Previous studies have shown that rainbow trout exposed to dieldrin via diet for 9 to 12 weeks increased biliary excretion of a subsequent dose of \([^{14}\text{C}]\)dieldrin by 500%. This was not explained by induction of the cytochrome P-450 (CYP) system involved in oxidative metabolism of these compounds. We hypothesized that epoxide hydrolase activity increased in dieldrin fed-fish. Epoxide hydrolase is an enzyme that catalyzes the hydrolysis of epoxide compounds to their corresponding diols. For instance, dieldrin is metabolized to 6,7 trans-aldrindihydrodiol. This study investigated the activity of epoxide hydrolase in microsomes and cytosol of rainbow trout fed a diet that contained 0 or 15 ppm dieldrin. Fish were fed control or dieldrin diet (0.324 ug/g body weight /day) for 3, 6, or 9 weeks. There was a small increase in mortality and decrease in body weight among dieldrin-fed fish after 9 weeks. After week 9, dieldrin-fed fish were fed a control diet for an additional 3 weeks because of these signs of toxicity. At week 12, the difference of body weight between control and treated was not significant. Microsomal
and cytosolic epoxide hydrolase activities were measured with a radiometric assay which
determined differential partitioning of the parent compound (epoxide) in dodecane and
the metabolite (diol) in the aqueous phase. Assays were run at optimal pH and
temperature using $[^3H]$trans-stilbene oxide (pH 7) as substrate for cytosol and $[^3H]$cis-
stilbene oxide (pH 8) as substrate for microsomes. In order to prevent competition for
reaction with stilbene oxide, depletion of glutathione was efficiently achieved by dialysis
at 4°C for 2 hours at room temperature in buffer [pH 7.5, potassium phosphate 10 mM,
KCl 0.15 M, EDTA 1 mM, BHT 0.1 mM, 0.1 mM PMSF]. Protein quantification was
determined by using BCA assay and concentrations were always between 5 and 25
ug/ml in the final assay volume. Epoxide hydrolase activities were not significantly
different in cytosol or microsomes from control and dieldrin-fed fish. Dieldrin residues
in liver were analyzed by gas chromatography with electron capture detection
(GC/ECD). The concentration in the liver increased with time of exposure and declined
markedly in fish fed dieldrin for 9 weeks and then fed control diet. No dieldrin was
detected in livers from control fish.
Dieldrin Pretreatment Does Not Induce Hepatic Microsomal and Cytosolic Epoxide Hydrolase Activities in Rainbow Trout (*Oncorhyncus mykiss*)

By
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A THESIS
submitted to
Oregon State University

in partial fulfillment of
the requirements for the
degree of
Master of Science

Presented April 30, 2002
Commencement June 2003
Master of Science thesis of Marie Victoire M. Rosemond presented on April 30, 2002.

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ACKNOWLEDGMENTS

“Gratitude is a memory of heart”

J.B. Massieu

I would like to express my deepest appreciation and gratitude to my advisor, Dr. Lawrence R. Curtis for his patient, resourceful guidance during my graduate studies and the course of my research. I thank him for his continued support. Without his patience, his input and revisions, the completion of this thesis would not have been possible. His professionalism will surely inspire me in my future career.

My thanks go to the following people who provided their expertise, assistance, time, or support during this research: Dr. Bruce Hammock (University of California, Davis), Dr. Ralph Reed, Dr. Kim Anderson, Dr. Philip Whanger, Eugene Johnson whom I will always remember his kindness and good mood, Rob Chitwood, Tamara Musafija, Doolalai Sethajintanin, Tamara Fraley, Lisbeth Siddens, Melanie Barnhill and staff at the Marine/Freshwater Biomedical Sciences Center.

I would also like to extend my thanks and appreciation to the members of my graduate committee for their input and participation: Dr. David E. Williams, Dr. Michael Schimerlik for his constant willingness to help, and Dr Alan Bakalinsky. Special Thanks to my fellow graduate students especially to Melanie Barnhill, her invaluable support, help will not go unappreciated. I appreciate the friendship of Doolalai, Yu Zhen, Ted, David, Adams, Wendy and my roommate Margaret Pratt.
Thanks to all the nice people I met in Corvallis and at OSU who offered me either their friendship or their smile that brightened many of my stressful days.

