assay

Microsomes were prepared by homogenizing the thawed livers in 2-4 ml stabilizing buffer (100 mM potassium phosphate buffer at pH 7.4, containing 20% glycerol, 1 mM dithiothreitol, 1mM EDTA, and 0.1 mM PMSF as anti-proteolitic agent) with a Brinkman Polytron homogenizer at the lowest possible speed, until no cell clumps were seen.
anymore. The homogenate was centrifuged two times in a Sorval RC28S ultracentrifuge at 12,000 g for 10 min to remove the cell debris, followed by a 100,000 g spin for 60 min to deposit the microsomal fraction. The centrifuge was cooled to 3°C, and between spins the samples were always kept on ice. The pellet was resuspended in 200 μl stabilization buffer and immediately frozen in liquid nitrogen. Protein content of the microsomal suspension was measured using the Bradford protein assay with BSA as protein standard. Duplicate samples of 5 μl suspension were dissolved in 95 μl deionized water, and 5 ml of Bradford dye reagent was added. After incubation for 10 min, absorbance was measured at 595 nm in a Gilford spectrophotometer, and recalculated to average protein concentration.

**Microsomal BP metabolism**

Most published methods for measuring BP metabolism by microsomes use at least 1 mg of microsomal protein (Nishimoto & Varanasi, 1985; Sikka et al., 1990; James et al., 1991). The fish used for the experiments described here were too small to yield 1 mg of microsomal protein per liver. Therefore an optimization experiment (experiment III) was performed to investigate if 0.5 mg protein would suffice, and the incubation time required for this amount of protein. Microsomal protein of 2 BP treated fish, and 3 control fish were pooled in two treatment batches. From each batch 5 samples of 0.5 mg protein were incubated in 0.5 ml buffer with 1 μCi 3H-BP. Buffer consisted of 50 mM phosphate buffer at pH 7.4, 4 mM MgCl2, 25 mM nicotinamide, 0.8 mM NADPH, and 1.0 mM NADH. 3H-BP was purified by HPLC using the procedure as described below for analysis of BP metabolites.

The samples were incubated in 15 ml glass disposable centrifuge tubes with teflon coated screw caps on a rotary shaker at 600 rpm in an incubator at 27°C. Every 10 min the reaction in one sample of each batch was stopped by placing the sample on ice and adding 0.5 ml ice cold acetone. The reaction mixture was extracted twice with 2 ml ethyl acetate, containing 0.01 M BHT to prevent oxidation of metabolites. The organic fraction was collected with Pasteur pipets in 20 ml glass scintillation vials, and blown to dryness.
with a gentle stream of purified N₂ gas, in a water bath at 40°C. The extracted compounds were redissolved in 150 μl methanol, and transferred to 200 μl glass inserts for HPLC autosampler vials.

Samples were analyzed on a HPLC configuration with two Waters 510 pumps, a Waters 717plus autosampler, Waters 486 absorbance detector, and a Radiomatic Flo- one\Beta, type A-100 radio detector with a 500 μl flow cell. The column was a reversed-phase 4.6 x 25 mm Partisil 10 ODS-2, kept at room temperature. A water-methanol non-linear gradient was used, starting with 55% methanol, at a flow rate of 0.8 ml/min. In a steep convex gradient the methanol content was increased to 100% in 15 min, and kept at 100% for 20 min, followed by a return to and re-equilibration at 55% in 10 min.

Based on the results of the pilot experiment an incubation period of 50 min. was chosen (Figure 4-1). After 50 min. not all parent compound was broken down, allowing variation in the results for different treatments, but enough was broken down to show anticipated inhibition of simultaneous Cd exposure.
Figure 4-1. Amount of BP and BP metabolites in microsome incubations over time. Microsomes (0.5 mg/ml) from BP injected fish (26.5 mg/kg), isolated 2 days post injection, incubated with 1 μCi ³H-BP/ml reaction mixture. Each bar represents one sample.
EROD assay and CYP1A amount

EROD activity was measured spectrophotometrically according to Klotz et al. (1984). Samples of 100 μg microsomal protein/ml buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 7.9) were incubated with β-NADPH (0.4 mM) and 7-ethoxyresorufin (2 μM). Formation of resorufin was measured at 572 nm over a 4 min. timespan. Microsomal CYP1A content was measured by immunoblot analysis, as described by Van Veld & Westbrook (1995). Microsomal proteins were separated on 12% polyacrylamide gels, and transferred to 20 μm nitrocellulose paper. Monoclonal antibody (Mab 1-12-3) was used as the primary antibody. The secondary antibody was alkaline phosphatase-conjugated goat anti-mouse IgG. Staining was performed using p-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP). Hepatic microsomal CYP1A from spot (Leiostomus xanthurus), previously calibrated against purified CYP1A, was used as a standard. The amount of stain was quantified with a Shimadzu CS-930 thin-layer chromato scanner.

Statistics

Data sets were analyzed statistically as described previously (Chapter 1).
Results

EROD and CYP1A measurements

The effects of Cd on the amount of CYP1A and on the CYP1A activity, as measured by the EROD assay, were investigated in two experiments. In the first experiment fish were sampled on days 3 and 7 post-injection. The results showed that there was no statistically significant difference between the Cd only treatment and the controls (Table 4-1). BP injection caused a tenfold increase in EROD activity (Figure 4-2) and a two orders of magnitude increase in the total CYP1A amount on day 3 (Figure 4-3).

This high induction rate was greatly reduced on day 7, though still significantly higher than the controls. When Cd was dosed together with BP, the amount and activity of the enzyme were not different from the BP alone treatment on day 3, but on day 7 the combined BP+Cd treatment had a significantly higher CYP1A activity than in the BP alone treatment. From this experiment it was concluded that CYP1A induction occurred rapidly in the first few days after injection, and trailed off towards day 7. Simultaneous Cd dosage delayed the return to base level amounts of CYP1A.

Because the induction of CYP1A appeared to occur in the first few days after injection, a second experiment was conducted with higher Cd doses, and sampling on day 2 post-injection. The results of this experiment showed that the amount of CYP1A was about half of the amount of day 3 in the first experiment, but the EROD activity was comparable (Table 4-2). There were no significant differences between the BP alone treatment and the treatments with increasing Cd amounts, both in EROD activity, and in the total amount of CYP1A. The effect of Cd on CYP1A induction became obvious only after the first 3 days post-injection, as seen in the first experiment.
Table 4-1. EROD and CYP1A measurements on day 3 and 7. N=3, but each sample composite of 4 livers. Standard deviation in brackets. EROD in pmol/min/mg protein, CYP1A in nmol/mg protein. A= significantly higher than controls on same day, B= significantly higher than BP alone treatment on same day.

<table>
<thead>
<tr>
<th></th>
<th>EROD</th>
<th></th>
<th>CYP1A</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 3</td>
<td>Day 7</td>
<td>Day 0</td>
</tr>
<tr>
<td>Blank</td>
<td>284  (65)</td>
<td></td>
<td></td>
<td>0.008 (0.003)</td>
</tr>
<tr>
<td>Saline</td>
<td>394  (142)</td>
<td>411 (145)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn oil</td>
<td>374 (62)</td>
<td>358 (100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sal.+oil</td>
<td>351 (35)</td>
<td>374 (48)</td>
<td>0.017 (0.001)</td>
<td>0.014 (0.001)</td>
</tr>
<tr>
<td>Cd</td>
<td>347 (33)</td>
<td>276 (73)</td>
<td>0.015 (0.002)</td>
<td>0.015 (0.004)</td>
</tr>
<tr>
<td>BP</td>
<td>3630 (481)(^A)</td>
<td>1013 (206)(^A)</td>
<td>0.926 (0.243)(^A)</td>
<td>0.155 (0.075)(^A)</td>
</tr>
<tr>
<td>BP+CdL</td>
<td>3230 (465)(^A)</td>
<td>2383 (210)(^A,B)</td>
<td>0.903 (0.173)(^A)</td>
<td>0.418 (0.235)(^A)</td>
</tr>
</tbody>
</table>
Figure 4-2. EROD activity (pmol/min/mg protein) of liver microsomes from fish with different treatments, sampled on 2 different days post injection. Each bar represents the average of 3 replicates, error bars are one standard deviation.
**Figure 4-3.** CYP1A content (nmol/mg protein) of liver microsomes from fish with different treatments, sampled on 2 different days post injection. Each bar represents the average of 3 replicates, error bars are one standard deviation.
Table 4-2. EROD and CYP1A after 2 days of exposure. N=5, composite of 2 fish, standard deviation in brackets. EROD in pmol/min/mg protein, CYP1A in nmol/mg protein. A= significantly higher than control, no significant differences between treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EROD</th>
<th>CYP1A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline + oil</td>
<td>557 (158)</td>
<td>0.007 (0.001)</td>
</tr>
<tr>
<td>BP</td>
<td>3604 (1754)\textsuperscript{A}</td>
<td>0.488 (0.269)\textsuperscript{A}</td>
</tr>
<tr>
<td>BP+Cd Low</td>
<td>4428 (1410)\textsuperscript{A}</td>
<td>0.525 (0.190)\textsuperscript{A}</td>
</tr>
<tr>
<td>BP+Cd Medium</td>
<td>3687 (898)\textsuperscript{A}</td>
<td>0.501 (0.132)\textsuperscript{A}</td>
</tr>
<tr>
<td>BP+Cd High</td>
<td>3195 (839)\textsuperscript{A}</td>
<td>0.406 (0.195)\textsuperscript{A}</td>
</tr>
</tbody>
</table>
**BP metabolism**

In experiment IV microsomes from all treatments were incubated for 50 min. The breakdown of BP and the production of the major metabolites over time was measured. The treatments with BP (with or without Cd) showed significant doubling of the metabolic activity, compared to the control on day 3 (Figure 4-4). For none of the sampling days was a significant effect of Cd dosage on the activity observed. Also, there was no significant difference between the control and the Cd only treatment. The expectation was that the combined BP+Cd treatment would have shown an effect, as it did in the EROD assay described above. Two factors were thought to be of influence: the incubation time, and the Cd dose.

The incubation time was initially set at 50 min. But this might have been too long. If Cd had caused a lower amount of CYP1A in the microsomes as compared to the BP alone treatment, this effect may have been masked in the BP metabolism experiment. Even a low amount of enzyme can metabolize all the substrate if it is incubated long enough. Therefore, in experiment V, using a subset of the microsomes from the previous experiment, an incubation time of 25 min was used. The results show that indeed a lower amount of 3H-BP has been metabolized by the microsomes from the BP treated fish (Figure 4-5). The microsomes from the combined BP+Cd treated fish generated a lower amount of metabolized 3H-BP on both day 7 and 14, though the difference with the BP only treatment was not statistically significant. Therefore a further experiment was performed with higher Cd doses.