Most importantly, I am grateful to the Latin American Scholarship Program for American Universities (Fulbright-Laspau) and the Oregon Agricultural Experiment Station. Without their financial support, I would not have been able to pursue a master’s degree in U.S.A.

I dedicate this thesis to my nephews and nieces: Gregory, Amy, Valerie, Stephanie, Sarah, Frederic and Melissa with all my love and affection.
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In loving memory of my parents
CHAPTER 1
INTRODUCTION

1.1 Dieldrin

Dieldrin, (1,2,3,4,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endoexo-5,8-dimethanonaphthalene) grouped within the cyclodienes, some of the most persistent chemicals known, was previously used as an organochlorine insecticide. It was used until nearly two decades ago in the U.S mainly in agriculture for crop protection from various soil dwelling pests and as protection against termite infestation [1]. Dieldrin was withdrawn from the market and its registration cancelled in the U.S in 1974 except for termite control. However, most remaining dieldrin products were canceled by 1987 and the last product was withdrawn in 1989 [1]. Dieldrin is one of the 12 compounds classified in the level-1 priority list of pollutant bioaccumulative toxicants (PBTs) of Environmental Protection Agency (EPA) [2] often called “the dirty dozen” [3]. Organochlorine insecticides are halogenated compounds that contain more than one chlorine atom and are characterized by low solubility in water and high solubility in lipids making them persistent and resistant to biodegradation. Its persistence along with its high lipid solubility allows dieldrin to bioconcentrate and bioaccumulate in the fatty tissues that serve as a storage compartment. Dieldrin was banned because it raised
concerns about hazards for human health and the environment. Those persistent organochlorine insecticides were recognized as an element of risk to one or more forms of life. For instance, it has been reported that dieldrin was responsible for the death of bats in Missouri [4]. Dieldrin has been associated with toxicity in dopamine neurons. Dieldrin was found in higher concentrations in brains of patients with Parkinson disease than in Alzeimer disease or control groups. Increased concentrations of dieldrin and lindane in substantia nigra could be linked to the reduced dopamine concentrations critical for the pathogenesis of Parkinson’s disease [5]. In Mexico, an evaluation of preschool children was done in a region where organochlorine pesticides are still applied. Children from the agrarian area were compared to children living in another area where pesticide use was avoided. There was a decrease in stamina, gross and fine eye-hand coordination, 30-minute memory, and the ability to draw a picture [6]. However, dieldrin and other organochlorine insecticides are still produced and world trade continues. It has been reported in a recent article that there were about 39 countries where companies report production of those compounds [7]. Although banned in the U.S more than two decades ago, dieldrin was recently detected at low concentrations potentially harmful to the environment. Dieldrin is also a primary degradation product of aldrin and its residues were detected at levels greater than 0.1 ug per g in areas where aldrin, its parent compound was used [8]. Dieldrin also accumulates in the liver. After low level environmental exposures to persistent organochlorines, concentrations of many of them, including dieldrin in liver exceed that in fat [9]. Upon trophic transfer to mammals or fish, dieldrin leads to bioaccumulation of
tissue concentrations much higher than found in the environment. Aquatic
organisms, including fish can accumulate organochlorine insecticides from water
through their respiratory system (gills) or from the food chain. Some workers have
reported that in some waters fish showed consistently high residues of dieldrin and
other organochlorines in drained agricultural or industrialized areas [7].
Environmental contamination has developed the need for a fuller understanding of
these persistent bioaccumulative insecticides in aquatic species, and fish in
particular. The biological activity of organochlorine insecticides remains
incompletely understood.