The results of experiment VI with higher Cd doses, revealed no significant differences between the BP alone treatment and the BP plus low or medium Cd dose, both on day 2 and day 4 (Figure 4-6). The highest Cd dose (3.2 mg/kg) appears to cause a decrease of BP metabolism, both on day 2 and day 4. On day 4 this was significant at p<0.10 level.
**Figure 4-4.** Amount of BP metabolized by microsomes from fish with different treatments, sampled on 3 different days post injection. Exposure of 0.5 mg microsomal protein to 1 μCi $^3$H-BP in 0.5 ml media for 50 min. Each bar represents the average of 3 replicates, error bars are one standard deviation.
Figure 4-5. Amount of BP metabolized by microsomes from fish with different treatments, sampled on 3 different days post injection. Exposure of 0.5 mg microsomal protein to 1 μCi ³H-BP in 0.5 ml media for 25 min. Each bar represents the average of 3 replicates, error bars are one standard deviation.
Metabolite production

A typical chromatogram for the ethyl acetate-extractable BP metabolites produced by microsomes shows three dominant peaks: BP-9,10-diol, BP-7,8-diol and 3-hydroxy-BP (Figure 4-7). Minor peaks were identified as tetrols, 4,5-diol and 9-hydroxy metabolites. No effects of Cd on the spectrum of metabolites could be identified from the chromatograms. As was demonstrated above there was a rapid biotransformation of the parent compound in the reaction mixture (Figure 4-1). But there was also a decline in the total recovery of ethyl acetate-extractable BP metabolites. This removal of the primary metabolites is most likely attributable to conjugating activity of UDP-glucuronyl transferases, which are also located on the microsomes (Burchell & Coughtrie, 1989).

Enzyme concentration-activity relation

The EROD activity is normally presented as catalytic activity per mg microsomal protein. In Figures 4-8 and 4-9 the EROD activity is presented as a function of the actual amount of CYP1A enzyme, for all treatments of two different experiments. If exposure of fish to a combination of Cd and BP would lead to a direct inhibition of the enzymatic activity of CYP1A by Cd, the data points for these combined treatments would drop significantly below the regression line. As can be seen from both figures the data points representing treatments with Cd do not show a lower EROD activity than the treatments without Cd. This would imply that Cd, when dosed i.p., does not have a direct inhibitive effect on the catalytic activity of CYP1A.
Figure 4-6. Amount of BP metabolized by microsomes from fish with different treatments, sampled on 3 different days post injection. Exposure of 0.5 mg microsomal protein to 1 μCi $^3$H-BP in 0.5 ml media for 25 min. Each bar represents the average of 5 replicates, error bars are one standard deviation.
**Figure 4-7.** Chromatogram by radiodetector from microsomal incubation with $^{3}$H-BP. Microsomes from BP injected fish (18 mg/kg), sampled 3 days post-injection. Incubation of 0.5 mg microsomal protein with 1 $\mu$Ci $^{3}$H-BP in 0.5 ml media for 25 min. Metabolites extracted with ethyl acetate. Identified peaks are: peak A: tetrols, peak B: 9,10-diol-BP; peak C: 4,5-diol-BP; peak D: 7,8-diol-BP; peak E: 9-OH-BP; peak F: 3-OH-BP; peak G: unmetabolized BP.
Figure 4-8. EROD activity (pmol/min/mg protein) of liver microsomes from fish with different treatments, sampled on different days, plotted as function of total CYP1A concentration for each sample (nmol/mg protein).
**Figure 4-9.** EROD activity (pmol/min/mg protein) of liver microsomes from fish with different treatments, sampled on different days, plotted as function of total CYP1A concentration for each sample (nmol/mg protein).
Discussion

Direct effects

In one of the first papers reporting a negative effect of i.p. injected Cd on P450 activity in mice, Unger & Clausen (1973) assumed that there is direct binding of Cd to cysteine thiol groups of the P450 enzyme. The same effect was reported for rats by Means et al. (1979), who documented that when microsomes are directly incubated with Cd, there is a direct dose-dependent inhibiting effect of Cd on P450 activity. It was also demonstrated that Cd caused a concentration dependent, increasing conversion of P450 into P420, accompanied by a loss of catalytic activity. The assumption that dosed Cd directly interferes with the active site on the P-450 enzyme, and thus affects the catalytic activity, has been tested by several researchers. The technique used by them was to incubate isolated microsomes in a media with heavy metals and a substrate. Forlin, et al. (1986), incubated microsomes with 5 μM Cd and found a 50 % reduction of P-450 activity. Bruschweiler et al (1996) show that especially Cd and Cu are very effective inhibitors, much better than Co, Ni, and Pb, which is confirmed for Cu by Stien et al. (1997). Viarengo et al. (1997) used the method to show the protective effect of glutathione on direct inhibition of EROD activity by copper and mercury in microsomal incubations.

The above mentioned studies, in which isolated microsomes were directly incubated with heavy metals, have caused considerable confusion. There is no doubt that heavy metals have a direct inhibiting effect on the CYP1A activity, when exposed in this way. However, it remains questionable how relevant this information is. The results presented in this chapter, combined with results of others, show that there is not an acute effect of Cd on P450 when dosed directly to complete, living cells or whole organisms. It appears that there is a buffering system in live structures that prevents acute effects. The effect of Cd on CYP1A in living structures occurs after a considerable time period (days), and is generally seen as an effect on enzyme production, not on enzyme activity.
Indirect effects

Schnell et al. (1979) link a reduced P450 activity in rats as a result from Cd exposure to a reduced amount of P450 enzyme, and not to a direct inhibition of enzymatic activity. They demonstrated that the catalytic activity, as expressed per amount of enzyme, did not change, while the total amount of enzyme did. The decline of P450 amount is thought to be a result of heme oxygenase activity, induced by Cd. This concept of an indirect effect has been followed by more researchers, and matches the results described in this chapter. Means et al., (1979) report a reduction of activity (80%) and total content of P450 (40%), 3 days after a 2 mg/kg Cd dose in rats. It was also demonstrated that Cd caused a concentration dependent, increasing conversion of P450 into P420, accompanied by a loss of catalytic activity. P420 is seen as the breakdown product of P450.

In a number of studies with fish, the reduction of EROD activity, combined with a loss of P450/CYP1A enzyme as a result of Cd exposure has been reported. George & Young (1986) studied the time related interactive effects of 3-methylcholanthrene, an artificial CYP1A inducer (10 mg/kg) and Cd (1 mg/kg) in plaice (Pleuronectes platessa). EROD activity doubled in 24 h, with a maximum (15 x control) at 10 days, and dropped to double the control value at day 14 in 3MC alone. Comparable observations were made by Sved et al. (1992): a rapid induction of hepatic EROD activity over the first two days when spot (Leiostomus xanthurus) were exposed to creosote contaminated sediment, followed by a decline to base level on day 7.

In the above described experiments by George & Young (1986), Cd abolished EROD activity immediately, to 10% of control at day 2 in combined 3MC-Cd. Forlin, et al. (1986) found that liver microsomes of rainbow trout 4 days after a double dose of 0.5 mg Cd/kg had a significantly lower ECOD activity, though the total P-450 amount was not lowered in this study. A direct dose-response relation for Cd - EROD activity was presented by George (1989): increasing Cd dose (0.01 - 1 mg/kg) in flounder resulted in decreasing EROD activity (to 20% of control), 6 days after Cd injection. Decrease is thought to be a result of less enzyme rather than direct inhibition by Cd because immunoassays showed not only a reduced P450 amount, but also a reduced amount of
A complicating factor in analyzing the total P450/CYP1A enzyme content is the method of detection. Spectrophotometric measurement of P450 is not specific for the BP metabolizing isozyme CYP1A, but measures all P450 isozymes. Reduction of the P450 amount, and increase of the breakdown product P420, indicates a general effect on the enzymes, either the removal of the heme group (causes the shift from P450 to P420?), or a general lower protein synthesis. Measurement of specific CYP1A by immunoassays is more specific for the actual induction of the BP metabolizing enzyme. However, the immunoassay also measures the apoenzyme, without the heme group. This means that there may occur discrepancies between EROD activity and CYP1A content (Forlin et al., 1986; Sandvik et al., 1997).

From all reported studies it becomes clear that time, dose and temperature are important variables that can influence the measured results. Induction of CYP1A is normally rapid, within the first day, and for some studies there is also a rapid effect of Cd (George & Young, 1986), while for most others a period of days is reported for the effect of Cd to become obvious. This may be explained by the amount of Cd dosed, and the binding capacity for Cd within the organism. It has been suggested that organisms can handle a certain amount of Cd, but when a threshold value is exceeded, acute effects will occur. For Cd the window between this threshold value and acute mortality may be
relatively small (George & Young, 1986). Temperature differences may also play an important role in comparing effects; studies were conducted at temperatures as low as 9°C (Sandvik et al., 1997) to 28° (Fair, 1989). The effect of temperature on BP metabolism in fish has been described before (Kennedy et al., 1989; Gill & Walsh, 1990).

In the experiments described in this chapter, fish were only dosed with a single Cd injection. This method was chosen to assure a consistent dosage per animal, which might be more difficult to achieve when fish are dosed through the water column. In three different studies effects of Cd on P-450 enzymes are reported for after exposure to aqueous Cd. Forlin, et al. (1986) exposed rainbow trout to 100 µg Cd/l for 4 weeks. This treatment led to increased liver P-450 content, but not ECOD activity, which might be explained by direct inhibition of the enzyme: it takes more enzyme to oxidize the same amount of substrate if the enzyme is partially defected. Lemaire-Gony & Lemaire, (1992, 1995) also exposed fish (eel and sea bass) for several weeks to Cd (5 and 40 µg Cd/l), before dosage of BP. The cadmium pretreatment caused an higher induction of EROD activity than the BP alone treatment. These studies show that, contrary to the results obtained in single dose studies, long term exposure to Cd has a stimulating effect on the P-450 system. This means that the modulating effect of Cd on P-450 induction and activity has a multitude of regulatory aspects.
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Chapter 5. Excretion of BP Metabolites through Bile, and Interactive Effects of Simultaneous Cd Dosage.

Introduction

In the previous chapters it was demonstrated that coadministration of Cd with BP had an inhibiting effect on the induction of CYP1A, and therefore caused a delay in the biotransformation of BP. The main route of excretion for BP metabolites is through the bile (Varanasi & Gmur, 1981), thus the question arose whether this inhibiting effect of Cd on BP metabolism would also be mirrored in the excretion of BP metabolites through the bile.

Biliary excretion

Many compounds are filtered from the blood plasma by the liver, and are subsequently removed from the liver in the bile. These compounds consist of natural breakdown products, like bile salts, cholesterol, biliverdin, steroids, and of xenobiotics, both organic compounds and heavy metals. Bile production is stimulated by a number of factors, of which bile acid accumulation in the liver is the most important. Some P450 inducers, like phenobarbital, also enhance the bile flow, while others, like 3-methylcholanthrene, fail to stimulate the biliary clearance of xenobiotics and endogenous metabolites in mammals (Klaassen & Watkins, 1984).

Conjugation is an essential step for excretion of waste products into the bile canaliculi. Conjugation increases the size and the polarity of the compounds, which appears to facilitate excretion. The molecular weight of a compound has to be over 800 for biliary excretion. Some compounds are excreted into the bile as glutathione conjugates, but most, like the steroids, bilirubin and a suite of xenobiotics, are conjugated to glucuronic acid. Only few compounds are excreted without conjugation. When conjugation is inhibited, either through removal of glucuronic acid or GSH, or through
inhibiting the activity of β-glucuronidase or glutathione transferases, hepatic accumulation will occur (Klaassen & Watkins, 1984). However, when Cd is dosed to MT-induced animals, the Cd will be bound to MT and accumulate in the liver. A reduction of 90% has been reported for the biliary excretion of Cd in MT-induced animals (Cherian, 1977).

Relatively few studies have described biliary excretion of BP metabolites in fish. Rapid uptake, metabolism and excretion of BP through bile was reported for English sole exposed to sediment associated BP (Stein et al., 1984, 1987). For southern flounder, BP metabolites were present in bile predominantly as glucuronide conjugates (James et al., 1991). The effects of different temperature regimes on BP metabolism and excretion of the metabolites through the bile has been described for gulf toadfish (Kennedy et al., 1989) and rainbow trout (Curtis et al., 1990). Temperature modulation affected the rate of excretion, but not the rate of metabolism, though higher temperatures appeared to cause a shift in the metabolite spectrum towards more carcinogenic intermediates.

The aromatic structure of PAHs and their metabolites makes them easily detectable by fluorescence detection. Since most PAH metabolites are excreted through the bile, fluorescence analysis of bile samples has become an accepted biomarker tool to access whether fish from field situations have been exposed to PAHs (Krahn et al., 1984; Deshpande, 1989; Lin et al., 1996).

**Objectives**

The objectives for the experiments described in this chapter were:

1) To measure the biliary excretion of BP metabolites over time.

2) To investigate potential effects of Cd on the excretion of BP metabolites.
Material and Methods

Experimental design

To study these aspects three experiments were performed. Radiolabeled BP was used as a tracer, allowing sensitive analysis for metabolites in bile, and in the surrounding water. The first experiment was designed as a pilot experiment to obtain an initial estimate of the rate of biotransformation and excretion. The second experiment was initiated to generate definitive estimates. This experiment was carried out in a flow-through system, allowing continuous removal of excreted radiolabeled material. The last experiment was performed to confirm the results of the second experiment, and to generate samples for metallothionein analysis (see Chapter 6). Unfortunately, the experimental set up for this last experiment had to be changed from a flow-through system to a static system because new regulations prohibit discharge of waste water containing any amount of radiolabeled compounds to the sewer system.

Experiment 1

For this pilot experiment, 10 fish (65-80 mm) were injected with BP, which was spiked with \(^3\text{H}-\text{BP}\), to give each fish a dose of 18 mg/kg BP and 5\(\mu\)Ci of the radiolabel. The fish were kept in a 10 gallon tank without water exchange. Water samples of 1 ml were analysed at least daily for radioactivity with a scintillation counter. All fish were sacrificed after 8 days, bile was collected and total radioactivity was measured by scintillation counter.

Experiment 2

Mummichog, 65-80 mm in length (average 76.4 ± 4.4 mm, 6.2 ± 1.1 g), were used for this experiment. The fish were collected, acclimated and injected as described before. The treatments consisted of BP alone, BP together with Cd, and the same Cd dose administered 4 days before the BP dose. The BP doses consisted of 10 mg/kg, the Cd doses consisted of 0.32 mg/kg. Sufficient \(^3\text{H}-\text{BP}\) was mixed in the stock solution of

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unlabeled BP to obtain a dose of 5 µCi per fish. The fish were housed in 20 gallon tanks, one tank per treatment. The tanks received a continuous flow of filtered York River water at 0.5 l/min. Water temperature was kept at 20°C. Water samples were collected from the tanks on a daily basis until no radioactivity above background could be measured. Fish were fed daily with dried fish food flakes at a rate of 4% of the dry body weight. Feed was supplied just after sampling, to ensure a sufficient bile accumulation in the gall bladder over the 24 hours before the next sampling moment. On days 1, 2, 4, 8, 16, 32, 64 and 96, five fish were sampled from each treatment. The fish were anaesthetized in MS-222 (200 mg/l), and the entire gall bladder was harvested, packed and frozen at -20°C for later analysis.

Experiment 3

For this experiment the following treatments were used: BP alone (18 mg/kg), BP with 1 mg/kg or 3.2 mg/kg Cd (further abbreviated to CdM and CdH), a control of 1 mg/kg Cd, and a treatment in which 1 mg/kg Cd was dosed four days before BP. The injected BP was spiked with ³H-BP to give each fish an amount of 5 µCi. Fish were kept in 20 gallon tanks, with water recirculating through a double-chambered Whisper filter with activated carbon in a filter bag. Water temperature was 22 °C throughout the experiment. Radioactivity in the water was monitored daily by analyzing 3 replicate samples of 1 ml in a scintillation counter.

Because the injected fish were kept in a closed system, the possibility existed that excreted radiolabeled material would be taken up again by the fish, and thus would complicate the interpretation of the results. To investigate if this effect would occur, uninjected control fish were kept in an enclosure in each treatment tank. Water circulation through the entire tank and the enclosure was maintained by the flow from the filter unit.

Bile was collected from the gall bladder of injected fish sampled on days 1, 2, 3, 4, 5, 6, 7, 11, and 14. Due to unexpected mortality, not all treatments could be sampled on days 11 and 14. Gall bladders from the control fish in the enclosures were collected on days 2, 4, 6, 11 and 14. Five replicates were collected per treatment per day. Some bile
samples were lost because the gall bladder was inadvertently punctured during dissection, which reduced the number of replicates for some sampling days. The entire gall bladder was removed and stored in 1 ml microcentrifuge tubes at 4°C until further analysis.

**Bile analysis**

For analysis of BP metabolites in the bile samples, 100 μl methanol was added to each sample, followed by squeezing the bile out of the gall bladder with a dissection needle, shaking the sample and centrifuging for 20 min at 14,000 rpm to pelletize the precipitated proteins and the gall bladder tissue (James et al., 1991). The supernatant was transferred to 200 μl HPLC autoinjector vials. Samples were analyzed on a HPLC configuration with two Waters 510 pumps, a Waters 717plus autosampler, Waters 486 absorbance detector, and a Radiomatic Flo-one\Beta, type A-100 radio detector with a 500 μl flow cell. The column was a reversed-phase 4.6 x 25 mm Partisil 10 ODS-2 kept at room temperature. Compounds were separated using a convex water-methanol gradient at a flow rate of 0.8 ml/min, starting with 60% water, changing to 100% methanol over 15 min, followed by 15 min of 100% methanol to remove any parent compound.

**Conjugate analysis**

To investigate if glucuronidated metabolites were present in the bile samples, several subsamples were incubated with β-glucuronidase to see if any of the peaks on the chromatograms would disappear. Fifty μl of bile was dissolved in 50 μl 0.2M sodium acetate buffer (pH 5.0). This sample was split in two subsamples, of which one was spiked with 500 units of purified β-glucuronidase (Type VIII from E. coli) in 10 μl acetate buffer. Both samples were incubated at 37°C for 24 h, and then treated and analyzed as described above for normal bile samples.

**Statistical analysis**

Data sets were analyzed for statistical differences as described in Chapter 1.
Results

Interpretation of chromatograms

In a typical bile chromatogram from fish injected with radiolabeled BP, 4 major peaks that are associated with radiolabeled compounds are found: a peak at the void volume (A), a peak at 8 min (B), a peak at 10.5 min (C), and a peak at 30 min (D, Figure 5-1). Peak D corresponds with the parent compound (nonmetabolized $^3$H-BP), as compared to reference samples. Incubation of bile samples with $\beta$-glucuronidase made peak C disappear to a large extent, indicating that this peak consists of glucuronic acid-conjugated metabolites (Figures 5-2A and 5-2B). Peaks A and B possibly contain sulfate and glutathione conjugates (James et al., 1991). Regression analysis showed that there was a good linear correlation ($r^2 = 0.95$) among the areas of the different peaks within one treatment. Further analysis of the data was therefore focused on the peak containing the glucuronidated metabolites.

To evaluate the effect of Cd dosage on the relative contribution of each (group of) metabolite(s) to the total radioactivity per sample, regression analysis was performed of peak area against total area. Regression equations with 95% confidence intervals for the slope coefficient were calculated for each treatment. For the BP alone treatment there was a significantly lower amount of peak A components and a significantly higher contribution of the glucuronides compared to the combined treatments. There were no significant differences between the treatments with Cd (Medium, High and Medium before). This indicates that Cd exposure causes less glucuronide conjugates to be excreted and more glutathione and/or sulfate conjugates.
Figure 5-1. Chromatogram of bile sample from fish injected with Cd (1 mg/kg) 4 days before $^3$H-BP (18 mg/kg). Overlay of radiodetector on UV detector. Peak A (UV detector) and peak B (radiodetector) were identified as BP-glucuronic acid conjugates, peak C is unmetabolized BP.
Figure 5-2. Chromatograms of 2 subsamples of bile sample, treated without (A) and with (B) 500 units of β-glucuronidase for 24 h at 37°C.
Figure 5-3. Percentage of injected radiolabeled material that was excreted over time into the surrounding water by 10 fish in static 10 gallon system. Each fish was injected with BP (18 mg/kg) which was spiked with $^3$H-BP to give a dose of 5 $\mu$Ci per fish.
Table 5-1. Concentration of BP-glucuronides in bile, flow-through experiment. In nCi/entire bile sample, standard deviation in brackets. N=3, except †: n=1, ‡ n=2. A=significantly increased compared to base levels as on days 16 and 32.

<table>
<thead>
<tr>
<th>Day</th>
<th>BP</th>
<th>BP+Cd Low</th>
<th>Cd before BP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.15 (0.97)^A</td>
<td>1.00 (0.45)^A</td>
<td>1.73 (0.24)^A</td>
</tr>
<tr>
<td>2</td>
<td>2.01 (0.30)^A</td>
<td>3.57 (1.21)^A</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4.08 (2.08)^A</td>
<td>5.12 (4.62)^A</td>
<td>2.54 (1.18)^A</td>
</tr>
<tr>
<td>8</td>
<td>0.99 (0.66)^A</td>
<td>0.79 (0.48)^A</td>
<td>2.79 (1.62)^A</td>
</tr>
<tr>
<td>16</td>
<td>0.22 (0.06)</td>
<td>0.06 (0.03)</td>
<td>0.19 (0.12)</td>
</tr>
<tr>
<td>32</td>
<td>0.05 †</td>
<td>0.58 (0.72)‡</td>
<td>0.13 (0.09)</td>
</tr>
</tbody>
</table>

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Experiment 1

The results of the pilot experiment showed that within 6 days almost 50% of the radiolabeled material was excreted (Figure 5-3). The amount excreted per day was highest on the second day after injection, and declined to zero after day 6. Analysis of BP metabolites in the bile samples by HPLC showed only minor amounts of radiolabel after 8 days, indicating that almost all of the BP metabolites had been excreted.

Experiment 2

The amount of radiolabeled glucuronide conjugates in the total bile sample as a function of time after injection increased significantly over the first 5 days (Table 5-1, Figure 5-4). After day 6 post-injection a rapid decrease was observed. By day 16 almost all radiolabeled compounds had disappeared from the bile. This pattern was seen independent of the Cd dose. The results further indicate that the excretion of BP metabolites is accelerated when the BP is dosed together with Cd. When Cd is dosed 4 days before BP, the excretion seemed to be slowed down, resulting in a longer excretion period. However, statistical analysis revealed that there were no significant differences between treatments on a given day. It needs to be noted that the data were highly variable, with a coefficient of variation up to 85% for some values, and some tested data sets did not show homogeneity of variance. Logarithmic transformation of these data sets did result in improved homogeneity, but did not result in the detection of significant differences between treatments.

Analysis of the effluent from the tanks in which the dosed fish were held showed a low, but measurable increase above background in the first few days (Figure 5-5). However, there were no obvious differences among the tanks.
**Figure 5-4.** Amount of radiolabeled glucuronide conjugates in bile samples from fish with different treatments, on days post-injection. BP dose was 10 mg/kg, spiked with $^3$H-BP to give a dose of 5 $\mu$Ci per fish. Cd dose was 0.32 mg/kg. Each data point represents average of 3 replicates.
Figure 5-5. Amount of radioactivity over time in effluent (0.5 l/min) from tanks that contained fish injected with BP and Cd. BP (10 mg/kg), spiked with $^3$H-BP to give a dose of 5 μCi per fish. Cd dose was 0.32 mg/kg. Each datapoint is a single measure per day per tank.
Experiment 3

The amount of BP-glucuronides in bile of fish that were kept in a closed system show a bimodal pattern; an increase over the first 3 days post-injection, a decrease towards day 5, followed by a steep increase again after day 5 (Figure 5-6). It is apparent that co-injection of Cd with BP increased the excretion of BP-glucuronides in the first 4 days, compared to the BP alone treatment (Table 5-2). After the third day the BP-glucuronide content decreases in fish from the combined treatments to almost the same level as seen in fish from the BP alone treatment. Metabolite levels after day 5 increased sharply for all treatments, but this effect was most predominant for the BP alone treatment.