1.2 Literature review and research objective

Rainbow trout (Oncorhyncus mykiss) is a variety of fish that has been
extensively used as a model in bioaccumulation studies with dieldrin or other
organochlorine insecticides. Previous bioaccumulation studies demonstrated that in
rainbow trout fed dieldrin for 2 to 8 weeks assimilation efficiency was about 23%.
At 16 weeks, the assimilation efficiency dropped by a factor of two. This indicated
fish were not at a steady state body burden of the chemical. Whole body
concentrations increased from 2 to 8 weeks, but at 16 weeks, dieldrin
concentrations decreased to levels comparable to those observed in trout exposed
for 2 weeks [10]. These changes were an adaptive response of rainbow trout to
chronic exposure to dieldrin that decreased bioaccumulation of this organochlorine.
Feeding rainbow trout a low concentration of dieldrin increased hepatic disposition
and biliary excretion of a single [$^{14}$C]dieldrin dose after 10 or 12 weeks [11]. At 10
weeks, the liver accumulated about 200% of control \[^{14}\text{C}]\text{dieldrin. In the bile, the changes were even more marked. Dieldrin increased the biliary excretion of the tracer dose up to five-fold. In addition to the stimulation of biliary excretion, distribution of the challenge dose increased in the fat by 500% at 10 weeks and 1200% at 12 weeks, but decreased in carcass lipid [10]. Therefore, there was a stimulated elimination of the contaminant not readily explained by induction of the cytochrome P-450 (CYP) system involved in oxidative metabolism of these compounds. Immunoquantification of CYP isozymes in isolated microsomes from control and dieldrin-fed fish revealed no differences [12]. The microsomes were incubated with benzo[a]pyrene or 7,2 dimethylbenz[a]anthracene (DMBA). There were no significant differences between control and dieldrin-fed fish in oxidative metabolism of these PAHs. The results provided evidence that dieldrin induced changes were not explained by CYP induction [12].

1.3  **Epoxide hydrolase enzyme**

Previous studies have shown that rainbow trout fed-dieldrin for 9 to 12 weeks increased polar metabolites excreted in bile, without CYP induction. Therefore, another metabolic pathway requires investigation. Normally, epoxide hydrolase catalyzes the hydrolysis of epoxides compounds by increasing the nucleophilicity of water and converts them to their vicinal diols. This enzyme plays an important role in detoxifying electrophilic epoxides that might otherwise bind to proteins and nucleic acids and cause cellular toxicity and genetic mutations [13]. The resulting metabolites, diols, represent a detoxification process. Epoxide
hydrolase activities occur in mice and other species including fish and a variation between species has also been shown [14]. Dieldrin is a three membered cyclic ether that bears an epoxide group which can be attacked by epoxide hydrolase converting it into its corresponding vicinal diol, 6, 7 trans-dihydroaldrindiol [fig 1]. When dieldrin is metabolized by epoxide hydrolase, the initial hydroxylation event and ring opening occur in which one of the C-O bonds is broken. The cleavage of epoxide occurs on opposite side of the molecule giving the dihydrodiol a trans-configuration [13]. Epoxide hydrolases occur in various tissues with the highest levels of the different forms found in the liver [15]. The activity in liver homogenates was located mainly in the microsomes and requires no cofactors [16]. Comparative studies have shown that levels of epoxide hydrolase in different tissues vary with the strain and species of animal being examined as well as the substrate used to quantify the activity [15]. There is substantial variation between compounds in their rate of hydration, for instance, HEOM an analogue of dieldrin is rapidly hydrated whereas dieldrin is hydrated slowly [14].
Figure 1.1. Metabolic pathways of dieldrin
1.4 Distribution of epoxide hydrolases in tissue