The levels of BP-glucuronides in bile from control fish kept in the enclosures were much lower than in those of the directly dosed fish. The maximum concentration was about 0.2 nCi/mg for indirectly exposed fish, whereas in the injected fish the lowest value was 0.8 nCi/mg (Table 5-3). Day 4 was the only time when there was a significant increase in one of the treatments (CdM) over the BP alone treatment (Figure 5-7). These results indicate that even though relatively large amounts of metabolites were excreted by the injected fish, as demonstrated by analysis of the surrounding water, these excreted metabolites were only absorbed at a low level by the uninjected fish in the enclosures.

The analysis of the recirculating water showed that radiolabeled metabolites were readily excreted in the first 4 to 5 days after injection (Figure 5-8). After the 5th day a steady state appears to have been achieved. On days 2, 3 and 4, fish exposed to BP+CdM seem to excrete more metabolites than fish injected with BP alone. This could not be statistically tested because of the unreplicated set-up. In contrast, fish treated with BP and a high Cd dose seem to excrete less metabolites than fish in any of the other treatments. Fish exposed to Cd before BP appear to excreted less metabolites than fish dosed with BP alone.
Table 5-2. Concentration of BP-glucuronides in bile, static experiment. In nCi/mg bile, ± standard deviation. N=4. A=significantly increased compared to BP alone on same day.

<table>
<thead>
<tr>
<th>Day</th>
<th>BP</th>
<th>BP+Cd Med</th>
<th>BP+Cd High</th>
<th>Cd before BP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.85 (0.17)</td>
<td>1.70 (0.88)</td>
<td>1.70 (0.26)</td>
<td>1.23 (0.18)</td>
</tr>
<tr>
<td>2</td>
<td>0.81 (0.10)</td>
<td>2.09 (1.35)</td>
<td>2.12 (0.59)</td>
<td>0.98 (0.32)</td>
</tr>
<tr>
<td>3</td>
<td>1.17 (0.20)</td>
<td>2.98 (1.31)</td>
<td>3.39 (1.12)</td>
<td>2.49 (0.84)</td>
</tr>
<tr>
<td>4</td>
<td>1.20 (0.39)</td>
<td>0.86 (0.31)</td>
<td>2.82 (0.47)</td>
<td>2.58 (1.39)</td>
</tr>
<tr>
<td>5</td>
<td>0.83 (0.16)</td>
<td>1.33 (1.48)</td>
<td>1.85 (0.40)</td>
<td>0.79 (0.51)</td>
</tr>
<tr>
<td>6</td>
<td>0.92 (0.02)</td>
<td>4.49 (0.37)</td>
<td>2.34 (0.97)</td>
<td>1.27 (0.83)</td>
</tr>
<tr>
<td>7</td>
<td>5.63 (2.57)</td>
<td>2.86 (1.69)</td>
<td>3.18 (1.20)</td>
<td>3.51 (1.32)</td>
</tr>
<tr>
<td>11</td>
<td>2.22 (0.62)</td>
<td>2.40 (0.34)</td>
<td></td>
<td>1.33 (0.48)</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>1.20 (0.48)</td>
<td></td>
<td>2.82 (0.18)</td>
</tr>
</tbody>
</table>

Table 5-3. Concentration of BP-glucuronides in bile of control fish from enclosures. In nCi/mg bile, ± standard deviation. N=3, except †: n=1, ‡ n=2. A=significantly increased compared to BP alone on same day.

<table>
<thead>
<tr>
<th>Day</th>
<th>BP</th>
<th>BP+Cd Med</th>
<th>BP+Cd High</th>
<th>Cd before BP</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.056 (0.019)</td>
<td>0.070 (0.002)</td>
<td>0.031 (0.008)</td>
<td>0.028 (0.005)</td>
</tr>
<tr>
<td>4</td>
<td>0.029 (0.013)</td>
<td>0.177 (0.060)</td>
<td>0.080 (0.020)</td>
<td>0.105 (0.030)</td>
</tr>
<tr>
<td>6</td>
<td>0.044 (0.008)</td>
<td>0.144 (0.062)</td>
<td>0.079 (0.057)</td>
<td>0.139 (0.091)</td>
</tr>
<tr>
<td>11</td>
<td>0.033 (0.022)</td>
<td>0.089 (0.055)</td>
<td>0.010 †</td>
<td>0.075 (0.080) ‡</td>
</tr>
</tbody>
</table>
| 14  | 0.073 (0.017) | 0.072 (0.046) | 0.005 †     | 0.043 †
Figure 5-6. Amount of radiolabeled glucuronide conjugates in bile samples (nCi/mg bile) from fish with different treatments, on days post-injection. BP dose was 18 mg/kg, spiked with $^3$H-BP to give a dose of 5 $\mu$Ci per fish. Cd doses were 1 (CdM) and 3.2 (CdH) mg/kg. Data points represent average of 4 replicates.
Figure 5-7. Amount of radiolabeled glucuronide conjugates in bile samples (nCi/mg bile) from uninjected fish that were held in enclosures in recirculation tanks with injected fish. BP dose of the injected fish was 18 mg/kg, spiked with $^3$H-BP to give a dose of 5 $\mu$Ci per fish. Cd doses were 1 (CdM) and 3.2 (CdH) mg/kg. Data points represent average of 4 replicates.
Figure 5-8. Amount of radioactivity over time in recirculation tanks that contained fish injected with BP and Cd. BP (18 mg/kg), spiked with $^3$H-BP to give a dose of 5 $\mu$Ci per fish. Cd doses were 1 (CdM) and 3.2 (CdH) mg/kg. Data points represent average of 3 replicates.
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Discussion

The chromatograms obtained from the bile samples reported here are very comparable to those reported by James et al. (1991) for southern flounder exposed to BP. For both species glucuronic acid conjugation appears to be an important route for biliary excretion. However, it is likely that GSH and sulfate conjugates are also involved. Glutathione-S-transferase activity has been reported to be 3-fold higher in mummichog from PAH contaminated field locations, indicating that GSH conjugation plays a distinctive role in hepatobiliary excretion of PAH metabolites (Van Veld et al., 1991). In rainbow trout exposed to dimethylbenz[a]anthracene (DMBA), conjugation to sulfate was significant over the first 12 h, but conjugation to glucuronic acid was much more important in the next 60 h (Schnitz et al., 1993).

The results of both experiment 1 and 2 suggest consistent trends for the influence of Cd dosage on BP metabolite excretion. When BP is dosed together with a small or intermediate dose of Cd, there is a faster excretion of the conjugated BP metabolites than in the BP alone treatment in the first few days. Combined with a high Cd dose, the excretion is much lower than in the BP alone treatment. When an intermediate Cd dose is administered four days before BP, the excretion of metabolites is reduced, but not as low as in fish that received the highest Cd dose.

Since conjugation of metabolites is essential for biliary excretion (Klaassen & Watkins, 1984), and because Cd affects the induction and availability of GSH as a conjugating compound (Singhal et al., 1987), the following model may explain the observed results. A moderate Cd dose will only have a moderate effect on the production of BP metabolites, but it will induce the production of conjugating compounds. This greater availability of conjugating compounds may then enhance the excretion of BP metabolites. Analysis of the chromatograms suggested indeed a larger contribution of GSH-conjugated metabolites in the combined BP-Cd treatments than in the BP alone treated fish. When the moderate Cd dose is given four days before the BP, Cd may have depleted the building blocks for conjugating compounds, leading to a reduced rate of
excretion of conjugated metabolites, as was seen in the aqueous samples from experiment 3. The high Cd dose may have reduced all biotransformation processes, and eventually caused mortality.

The above described processes seem to be consistent with most of the data. However, statistical analysis of the results showed a large variability in the data. Obviously there are considerable individual differences within any one sampling group, which hampers interpretation of the results. Sampling an entire bile sample on a fixed moment in the day did not give a clear picture of treatment differences. Analysis of the water samples proved to be much more consistent, but is an indirect approach to measuring metabolite excretion. In an effort to reduce the variability observed in experiment 2, the fish were fed right after sampling in experiment 3, allowing a 24 h build up of bile in the gall bladder before sampling again. This obviously did not reduce the variance in metabolite concentrations, as wide range of bile volumes was observed within sample groups in experiment 3 (7 - 66 mg).

The use of a natural marker in bile was considered to independently measure the excretion rate of bile. If this would have been feasible, the amount of BP metabolites could have been related to a known excretion rate. This might have reduced the variation caused by variable bile volumes. Analysis of the UV chromatograms revealed a few peaks that were not related to the BP metabolites and that might serve as internal markers for bile production. However, expression of the metabolite amounts as a fraction of these markers did not reduce the variation in the results.

Another approach would have been to inject an inert standard into the fish together with the BP dose. Phenol red has often been used to measure the passage time of metabolites through the liver, both in mammals (Collado et al., 1988; Kakutani et al., 1992) and fish (Guarino, 1986; Plakas et al., 1992). The problem with coadministration of phenol red and BP is that both compounds induce the P450 system, and phenol red might thus be metabolized and excreted faster when dosed together with BP than when dosed alone. Therefore, phenol red would not have been a reliable internal standard for the rate of BP excretion through the bile, and was not used.
An interesting phenomenon was observed in the static experiment: after an initial increase in bile glucuronides, concentrations dropped around day 4, but rapidly increased again after day 5. This may be attributed to entero-hepatic cycling (Klaassen & Watkins, 1981). Normally, conjugated metabolites, which are excreted through the bile into the intestine, will not be lipophilic enough to be reabsorbed from the intestine. However, intestinal bacteria can break down the conjugates (through β-glucuronidase and GST activity), and make the metabolites available for reabsorption. This process has been described for BP-metabolites in rats (Chipman et al., 1981). Since it was shown, using control fish in enclosures, that secondary uptake of metabolites was trivial, the sudden increase in bile metabolites in BP injected fish is unlikely to be attributed to secondary uptake of excreted metabolites from the surrounding water. Therefore the hypothesis of entero-hepatic cycling to explain the bimodal pattern of bile metabolites over time is a reasonable explanation.
References


Chapter 6. Metallothionein Induction by Cd, and Interactive Effects of Simultaneous BP Dosage.

Introduction

Initial phases of this research confirmed induction of BP metabolism in mummichog and demonstrated the interactive effects of Cd on the induction of CYP1A, on the biotransformation of BP and on the excretion of BP metabolites. The mortality experiments suggested that at certain dose combinations, BP exposure may also have an effect on the toxicity of Cd. The last part of this research project was focused on the mechanisms that may explain why BP has an synergistic effect on Cd toxicity.