There are immunologically 3 distinct forms of epoxide hydrolase: one in the cytosol, which is the soluble form cEH, and two in the endoplasmic reticulum. One of the microsomal enzyme hydrates cholesterol 5, 6α oxide but has no capacity to detoxify xenobiotic oxides [13]. The form present predominantly in the microsomal and nuclear subcellular fractions is described as the microsomal epoxide hydrolase and the form present in the soluble and mitochondrial fractions is referred as the cytosolic epoxide hydrolase. However, ongoing research supports the existence of several forms of epoxide hydrolase [16]. Both cytosolic and microsomal epoxide hydrolase have different substrates specificities and can be distinguished by their pH optima, molecular weight and response to inhibitors. The microsomal epoxide hydrolase hydrates the cis-stilbene oxide at basic pH whereas the cytosolic preferentially hydrates the trans-stilbene oxide at neutral pH as shown in figure 2 from Cassarett & Doull’s (fifth edition).
figure 1.2. Stereoselective hydroxylation of stilbene oxide by microsomal and cytosolic epoxide hydrolase. These two forms of epoxide hydrolase are distinct genes products and have different substrate specificities (13).
Mammalian liver microsomal epoxide hydrolase (mEH) has been shown to display high enantioselectivity towards a wide range of substrates. Studies have emphasized that because of the steric and electronic nature of the catalysts, the best substrates are cis-alkenes conjugated with aryl, acetylenic or alkenyl groups [17]. Epoxidation of trans-alkenes proceeds with only moderate enantioselectivity. Generally, mEH shows greater enantioselectivity than the related cEH and has been the most used as a potential asymmetric catalyst. In their structure-activity relationships for substrates of mEH, using partially purified mEH of guinea pig liver, Oesch concluded that monosubstitued oxiranes with large hydrophobic substituents were excellent substrates for mEH; cis-disubstitued and 1,1 disubstitued, trisubstitued, and tetrasubstitued epoxides were extremely poor substrates for the enzyme [17]. It appears that hydrolysis occurs via attack at the less hindered side of the epoxide. This lack of steric freedom on the front, leftside prevents trans-epoxides from being hydrolyzed readily [17]. Hammock and Hasagawa compared the hydration of a variety of substrates by enzymes in the cytosol and microsomes of mouse liver. They found that cyclodiene epoxides were not metabolized in the cytosol [18].

1.5 Research objective

The primary objective of this study was to test the hypothesis that dieldrin-stimulated biliary excretion of [14C]dieldrin observed in rainbow trout was related to induction of mEH. Cytosolic and mEH modulation by organochlorine insecticides have not been investigated in fish. To supplement the understanding of
the metabolism and biological persistence of dieldrin, this study focused on hepatic cEH and mEH activities in rainbow trout (Oncorhyncus mykiss) after dietary exposure to dieldrin for 3 to 9 weeks. Hepatic microsomal and cytosolic epoxide hydrolase were determined with a radiochemical assay. The principal was differential partitioning of the epoxide in dodecane and the metabolite diol in the aqueous phase. Assays were optimized for pH and temperature using $[^3H]$ cis-stilbene oxide as substrate for mEH and $[^3H]$ trans-stilbene oxide as substrate for cEH. Depletion of glutathione was successfully achieved by dialysis to prevent conjugation reaction. Dieldrin residues in fish livers fed dieldrin and control diet were analyzed by gas chromatography with electron capture detection (GC/ECD).
2.1 Chemicals

Dieldrin (99% pure) was purchased from AccuStandard Inc. (New Haven, CT). Radiolabeled substrates, [3H]cis-stilbene oxide and [3H]trans-stilbene oxide, were obtained from Dr. Bruce Hammock (University of California, Davis). Substrates (1 mCi/mmol) were delivered in 1 ul of ethanol with a Hamilton syringe. BCA protein assay reagents were purchased from Sigma Chemical Company (St Louis, MO). Other chemicals were reagent grade and commercially available.

2.2 Animals exposure

Juvenile Shasta strain rainbow trout (Oncorhyncus mykiss) (~2g) were provided by the Marine Freshwater Biomedical Sciences Center core facility at Oregon State University (Corvallis, OR). Fish were housed within circular tanks (89 L) in continuous flow with an appropriate rate of 6 L/min at 13 degrees C. Fish (80/tank) were maintained on a 15 hr light/hr dark photoperiod during the period of treatment. Fish were fed 4% growth ration Oregon Test Diet that contained 0 or 15 ppm dieldrin (0.324 ug/g wet body weight). Uniformity of the mixture was made by dissolving the dieldrin in menhaden oil prior to incorporation into the diet. After week 9, fish fed dieldrin were fed control diet for an additional 3 weeks at a reduced 2% maintenance ration because they showed signs of toxicity.
2.3 **Preparation of hepatic microsomes and cytosol**

Control and treated fish were euthanized with tricaine methane sulfonate (200 mg/L), weighed and killed at 3, 6, 9 or 12 weeks. Livers were excised, weighed, minced, and homogenized with a Dounce homogenizer in 3 volumes buffer [potassium phosphate 10 mM, pH 7.5, KCL 0.15 M. EDTA, 1 mM. BHT, 0.1 mM . PMSF, 0.1 mM]. Microsomes and cytosol were obtained by centrifugation at 10,000 x g for 23 minutes followed by a second centrifugation at 100,000 x g for 90 minutes. The final supernatant fraction was the cytosol. The 100,000 x g pellets (microsomes) were resuspended in 0.1 M potassium phosphate, pH 7.4, 20% glycerol, 1 mM EDTA and 0.1 mM PMSF. Fractions of cytosol and microsomes were frozen at -80° degrees for further analysis.