It is known from a wide variety of invertebrate and vertebrate species that exposure to heavy metals, and especially cadmium, induces the production of metal binding proteins (Roesijadi, 1992). Analysis of cytosolic fractions, prepared from the livers of metal exposed animals, has revealed that there are 3 groups of metal binding proteins: a high molecular weight fraction (>20 kD), a low molecular weight fraction (5-15 kD) and a very low molecular weight fraction (<1 kD). The metal binding proteins in the low molecular weight fraction are generally called metallothioneins (MT). This fraction can be measurably increased after exposure to metals. Apart from metals, of which Cd and Zn are the strongest inducers, MT can also be induced by oxyradical producing agents, like UV light, nitrosamines, hydrogen peroxide, and dexamethasone. It has been demonstrated that Cd exposure also induces the production of glutathione (GSH), heat shock proteins (HSP) with molecular weights of 70, 90 and 110 kD, and heme oxygenase (Beyersmann & Hechtenberg, 1997).

Some of the heavy metals, like Cu, Fe, and Zn, are essential for biological/enzymatic processes in the cell. However, the free ion form of these metals can be highly toxic inside the cell. Distribution of iron inside the cell is mediated through an iron-binding protein, ferritin, and a receptor (transferrin) that can release Fe from the
protein. For Cu and Zn, metallothioneins appear to be the detoxifying, binding protein, comparable to ferritin for Fe. However, it is still controversial whether the metals bound to MT are available for copper or zinc dependent processes (Brouwer, 1995).

Analysis of the structure of MTs from different species has revealed that they are very conservative in structure. There are generally 60 to 61 amino acids, of which 20 are cysteine. The position of the cysteine residues within the protein is highly conserved. In most species at least two isoforms are present (MT I, MT II, etc), as analyzed by anion-exchange chromatography. Functional differences have not been described for these isoforms. Analysis of the secondary structure of MT has shown that the protein consists of two domains, named the α-domain and the β-domain, both consisting of 30 amino acids. The α-domain can hold four metal ions, while the β-domain can bind three. The metals are exclusively associated with the cysteine residues through tetrahedral thiol-bonds. The cysteine-metal bonds are considered very stable, but metals can be released in a low pH environment. The stability of metal saturated MT is used during purification: heat denaturation at 60°C precipitates most proteins, but not MT. MT induction is temperature dependent, and generally slower in fish than in mammals. In rainbow trout, a sharp increase in MT mRNA was observed 6-12 h post injection, but dropped soon after that. MT protein levels stayed high, but were organ specific. The half life of MT in fish is around 30 days. MT levels show seasonal variation in female fish, related to egg production. Vitellogenesis requires an increased level of Zn, which obviously triggers an increased MT level during egg production (Olson, 1993).

Human and rainbow trout metallothioneins show differences in copper binding capacity (12 and 10 g/mol respectively), but no differences in Cd binding capacity (7 g/mol). Recombinant metallothionein proved that the differences originated in the α-domain of the protein. The “mermaid” form of the recombinant metallothionein, with an α-domain (the C-terminal, or tail) originating from the fish genome, and the β-domain (the N-terminal, or head) originating from the human genome, had a lower Cu binding capacity (10 g/mol) than the “fishman” form, with an α-domain originating form the human genome, and the β-domain originating from the fish genome (12 g/mol) (Kille et al., 1993).
The specific effects of Cd exposure in mammalian cells were reviewed by Beyersmann & Hechtenberg (1997). Cd exposure induces not only MT synthesis, but also increases cysteine uptake and glutathione synthesis, which makes the analysis of Cd uptake kinetics a complex task. Time is an important factor in the process: a single dose or a long-term, low dose exposure will lead to total MT binding of the Cd. After a single dose there is immediate accumulation of "free" Cd in the cytosol and the nucleus. After 24 h, all Cd is bound to MT in cytosol. Cellular Cd toxicity is probably an indirect effect, caused by the generation of oxyradicals. No acute cytotoxic effect could be observed when antioxidants are available, and peroxide-resistant cells were also Cd resistant. Beyersmann & Hechtenberg (1997) also address the conflicting evidence of Cd effects on DNA/RNA synthesis and protein metabolism. Free Cd can inhibit all of these processes, but several studies have reported that when Cd is bound to cysteine residues, either in MT or GSH, it can still induce the synthesis of more metal binding proteins. Stimulated DNA/RNA synthesis under Cd stress is explained as an effect of cell damage caused by Cd, which induces repair mechanisms.

MT induction in mummichog was first described by Pruell & Engelhardt (1980). They exposed mummichog to aqueous Cd (10 and 25 mg/l for 96 h), and found elevated Cd binding protein levels at the higher dose. The molecular weight of these proteins were estimated to be 7000 to 10,000. They also found evidence that preexposure to a low metal dose induces a very low molecular weight (<1000) Cd binding protein, which may be glutathione. An observed increase in cytosolic Cu is explained to be a result of competition between Cu and Cd for binding sites on the available MT. Cd is know to have a stronger affinity for MT than Cu, and will therefore replace Cu. Weis (1984) measured binding of mercury (Hg) to MT in mummichog. Hg did not induce MT levels in embryos. He found considerable variation in MT levels in embryos, which proved predictive for the MT levels at later age.

In the mortality experiments described above (Chapter 2), it was demonstrated that a sublethal dose of BP, together with sublethal amounts of Cd, significantly increased the
mortality above the level that could be expected for linear additivity of Cd and BP. This observation implies that BP or its metabolites intervene with processes in the cells that would normally reduce the toxicity of Cd. Given that some intermediate BP metabolites can be highly reactive and bind to DNA and other cellular proteins, they may also bind to the cysteine residues on MT, thus competing for binding places with Cd. This would mean that there is direct competition between Cd and BP metabolites for binding sites on MT. Another possible mechanism is that BP metabolism interferes with MT synthesis, either through effects on the transcription/translation process, or by competition for protein residues. Primary BP metabolites are in part conjugated to glutathione (GSH). Cd exposure induces GSH production, but when GSH, and thus one of its building blocks, cysteine, is being scavenged by BP metabolism, there will be less cysteine available for MT synthesis.

The questions that were addressed in the experiments described in this chapter are:

• How do different Cd doses induce MT in mummichog over time?
• Does BP have an effect on MT induction when dosed together with Cd?
• Do BP metabolites bind to MT?
• If BP metabolites bind to MT, will the Cd binding capacity of MT be reduced?
Material and methods

Experimental design

Three experiments were designed to study induction of MT, and to investigate effects of BP on MT induction and binding. The first experiment was a pilot experiment designed to measure the induction of MT by Cd. Fish were injected with a low (0.32 mg/kg) and a high (3.2 mg/kg) Cd dose, together with a control group treated with solvent only. The fish were kept in a flow through system. Four replicate fish were sampled from each treatment on days 4, 7 and 14. The livers were harvested, frozen in liquid nitrogen, and stored at -80°C until further analysis.

In the second experiment the effect of combined Cd and BP exposure on MT induction was studied. Five treatment groups were incorporated: Cd alone (1 mg/kg), BP alone (18 mg/kg for all treatments), BP with a medium Cd dose (1 mg/kg), BP with a high Cd dose (3.2 mg/kg) and a medium Cd dose 4 days before BP. The BP solution was spiked with $^3$H-BP, so that each fish would receive a dose of 5 μCi. Radiolabeled BP was used to analyze if BP metabolites would bind to MT after in vivo exposure. Four replicate fish were sampled from each treatment on days 1, 2, 4, 7 and 11. The fish in this experiment had to be kept in a closed system, because excreted radioactive material had to be contained. To investigate if potential excretion and reabsorption of Cd would influence the results, uninjected control fish were kept in an enclosure inside the holding tank. These uninjected control fish were sampled on days 2, 4, 6 and 11. A third experiment was designed to study if BP metabolites, generated by isolated microsomes from radiolabeled BP, would bind to purified MT in vitro.

MT purification

Frozen livers were thawed, weighed and homogenized with a Polytron homogenizer in 2 ml 10mM Tris buffer (pH 8.5), containing 0.5 mM phenylmethyl sulfonyl fluoride (PMSF) as an antiproteolitic agent and 0.01% mercaptoethanol as a reducing agent. A cytosolic fraction containing dissolved MT was prepared by
centrifugation at 100,000 x g for 60 min at 4°C. A subsample of 1.5 ml supernatant was collected in a microcentrifuge tube, spiked with 0.24 mM Cd\(^{2+}\), and incubated for 10 min to saturate the MT with Cd, and thus avoid polymerization during the following denaturation step. The samples were heated in a heating block for 15 min at 60°C to denature the non-metallothionein proteins, followed by cooling down in ice water and centrifugation for 20 min at 10,000 g to spin down the denatured proteins. The MT containing supernatant was collected in 1 ml autosampler vials and stored at 4°C until analysis by HPLC.

**MT analysis**

Metallothionein was quantified by HPLC-gel filtration chromatography (Jin et al., 1993). This method was chosen because it would allow simultaneous detection of MT and radiolabeled BP metabolites with UV and radiodetector. A Superdex 75 HR 10/30 gel filtration column was coupled to a HPLC configuration with two Waters 510 pumps, a Waters 717plus autosampler, Waters 486 absorbance detector, and a Radiomatic Flo-one\Beta, type A-100 radio detector with a 500 \(\mu\)l flow cell. The mobile phase consisted of 0.1 M sodium phosphate buffer (pH 7.4) with 0.2 M NaCl, at a flow rate of 1 ml/min. MT was measured in 200 \(\mu\)l subsamples with the UV detector set at 250 nm. The retention time for MT under these conditions was 13.8-14.1 min. Molecular weight of the assumed MT peak was established from a calibration curve using the following molecular weight markers: Aprotinin (Mol.Wt. 6500), Cytochrome C (12,400), Carbonic Anhydrase (29,000), and Albumin (66,000). Dextran Blue (2,000,000) was used to measure the void volume of the column (Figure 6-1).

A second calibration curve was produced to relate peak heights on the chromatograms to MT weight units. A seven step dilution series (100% to 1.56%) was made from a stock solution of 0.667 mg/ml purified rabbit MT. There was a linear regression between MT concentration and peak height, with a correlation coefficient of 0.9998. Peak heights of analyzed samples (10,000 - 160,000) fell well within the range of the calibration curve, therefore no extrapolations needed to be made.
During the initial phase of method development, MT was measured in a series of cytosolic fractions that were collected during microsome preparations (see Chapter 4). However, the chromatograms from these samples were not suitable for reliable MT quantitation; the MT peak was either very broad, or coeluting with another protein peak. The stabilization buffer used for microsome preparations was obviously not appropriate for MT extraction, possibly because the lack of a reducing agent like mercaptoethanol. In an oxidative environment MTs tend to polymerize, creating a broad peak (Roesijadi & Drum, 1982). Reducing agents can not be used during preparation of microsomes, because of potential negative effects on P450 activity.

Initially, MT content was expressed as μg per mg cytosolic protein. However, the gel filtration chromatograms showed that there was considerable variation in denatured protein content, resulting in a wide range of coefficients of variation. Expressing MT per wet liver weight proved to be a more consistent way, with lower coefficients of variation. Wet liver weights of the fish from the selected experimental size range fell in a narrow weight range (0.15-0.30 mg, with only a few outliers in a sample of 47, avg 0.23 g, sd = 0.08). Therefore the average wet liver weight of these fish was used to normalize MT amounts in livers from fish for which the liver weight was not measured.
Figure 6-1. Calibration curve to establish molecular weight of compounds separated by HPLC-gel filtration column. Ve is the elution volume for each compound, V0 is the void volume of the column, as measured with dextran blue. Regression equation is: mol weight/1000 = -1.575*Ve/V0 + 6.61. R² for regression was 0.989
In vitro experiment

Microsomes were used from BP treated fish, two days after injection, with maximal CYP1A activity. One mg of microsomal protein in stabilization buffer was added to 1 ml of incubation buffer (see Chapter 4 for details). Aliquots of 67 μg MT in Tris buffer with 0.01% mercaptoethanol were added, together with 5 μCi ³H-BP in a small amount of methanol. The reaction mixture was incubated at 27°C, and was sampled at 3, 6, 12, and 24 h after the start of the reaction. Subsamples of 225 μl were taken for MT analysis, and 75 μl samples were analyzed for BP metabolism. Metallothionein samples were prepared and analyzed as described above. The subsamples for BP analysis were mixed with 125 μl methanol, containing BHT as an anti-oxidant, followed by centrifugation (14,000 g for 20 min) to spin down precipitated proteins. The supernatant was transferred to 200 μl autosampler vials, and analyzed by reversed phase HPLC as described in Chapter 4. The pellet was resuspended in 2*200 μl methanol, and was counted by liquid scintillation counter.