2.4 **Depletion of glutathione by dialysis**

Microsomes and cytosol were dialyzed in order to deplete glutathione and prevent conjugation of stilbene oxides. Samples (0.5 ml) were injected in dialysis cassettes with an 18 gauge needle and 1 ml syringe and incubated in buffer [pH 7.5, potassium phosphate 10 mM. KCL, 0.15 M. EDTA, 1mM. BHT, 0.1mM] at 4° for 2 hr. After dialysis, samples were removed from the dialysis cassette and levels of GSH were measured by high pressure liquid chromatography UV detection after derivatization with 2, 4-dinitrofluorobenzene( HPLC-UV/FDNB).
2.5 Measurement of GSH and GSSG by HPLC-UV/FDNB

Glutathione levels were measured by high pressure liquid chromatography with UV detection and 2, 4 dinitrofluorobenzene (FDNB) [19]. Dialyzed and non-dialyzed samples were derivatized with initial formation of S-carboxymethyl derivatives of free thiols by a reaction with iodoacetic acid. This was followed by a second derivatization with addition of Sanger’s reagent, 1-fluoro 2, 4-dinitrobenzene (FDNB) that converted the primary amines to their 2, 4 dinitrophenyl (DNP) derivatives. Reverse phase ion-exchange HPLC with a 3-aminopropyl column separated the amino-acid DNP derivatives. The eluted derivatives were measured by ultraviolet (UV) detection at 365nm. HPLC column: 20 cm x 4.6 mm-i.d. column with 5-um Exsil silica derivatized with 3-aminopropyltriethoxy silane. HPLC system: Pump with gradient capability (Spectra-Physics P200, Spectra-Physics); Flow rate: 1.0 ml/min. UV detector (365 nm; Alltech 200, Alltech), Recording integrator (Hewllett Packard 3390, Alltech). Hamilton gas-tight syringe.

2.6 Protein determination

Protein concentration was determined using BCA assay. Bovine serum albumin served as the standard [20]. A standard curve was prepared by plotting the net absorbance at 562 nm against the known added protein standard. The standard curve was used to determine the amount of protein in the microsomes and cytosol.
2.7 Enzyme assays

Enzymatic activity assays for microsomal and cytosolic epoxide hydrolase were measured radiometrically utilizing differential partitioning of the epoxide into dodecane and the diol metabolites into the aqueous phase [21]. Assays were optimized for pH and temperature. \([^3\text{H}]\)trans-stilbene oxide was used as substrate for cytosol at pH 7 and \([^3\text{H}]\)cis-stilbene oxide was used as substrate for microsomes at pH 8. Frozen microsomes and cytosol were thawed and dialyzed for 2 hours at 4°C in appropriate buffer prior to enzymatic activity assay. After dialysis of samples and protein quantification, assays were conducted in duplicate in 2 ml polypropylene microcentrifuge tubes. The incubation mixture contained 1 ml of buffer [0.05 M tris-HCL, 0.25 M sucrose, 1mM EDTA, 1mM dithiothreitol] at a protein concentration in the range of 5 to 25 \(\mu\)g/ml in the final assay volume, and 1 ul of TSO or CSO in ethanol (5 \(\times\) 10\(^{-5}\) M final concentration). After incubation for 30 min, reactions were ended by addition of 200 ul of dodecane. The tubes were vortexed for 20 seconds and centrifuged for 5 min to separate the 2 phases. Duplicate 0.2 ml samples of the aqueous phase, which contained the diols, were added to a 8 ml liquid scintillation solution. Scintillation vials containing the mixture were let in the dark overnight to prevent chemiluminescence and then counted by liquid scintillation counting (LSC). Counts were in dpm and specific activity was expressed in nmol per mg protein.
2.8 Verification of diol formation

Differential extraction with various solvents was used to determine the partitioning of stilbene oxide, stilbene diol, and the GSH conjugate of stilbene oxide. After a first extraction with dodecane that separated the parent compound (epoxide) in the organic phase, hexanol was added to the remaining aqueous phase that contained the metabolite diol. Then, the organic phase was concentrated under nitrogen. 25 ul of the concentrate was counted for diols by LSC and 50 ul was applied onto the silicate pre-layered plates, developed in toluene:n-propanol (19:1) and let dried overnight. Bands of silica-gel plates (1cm) were scraped, added to an appropriate volume of liquid scintillation solution and counted by liquid scintillation counting that showed a single broad spot. Presence of mercapturate conjugates was assessed by LSC of the remaining aqueous phase after extraction with hexanol. No GSH conjugate was detectable.

2.9 Analysis of dieldrin residues in liver rainbow trout

Dieldrin concentration was determined in the liver of fish fed 0 or 15 ppm dieldrin for 3, 6, or 9 weeks. Liver dieldrin concentrations of fish fed control diet for 12 weeks or dieldrin for 9 weeks followed by 3 weeks on control diet were also determined. Samples were analyzed by gas chromatography with electron capture detection (GC/ECD). The identity of a chromatographic peak was determined by comparison of its relative retention time with those determined from the standard. The concentrations were determined based on peak areas in both samples and
standard. For the purpose of analytical quality assurance, samples of liver tissue were spiked and analyzed to calculate recovery values.

2.10 Samples preparation and analysis

Livers (0.5-1.0 g) were weighed into a glass mortar and 5 g sodium sulfate was added. The sample mixture was ground with a glass pestle and the free-flowing powder obtained was poured into a chromatographic column plugged with glass wool and filled with sodium sulfate and dichloromethane:hexane (1:1). An additional 2 g sodium sulfate was mixed into the mortar (to remove residual sample) and added to the column. The mortar was rinsed with the solvent mixture that was applied to the column. After one hr, the column was eluted with a total volume of 62 ml dichloromethane:hexane (1:1). The eluate was collected in a 250 ml round bottom flask at a nominal flow rate of 5 ml/min and rotaevaporated to approximatively 1-2 ml. The concentrate was transferred to 5 ml centrifuge tube, rinsed with hexane, and evaporated to 0.5 ml in a Zymark TurboVapR L.V. evaporator. Samples were processed by gel permeation chromatography (GPC) for separation with a Waters 717 plus Autosampler, with 2487 dual absorbance detector connected to Waters 515 HPLC Pump with flow rate 5 ml/min. The eluates were collected from disposable culture tubes (20x150 mm) in the fraction collector. Eluates were evaporated to 1 ml and a solvent exchange dichloromethane/hexane was performed to eliminate dichloromethane. Volume was brought to 10 ml with hexane and diluted by a factor of 10. After dilution, 2 ul samples were injected into a GC/ECD, Varian Star 3400 Cx, Autosampler 8200,
single injection split dual analysis using a ramped temperature program: Initial GC temperature was 250° C, initial column temperature was 150° C for 2 min, 255° C for 14 min, then a final temperature 270° C for 15 min. DB-17 ms and DB-xlb (J&W), each 30 mx 0.25 l.D. x 0.25 um film thickness.

2.11 Statistical analysis

Statgraphics Plus 5.0 was used for all statistical analyses. Two-way analysis of variance compared multiple means and determined significant time or treatment effects. A two-sample comparison, or t-test, was used when comparing two means for significant treatment effects. Significance was determined using a 95.0% confidence level (p<0.05). If assumptions of either test were violated and transformation of the data did not correct the problem then non-parametric methods (e.g., Kruskal-Wallis test) were used to compare medians rather than means.
3.1 Animals