Statistics

Statistical analysis of the data was performed as described in Chapter 1.
Results

**MT induction at different Cd levels**

A typical HPLC-gel filtration chromatogram for analysis of MT shows a MT peak at 14 min (Peak B in Figure 6-2). Confirmation that peak B was indeed MT was obtained by comparing the retention time of this peak with the MT standard used for creating the calibration curve described above. Also, the estimated molecular weight (12,000) of the compound in peak B, as obtained from the calibration curve in Figure 6-1, falls within the reported range for MT. Finally, the retention time of peak B is comparable with the MT peak described by Jin et al. (1993), who used the same column under the same conditions as described here.

At the lowest Cd dose used (0.32 mg/kg), no induction of MT could be measured, i.e. there were no significant differences at any time when compared to the control, (Table 6-1, Figure 6-3). The highest Cd dose (3.2 mg/kg) resulted in 2.1 mg MT/g wet liver, a 4 fold induction on day 7 post-injection. The induction persisted at this level until at least day 14. At the intermediate Cd dose of 1 mg/kg, which was used in the second experiment, there was a slightly lower MT induction of 1.5 mg MT/g liver (Figure 6-4).

**MT induction in combined exposure**

The results of MT analyses in the uninjected control fish are shown in Table 6-2 and Figure 6-5. MT levels were low in all treatments (< 1.2 mg/g) on all sampling days. On day 2 there seems to be a slight induction of MT in the Cd only treatment, and in the combined BP+Cd treatments there seems to be a delayed induction towards day 11. However, none of the inductions proved to be significant in any of the treatments, on any of the sampling days. This means that it can be assumed that potential recirculation of excreted Cd in a closed system did not have an enhancing effect on MT induction in the injected fish that were kept in this system.
Figure 6-2. Gel filtration chromatogram of liver cytosol sample from fish treated with Cd (3.2 mg/kg) and BP (18 mg/kg), after heat denaturation, measured with UV detector (250 nm). Peak A corresponds with metallothionein standard, other peaks were not identified.
Figure 6-3. Amounts of hepatic MT (mg/g liver) in fish injected with a low (CdL) and high (CdH) dose of Cd (0.32 and 3.2 mg/kg), at different days post injection. Data points represent average of 4 replicates.
Figure 6-4. Amounts of hepatic MT (mg/g liver) in fish injected with a medium (CdM) and high (CdH) dose of Cd (0.32 and 3.2 mg/kg) combined with BP (18 mg/kg), at different days post injection. Data points represent average of 4 replicates.
MT (mg/g)

0.5
1
1.5
2
2.5
3
3.5

0 2 4 6 8 10 12

days post-injection

BP BP+CdM BP+CdH CdM_BP CdM

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Table 6-1. Metallothionein content in fish in flow-through experiment. Average values, with standard deviation, n=4, except for †: n=3. A=significantly different from other treatments on the same day, B=significantly different from blank.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 0</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0.91 (0.21)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvent contr.</td>
<td></td>
<td>0.34 (0.02)</td>
<td>0.70 (0.19)</td>
<td>1.00 (0.71)†</td>
</tr>
<tr>
<td>BP</td>
<td></td>
<td>0.49 (0.02)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cd Low</td>
<td></td>
<td>0.67 (0.24)</td>
<td>0.71 (0.17)</td>
<td>0.70 (0.07)</td>
</tr>
<tr>
<td>Cd High</td>
<td>1.56 (0.16)A</td>
<td>2.06 (0.53)A,B</td>
<td>2.11 (0.17)A,B</td>
<td></td>
</tr>
</tbody>
</table>

Table 6-2. Metallothionein levels (mg/g wet liver weight) in control fish from enclosures. Mean values, with standard deviation, n=3. No significant differences were detected.

<table>
<thead>
<tr>
<th>Day</th>
<th>Blank</th>
<th>Cd Med.</th>
<th>BP+Cd Med.</th>
<th>BP+Cd High</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.67 (0.21)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.17 (0.17)</td>
<td>0.77 (0.39)</td>
<td>0.57 (0.04)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.88 (0.25)</td>
<td>0.80 (0.09)</td>
<td>0.89 (0.89)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.77 (0.14)</td>
<td>0.64 (0.15)</td>
<td>0.87 (0.09)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.68 (0.18)</td>
<td>1.10 (0.10)</td>
<td>1.19 (0.46)</td>
<td></td>
</tr>
</tbody>
</table>
In the second experiment, in which the effects of BP on MT induction were studied, the BP alone treatment was used as control group (Figure 6-4). Statistical analysis showed that there were no significant changes in the MT levels over time for the BP alone treatment when compared to the base line levels (day 0) before injection (Table 6-3). Therefore, the BP alone treatment was used as the control group. After day 1 post-injection, all other treatments in this experiment had significantly higher MT levels than the BP alone treatment, on all days.

The treatment with the intermediate dose of 1.0 mg/kg Cd alone was used in this experiment as a reference for comparison with combined dosages. For this Cd alone treatment the maximum MT level was reached by day 1. When BP was dosed together with Cd at intermediate and high level, a slower induction of MT was observed; the maximum MT level was reached on day 2 for the highest Cd dose, and around day 4 for the intermediate Cd level. It appears that BP had an inhibiting effect on the MT induction in the first days after injection when compared to the treatment were only Cd was dosed.

For the combined BP+Cd High treatment, a rapid induction of liver MT was found after day 1; the maximum MT concentration of 3.0 mg/g was reached on day 2 post-injection (Figure 6-4). This is consistently higher than in the first experiment, where the maximum MT concentration was 2.1 mg/kg in the high Cd alone dose. It appears that the simultaneously dosed BP increases and accelerates the MT induction after the first day.

The initial inhibition of MT induction by BP is also seen at the intermediate Cd dose levels. On day 1 there is a significantly lower amount of MT in the combined exposure than in the Cd alone treatment. Though not statistically significant (p<0.05) there was more MT in the combined treatment than in the Cd alone treatment on each sampling day. This is consistent with the results for the highest Cd dose when the Cd alone treatment is compared with the combined treatment, as described above. It appears that initially BP inhibits the induction of MT, but after a few days the induction is actually enhanced.
Figure 6-5. Amounts of hepatic MT (mg/g liver) in uninjected fish that were held in enclosures in recirculation tanks with injected fish. Cd doses in the injected fish were 1 (CdM) and 3.2 (CdH) mg/kg, BP dose of the injected fish was 18 mg/kg. Data points represent average of 4 replicates.
Table 6-3. Metallothionein content (mg/g wet liver weight) in static experiment. Mean values, with standard deviation. N=4, except for †: n=3, ‡: n=6. A=significantly higher than other treatments on the same day, B=significantly different from blank, C= significantly lower than other treatments on same day.

<table>
<thead>
<tr>
<th>Day</th>
<th>Blank</th>
<th>BP</th>
<th>Cd Med.</th>
<th>BP+CdM</th>
<th>BP+CdH</th>
<th>Cd bf. BP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.83 (0.28)‡</td>
<td>0.56 (0.10)</td>
<td>1.53 (0.33)\textsuperscript{A,B}</td>
<td>0.98 (0.21)</td>
<td>1.17 (0.24)\textsuperscript{A}</td>
<td>0.98 (0.13)</td>
</tr>
<tr>
<td>1</td>
<td>0.40 (0.19)\textsuperscript{C}</td>
<td>1.51 (0.14)\textsuperscript{B}</td>
<td>1.09 (0.20)</td>
<td>2.96 (0.42)\textsuperscript{A,B}</td>
<td>1.18 (0.44)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.55 (0.33)\textsuperscript{C}</td>
<td>1.43 (0.22)\textsuperscript{B}</td>
<td>1.66 (0.49)</td>
<td>3.11 (0.33)\textsuperscript{A,B}</td>
<td>1.80 (0.17)\textsuperscript{†B}</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.76 (0.14)</td>
<td>1.54 (0.17)\textsuperscript{B}</td>
<td>1.94 (1.07)\textsuperscript{B}</td>
<td>2.95 (0.64)\textsuperscript{B}</td>
<td>1.70 (0.45)\textsuperscript{†B}</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1.01 (0.49)\textsuperscript{C}</td>
<td>1.68 (0.21)\textsuperscript{B}</td>
<td>1.89 (0.38)\textsuperscript{B}</td>
<td></td>
<td></td>
<td>1.94 (0.26)\textsuperscript{†B}</td>
</tr>
</tbody>
</table>
A special effect was observed when Cd was dosed 4 days before BP. Assuming (because it was not measured) that there was a rapid induction of MT in those 4 days, as in the Cd only treatment, surprisingly, MT levels are significantly lowered one day after the BP injection, and then rise to a maximum on day 4 (Figure 6-4).

**Binding of BP to MT**

*in vitro* experiment

Potential binding of BP metabolites to MT was studied first in an *in vitro* system. Microsomes with a high CYP1A activity were isolated from BP treated fish. These microsomes were incubated with radiolabeled BP and standard rabbit MT. The reaction mixture was analyzed for MT by gel filtration HPLC with both a UV detector and a radiodetector. Chromatograms from the UV detector were overlain with those from the radio detector to identify if any radioactivity was associated with the MT peak (Figure 6-6). The UV detector shows several peaks, with the MT peak at 14 min. The radiodetector shows only one peak at 21 min.

Obviously there was no radioactivity associated with the MT peak. This was consistent for all chromatograms examined for this experiment. A subsample from the reaction mixture was also analyzed by reversed phase HPLC, to investigate the nature of the radioactive 21 min peak on the gel filtration chromatogram. The chromatograms of this reversed phase analysis show that most radioactivity is associated with the void volume peak, which contains conjugated BP metabolites (Figure 6-7). Apart from the conjugated metabolites, detectable amounts of 7,8-diol, 9,10 diol and tetrols were present.
Figure 6-6. Gel filtration chromatograms of reaction mixture with microsomes from induced fish, $^3$H-BP and purified rabbit MT, 24 h after start of reaction. Overlay of radiodetector on UV detector (250 nm). Peak A (UV detector) coelutes with MT standard. Peak B (radiodetector) and Peak C (UV detector) were identified as BP metabolites, predominantly in conjugated form.
**Figure 6-7.** Reversed phase radioactivity chromatogram of ethyl acetate extracted reaction mixture containing microsomes from induced fish, $^3$H-BP and purified rabbit MT, 24 h after start of reaction. Peak A coelutes with tetroles, peak B is 9,10-diol-BP, peak C is 7,8-diol-BP.
-in vivo experiment

Binding of radiolabeled BP metabolites to MT was also analyzed in a subset of samples used to identify the effect of BP on MT induction, as described above. One sample from each treatment (BP alone, BP+CdM, BP+CdH, Cd before BP) for each sampling day (day 2, 4, 7 and 11) was measured simultaneously with a UV detector for MT content and a radiodetector for radiolabeled compounds. Separate analysis on a scintillation counter of an equal amount of sample as injected into the HPLC (200 μl), showed a radioactivity of 365 - 7577 dpm. However, no quantifiable peaks showed up on any chromatogram generated by the radiodetector. Obviously the radioactivity in the cytosolic fraction was below the detection limit of the radiodetector, because even if there was no binding of BP metabolites to MT, at least one peak for the conjugated metabolites should have been evident in some samples, as was observed in the in vitro experiment described above.

Mortality

During the second experiment, unexpected mortality occurred (Figure 6-8). These results cannot be compared directly to those obtained in the mortality experiments described in Chapter 2, because of differences in experimental set up (flow-through vs. static), and because fish were sampled and sacrificed at regular intervals in the last experiment. Nevertheless, the trends in mortality are comparable: no mortality in the Cd alone treatment (1 mg/kg), an intermediate mortality at the BP alone treatment (18 mg/kg), high mortality in the BP+CdHigh (3.2 mg/kg) treatment, and most interestingly, in both treatments were BP was combined with intermediate Cd dose (1 mg/kg) mortality was lower than in the BP alone treatment. This is consistent with the earlier observation that intermediate Cd doses have an antagonistic effect on BP toxicity.
**Figure 6-8.** Cumulative mortality over time post injection of fish injected with a medium (CdM) and high (CdH) dose of Cd (0.32 and 3.2 mg/kg), combined with BP (18 mg/kg). Each treatment consisted of 50 fish each at day 0. On days 1, 2, 4, 7 and 11 five live fish were sampled and sacrificed from each treatment for bile and liver analysis.
Discussion

MT levels in other species

The measured levels of MT in this study range from a background level of 0.8 mg/g wet liver weight in control fish to 1.5 - 3 mg/g in Cd dosed fish. Romeo et al. (1997) reported comparable MT levels of 1.7 - 4.3 mg/g wet liver in sea bass, 24 h after injection with 0.5 mg/kg Cu. George & Young (1986) report 0.05 - 0.3 mg/g wet liver in plaice, 14 days after a single dose of 1 mg/kg Cd. These values may be underestimated as a result of a different method for MT analysis, the \(^{109}\)Cd saturation method, according to Jin et al. (1993), who compared the saturation technique with the gel filtration chromatography method used in this study.

Molecular weight

Metallothioneins are low molecular weight (6,000-15,000 D) proteins. The following molecular weights have been reported for fish species: 10,000 for carp (Yamamoto et al, 1978), 11,000 ±500 for goldfish (Carpene et al.,1987), 15,000 for plaice (Overnell & Coombs, 1979), and for mummichog 7-10,000 (Pruell & Engelhardt, 1980) and 14,000 (Weis, 1984). The molecular weight of 12,000 as measured in this study falls well within this range. Variations in molecular weight are likely a result of the analytical method used. Gel filtration chromatography tends to yield higher molecular weights than gel electrophoresis because of the non-globular shape of the MT protein (Overnell & Coombs, 1979).

Induction over time

Maximum MT induction was observed at day 7 for the highest Cd dose, and this high level persisted until at least day 14. Olson (1993) reported a half life of 30 days for MT in fish, indicating that breakdown or excretion of MT is slow. For the medium Cd dose, maximum MT was already achieved after 24 h. George & Young (1986) reported the first measurable induction of MT 6 days after injection of 1 mg/kg in plaice. This
difference in induction time may be species specific, or it can be a temperature effect. George & Young (1986) kept their fish at 10°C, while the experiments described here were performed at 18-22°C. Induction times for MT mRNA are highly temperature dependent, ranging from 192 h at 6°C to 3-6 h at 18-20°C as shown in rainbow trout (Olson, 1993). Carpene et al (1987) show for goldfish that 10 h after injection (4 mg/kg, 3 times) Cd is bound to a macromolecular fraction, an MT fraction and a very low molecular weight, or free ion fraction. This last fraction disappears over time (20 days), while the MT fraction substantially increases. They also note a slow turn-over of the MT fraction (> 30 days).

Dose response relations

The results from the experiments described here show that MT is significantly induced in mummichog at dose levels of 1 and 3.2 mg/kg, but not at not 0.32 mg/kg. George et al. (1996) describe a linear dose-response induction for MT in turbot, for Cd doses below 0.2 mg/kg. Several authors have described that above a certain metal dose, there is no further increase of MT levels (cited in Olson, 1993). Apparently there is a "critical concentration", above which the metals interfere directly with MT synthesis, or indirectly inhibit metabolic processes that are essential for MT production. George et al. (1986, 1996) demonstrated the "critical concentration" concept for plaice and turbot, Harrison & Lam (1986) describe it for Cu in bluegills, and Ueng et al (1996) observe the same effect in tilapia with no further increase of MT induction at Cd doses higher than 2 mg/kg.

George (1989) argues that for plaice direct inhibition of protein synthesis occurs at Cd doses of 0.5 mg/kg and higher. His calculations show that for plaice injected with up to 0.1 mg/kg Cd there was enough MT to bind all Cd to MT. At higher doses (0.5 and 1 mg/kg Cd) there was not enough MT to bind all Cd. He demonstrated that there was enough cysteine and GSH to synthesize more MT, which obviously did not happen. He therefore concluded that at high doses, Cd inhibits protein synthesis, and specifically MT synthesis.
Other interaction papers

From the results presented here it appears that BP dosage initially has a inhibitory effect on MT synthesis, but after 1-4 days MT synthesis is enhanced. In a comparable study, sea bass (*Dicentrarchus labrax*) were injected intraperitoneally with 0.5 mg/kg copper and/or 20 mg/kg BP (Romeo et al., 1997). The Cu-only treatment did not significantly alter the hepatic MT content (1.7 mg/g), but the BP treatment significantly lowered the MT level (0.4 mg/g) while the combination of Cu and BP significantly increased the MT amount (4.3 mg/g). That copper alone did not increase the MT level is speculated to be caused by a too high Cu dose, which may have inhibited protein synthesis, as argued above. The lowering of MT synthesis by BP may be explained as a result of competition for cysteine residues by glutathione synthesis, induced by BP metabolism. Glutathione is a cysteine containing tripeptide (glu-cys-gly). The authors did not have data to explain the significant increase in MT levels in the combined treatment, but hypothesize that increased production of oxyradicals may have enhance the MT induction. Reactive oxygen species have been reported as a by product of incomplete oxygenation of organic contaminants by P450 monooxygenases (Di Giulio et al., 1995).

Brown et al. (1987) suggest three possible explanations for low liver metal concentrations in fish from heavily polluted sites: 1) reduction of MT synthesis due to competition for cysteine by induced GSH synthesis, which is used for detoxification of organic metabolites. (This was supported by higher GSH levels in fish from contaminated sites); 2) reduced binding of metals to MT because of oxidation of the thiol (-SH) groups on the MT by hydroxyl radicals, which can be formed during metabolism of organic contaminants; 3) reduced binding of metals to MT because of competition for metal binding sites by organic metabolites.

Even though the mechanism has been suggested by Klaassen & Cagen (1982), no binding of BP metabolites to MT could be demonstrated in this study. This would mean that enhanced toxicity of Cd by coadministration of BP, as shown in the mortality experiments, is more likely a result of the inhibition of MT synthesis, than of interference of BP metabolites with the binding of Cd on MT.
So far there is no indication that BP interferes with binding of Cd to MT. The higher MT levels in combined treatments than in Cd alone treatments seem contradictory with an increased mortality in the combined treatments. The high levels of MT supposedly should protect against Cd toxicity, and not enhance toxicity. Still, the combination of BP with sublethal doses of Cd (both 1 and 3.2 mg/kg) causes dramatic mortality. One explanation may be that the crucial time period for the eventual effects is within the first hours to days after the injection. If the observed delayed MT induction in the first days, as a result of BP metabolism, allows unbound Cd to cause irreversible cell damage, than even the high MT levels after several days may not be able to ward off the damage, which causes mortality. It is also possible that because of cell damage, MT production is induced to scavenge free radicals that have been produced (Klaassen & Liu, 1997).
References


George, S. G., Young, P. (1986). The time course of effects of cadmium and 3-methylcholanthrene on activities of enzymes of xenobiotic metabolism and metallothionein levels in the plaice, Pleuronectes platessa. Comp. Biochem. Physiol. 83C: 37-44


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Overview of obtained results

This study was designed to investigate the interactive effects of Cd and BP in mummichog. These effects were to be studied at different organizational levels in the organism: whole animal mortality, BP metabolism in isolated hepatocytes and liver microsomes, BP metabolite excretion through bile and MT levels in liver cytosol. It was expected that this multi-level approach would generate more insight in the underlying mechanisms for interaction of BP and Cd, and help to elucidate how biochemical effects at subcellular level can be translated to the cellular and organismal level.

The results from the mortality studies showed that at some dose combinations a synergistic effect occurred, while at other dose combinations an antagonistic effect was observed (Chapter 2). These results guided the design of experiments to study questions on how Cd interferes with BP metabolism, and how BP exposure may have an effect on Cd toxicity.

The experiments investigating BP metabolism in isolated hepatocytes from preexposed fish demonstrated that Cd has an inhibiting, or delaying effect on the induction of BP metabolism (Chapter 3). The first indications were found for an effect of Cd on the production of BP metabolizing enzymes, and not on a direct inhibition of native enzyme. When BP-induced hepatocytes were incubated with Cd there was no acute effect of Cd on the capacity of the cells to metabolize BP. Analysis of the primary metabolites formed did not show a change under influence of Cd exposure. If Cd would have directly inhibited CYP1A, other biotransformation pathways might be enhanced, resulting in a different metabolite spectrum.

Further evidence for an indirect effect of Cd on the BP-metabolizing CYP1A enzyme was provided in the experiments investigating the catalytic activity by liver
microsomes from pretreated fish (Chapter 4). In livers from fish that were exposed to a combination of Cd and BP, the induction of CYP1A was inhibited. This was confirmed by a lower EROD activity and lower BP metabolism in microsomes from fish treated with a combination of Cd and BP, compared to BP alone treatment. Analysis of the catalytic activity per unit of enzyme revealed that the enzyme itself was not affected, which excludes a direct inhibiting effect of Cd on CYP1A.

Conjugated BP metabolites are predominantly excreted through the bile. Glucuronidation appears to be the predominant conjugation pathway for BP metabolites in mummichog (Chapter 5). Cadmium exposure accelerated the excretion of BP metabolites, possibly by inducing the production of conjugating compounds.

Injection of mummichog with Cd resulted in a measurable induction of MT (Chapter 6). Coexposure to BP had an inhibiting effect on MT induction. The original hypothesis was that BP metabolites that are generated during biotransformation of BP would bind to MT, and thus inhibit the binding of Cd to MT. No evidence could be found in in vitro or in vivo experiments to support this hypothesis. The synergistic effect of BP on Cd toxicity may therefore be sought in an inhibition of MT synthesis by BP metabolites, slowing down the detoxification of Cd, and giving it more time to exert toxic effects. Inhibition of MT synthesis by BP could be a result of competition for cysteine, which is one of the building blocks for both MT and for BP conjugating compounds (glutathione).
Mechanisms for interactive Cd-BP effects.

The following scenario is proposed to provide a consistent interpretation of the data presented herein, combined with data from the literature.

**BP metabolism**

Mummichog has a measurable amount of native CYP1A. When uninduced fish are exposed to BP, this base level amount of CYP1A will start metabolizing the administered BP. Meanwhile, BP that enters the liver cells will bind to the AH receptor, be transported to the nucleus, bind to the ARNT receptor, and thereby induce DNA transcription into mRNA production. This CYP1A-mRNA will produce the CYP1A apo-enzyme in the cell plasma, which will in turn be transformed into the holo-enzyme after incorporation of the heme group on the active site of the enzyme (Stegeman & Hahn, 1994).

**Indirect effect of Cd on BP metabolism**

The experiments described here, supported by published results (George, 1989; Bruschweiler et al., 1996), show no direct inhibition of the CYP1A enzyme when Cd is dosed to live fish or to live, isolated hepatocytes. This observation seems to conflict with reports of direct inhibition of CYP1A by Cd (Means et al., 1979; Bruschweiler et al., 1996). However, the experiments in which direct inhibition was observed were all done with isolated microsomes. Obviously, when microsomes are isolated from the protective mechanisms in the living cell, direct inhibition by Cd ions can occur.

In living cells, the effect of Cd on BP metabolism consists of an inhibition of the production of new enzyme. This can be caused by a variety of interactions. Cadmium could interfere with the binding of BP to the AH receptor, or the nuclear ARNT receptor. However, Bruschweiler et al. (1996) consider binding of Cd to the AH receptor as an unlikely scenario, because, despite the inhibiting effect of of heavy metals, at least some CYP1A induction has been observed in the presence of metals. The Cd effect is therefore most likely to occur in the process of protein synthesis. George (1989) suggested a
general shut down of protein synthesis at high Cd doses (1 mg/kg in flounder), which is reflected in reduced mRNA amounts. Another often cited possibility is a shortage of heme for incorporation into the CYP1A enzyme (Eaton et al., 1980; Ariyoshi et al., 1990).

**Heme oxygenase**

P450 enzymes belong to a group of heme containing proteins. Other compounds belonging to this group are hemoglobin, catalase, and cytochromes b and c. The important role of these proteins and enzymes make heme metabolism an important cellular process.

A heme group consists of a divalent metal ion in a porphyrin ring (Tenhunen et al., 1969). Synthesis of heme is regulated by aminolevulinate (ALA) synthetase. Surplus heme is degraded by heme oxygenase (HO), which removes the ion from the porphyrin ring to create biliverdin, which may be further degraded to bilirubin and excreted as a glucuronide conjugate. The cycle of synthesis and degradation is self regulating: excess heme will inhibit ALA synthetase and induce HO. The active regulator is the metal ion in the heme group, and the process is not very metal specific. That is probably the reason why exposure to exogenous metals interferes with heme homeostasis, and thus induces HO activity (Maines & Kappas, 1977).

Heme oxygenase does not interfere directly with the heme group of intact P450. Instead, the P450 first needs to be denatured to P420, to allow HO to use the heme group as a substrate for oxidation (Kutty et al, 1988). This suggests that the mechanism for interaction of HO on P450 is proactive: HO activity does not break down P450, but reduces the cellular pool of heme, hampering the incorporation of heme into the P450 apoenzyme to form the holoenzyme.

Induction of HO by exposure to Cd and other heavy metals has been well documented, and is normally accompanied by a decrease of P450, both in mammals (Maines & Kappas, 1977; Eaton et al., 1980) and in fish (Ariyoshi et al., 1990a). Schlenk et al. (1996) even report an inverse relationship between CYP1A and HO in largemouth bass taken from a pollution gradient in the field. The induction of HO by Cd may also explain the inhibition (by breakdown) of catalase, another heme containing enzyme, as
reported for mummichog by Jackim et al. (1970) and Pruell & Engelhardt (1980).

**P450-reductase**

While Cd may not have a direct effect on the catalytic activity of P450, it may have a direct effect on NADPH-cytochrome P450 reductase. This reductase restores CYP1A to the reduced state after its catalytic action. A negative effect of thallium on NADPH cytochrome P450 reductase was documented by Woods & Fowler (1984) in rats. However, the potential for Cd to produce this effect was not found in rats (Schnell et al., 1979; Means et al., 1979), indicating that Cd does not have an effect on electron-transporting capacity of NADPH-cytochrome P450 reductase.

**Dose effects**

From several studies it has become obvious that Cd dose is an important variable affecting the effects on both P450 and HO amounts and activities. It appears that there is a threshold level for Cd, below which no effects are observed, but above which there is only a small range of tolerance before severe cytotoxic and lethal effects occur (George, 1989; Ariyoshi et al., 1990a). Two predominant mechanisms have been described to explain detoxification of Cd, limited by a threshold: binding to glutathione and binding to metallothionein.

**Cd sequestering**

When animals are exposed to Cd, processes are induced that immobilize Cd to prevent direct harm to the cell. The first line of defense is the induction and scavenging of Cd by glutathione (GSH). Glutathione is recognized as an important compound in protection against harmful toxicants (Singhal et al., 1987; Viarengo et al., 1997). Glutathione is a tripeptide, with cysteine as one of the amino acids. The SH group on the cysteine is an important scavenger for divalent metals and oxygen radicals, which can be formed by chemical reactions or radiation. Maines & Kappas (1977) mention that the effect of metals on heme metabolism can be eliminated when those metals are bound to...
SH groups in cysteine or glutathione. Removal of SH groups by degrading GSH with diethyl maleate (DEM) greatly enhanced HO induction by metals. Singhal et al. (1987) decreased GSH levels in mice, which made the animals much more sensitive to Cd. When sensitized mice (with low GSH levels) were treated with the GSH precursor, the toxic effects were diminished. The authors argued that the acute binding of Cd to GSH bridges the time period needed to induce MT production, and thus prevents early toxic effects. Protective effects of GSH against Cd toxicity have also been described for human cell lines, using the same method of artificially decreasing and increasing GSH levels (Keogh et al., 1994; Cookson & Pentreath, 1996).

In rats, concentrations of GSH follow a biphasic pattern upon metal exposure: a strong decline in the first 6-16 hours after dosing was followed by an increase to normal levels after 48 h. (Maines & Kappas, 1977; Ossola & Tomaro, 1995). This supports the model that GSH immediately binds Cd, followed by an induction to produce new GSH.

Effects of Cd on GSH levels have also been described for fish. In tilapia, GSH levels decreased within 24 h after exposure, stayed low for a week, and were at normal level again after 140 days (Allen, 1993, 1995). In striped mullet, GSH levels were increased after 4 weeks exposure to aqueous Cd, though all hepatic Cd was bound to MT and not to GSH (Wofford & Thomas, 1984). Viarengo et al. (1997) described a protective effect of GSH in microsomes from European sea bass. Microsomal EROD activity was greatly inhibited in media with Hg^{2+}, but when GSH was added at normal intracellular concentrations, the inhibition was reversed.

Metallothionein

The second important metal binding component is metallothionein (MT). Metallothionein is rich in cysteine, which provides the SH groups for binding divalent metals. Metallothionein is an effective metal binder: 7 Cd ions can be bound on one molecule. Induction of MT is a relatively slow process, which generally takes days rather than hours. The reason may be that Cd is initially bound to GSH as a first step in detoxification, which slows down the induction of MT.
Supposedly, induction is caused by binding of free ions to a receptor, which triggers the transcription/translation sequence (Roesijadi, 1992). Also, GSH may be an important cysteine source for MT production. So, if the cellular GSH pool is depleted by scavenging free Cd, less GSH will be available for MT production.

George et al. (1996) described the initial effect of nonspecific Cd-binding to intracellular ligands (GSH, proteins) as a toxic interaction. Detoxification of Cd then consisted of transfer of Cd to newly synthesized MT, which they called a "rescue phenomenon". For turbot, it took 9 days to synthesize enough MT to sequester a dose of 0.075 mg/kg. For 0.5 mg/kg it would take 30 days, assuming a linear increase for MT synthesis.

Most MT-sequestered Cd is stored in the liver. George et al. (1996) reported that 90% of Cd is stored in liver tissue. Wofford & Thomas (1984) almost no Cd excretion in the first 8 h after striped mullet was injected with radiolabeled Cd. The small amount of Cd that was excreted into the bile was bound to high molecular weight compounds, not to GSH.

**Phase II conjugation**

From the information above it becomes clear that glutathione plays an important role in the detoxification of Cd. It serves as a first line of defense by sequestering free Cd ions, but it may also serve as a source of cysteine for production of newly synthesized MT. This means that there is a demand for GSH when animals or cells are exposed to Cd. However, GSH is also one of three compounds that are involved in conjugating BP metabolites. This means that when organisms are exposed to a combination of Cd and BP, there will be a large demand for GSH. It may be that because of the Cd stress, there is less GSH available for BP metabolite conjugation. This could be circumvented by conjugating BP metabolites to glucuronic acid, which seems to be the predominant route anyway according to the bile analyses presented here. Nevertheless, the activity of glutathione-S-transferase in mummichog liver is conspicuous, and even induced in fish from PAH contaminated environments (Van Veld et al., 1991).
It was shown in the mortality experiments that simultaneous exposure to Cd and BP not only could influence BP metabolism, and thus reduce the toxicity of BP metabolites, but also that BP exposure could enhance Cd toxicity. The hypothesis that reactive BP metabolites would bind to SH groups on MT could not be verified. Therefore another interaction must take place. An indication is given by a slower induction of MT when BP is present. This could mean that competition for GSH by BP metabolite conjugation would slow down sequestering of Cd. However, these results do not explain why excretion of conjugated BP metabolites into the bile is enhanced by Cd exposure, as was shown in the experiments described in this study.

**Continuous Cd exposure**

In the experiments described here, fish were only dosed with a single Cd injection. This method was chosen to assure a consistent and known dose per animal, which might be more difficult to achieve when fish are dosed through the water column. In three different studies, effects of Cd on P450 enzymes are reported after continuous exposure to aqueous Cd. Forlin, et al. (1986) exposed rainbow trout to 100 μg Cd/l for 4 weeks. This treatment did increased the liver P450 content, but not the ECOD activity, which might be explained by direct inhibition of the enzyme (it takes more enzyme to oxidize the same amount of substrate if the enzyme is partially defected). Lemaire-Gony & Lemaire, (1992, 1995) exposed fish (eel and sea bass) to Cd (5 and 40 μg Cd/l) for several weeks before dosing with BP. The cadmium pretreatment caused a greater induction of EROD activity than the BP alone treatment. These studies show that, contrary to the results obtained in single dose studies, long term exposure to Cd has a stimulating effect on the P450 system. This suggests that the modulating effect of Cd on P450 induction and activity has a multitude of regulatory implications. Long term exposure to dietary Cd also increased hepatic MFO activity in rats (Wagstaff, 1973). Schnell et al. (1979) compared i.p. dosage with oral dosage of Cd. Only 1-2% of orally dosed Cd is absorbed, which has major consequences for environmental interpretation of data on Cd-BP interactions. Aqueous exposure can be translated to body burden, and even to tissue concentrations (Forlin et al.,
1989). But if the uptake of Cd from food or the aqueous environment is slow, and MT induction and Cd sequestering can keep up with the Cd influx, then values of 1 mg/kg in liver tissue obtained after aqueous exposure (Forlin et al., 1989) cannot compare in effects to a single i.p. dose of 1 mg/kg.

In conclusion, the mechanisms for effects of Cd on BP metabolism appear to be reasonably well understood, while the effects of BP exposure on Cd detoxification remain unclear. Several possible mechanisms for the interactive effects of BP on Cd toxicity have been presented here. Further experimental research is encouraged to generate more insight into mechanisms by which BP inhibits detoxification of Cd.
References


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