Fish fed dieldrin showed signs of toxicity between 6 and 9 weeks of treatment. There was an increase in mortality among fish fed dieldrin during the time of exposure (2.1% in control and 3.8% in treated). From 3 to 9 weeks, treated fish received a daily dose of 0.324 ug dieldrin/g body weight (15 ppm). A decrease in body weight was observed between week 6 and week 9 in dieldrin fed-fish (fig 3.1). Thus, treated fish were fed a control diet after week 9 for an additional 3 weeks and the initial 4% growth ration was reduced to 2% maintenance ration. At week 12, fish started to recover and the difference in body weight between control and treated fish was not significant (fig 3.1).
Figure 3.1. Weight gain in rainbow trout (initial wt ~2 g) following dieldrin exposure for 9 weeks. The body weight of dieldrin-fed fish was 75% of control fish at wk 9 in the first experiment. Therefore, dieldrin was removed from the diet and fish were switched from a 4% growth ration to a 2% maintenance ration. All fish received control diet for an additional 3 wks. Values are means ± SE. (wk 3 and 6: n=9; wk 9: n=18; wk 12: n=33). At wk 12 the SE is based on a sample size of 9. Individual weights of 24 fish were not available because their weights were pooled.
3.2 Optimization Assays for pH and Temperature

Effect of pH.

Enzyme activities were measured with pH ranging from 6.5 to 9.5 at room temperature for 30 minutes using $[^3]H$ cis-stilbene oxide as substrate for microsomes and $[^3]H$ trans-stilbene oxide as substrate for cytosol. Results from liquid scintillation counting were calculated as dpm. Specific activity was calculated based on the volume of aqueous phase counted in LSC, the incubation time, and the substrate specific activity. Activity was expressed in nmol/min/mg protein. Protein concentration was always between 5 and 25 ug/ml in the final volume assay. Cytosol showed a broad pH optimum in the optimum range of neutral pH (fig 3.2). Microsomes showed a broad pH optimum in the range of basic pH. (fig 3.3).
Figure 3.2. Effect of pH on cEH activity using TSO as substrate for cytosol. Assays were in duplicate at room temperature over a pH range of 6.5 to 9.5.
Figure 3.3. Effect of pH on mEH activity using CSO as substrate for microsomes. Assays were in duplicate at room temperature for 30 min over a pH range of 6.5 to 9.5.
Effect of temperature. Cytosolic and microsomal epoxide hydrolase activities were measured with 4 different temperatures: 4°, 15°, 24° (room temperature), 30°. Trans-stilbene oxide was used as substrate for cytosol at pH 7 and cis-stilbene oxide was used as substrate for microsomes at pH 8. Activities were higher at 15° and room temperature for both cytosol (fig 3.4) and microsomes (fig 3.5).

Figure 3.4. Effect of temperature on cEH using trans-stilbene oxide (TSO) as substrate for cytosol. Assays were in duplicate at pH 7 for 30 min at 4°, 15°, 23°, 30°.
Figure 3.5. Effect of temperature on mEH using cis-stilbene as substrate for microsomes. Assays were in duplicate at pH 8 for 30 min at 4°, 15°, 24°, and 30° C.
3.3 Analysis of GSSG and GSH in cytosol and microsomes after dialysis by HPLC-UV/FDNB

Glutathione was efficiently depleted by dialysis. The concentration of glutathione in liver is ~10 mM [19]. Dialyzed and undialyzed samples were analyzed by high performance liquid chromatography (HPLC) systems that separated the cellular thiols. There was initial formation of S-carboxymethyl derivatives of free thiols by a reaction with iodoacetic acid. The addition of FDNB reagent converted the primary amines group to their 2,4-dinitrophenyl (DNP) derivatives. Levels of glutathione in cytosol were reduced up to 80% (fig 3.6) and 60% in microsomes (fig 3.7).
Figure 3.6. Levels of GSH in dialyzed cytosol vs undialyzed cytosol measured by HPLC. Depletion of GSH was up to 80%. Values are means ± SE.
Figure 3.7. Levels of GSH in dialyzed microsomes vs undialyzed microsomes measured by HPLC. Depletion of GSH was up to 60%. Values are means ± S.E.
3.4 Enzyme assay

Enzyme activities for cEH and mEH were measured at week 3, 6, 9, and 12 at the pH optimum and at room temperature. Specific activities were measured with a radiometric assay whose principal was a differential partitioning of the \(^3\text{H}\)epoxide in dodecane and the \(^3\text{H}\)metabolite diol in the aqueous phase. Duplicate 200 ul of the aqueous phase were analyzed by LSC. Counts in dpm was used to calculate specific activity. At the end of the incubation 90% of substrate was available showing that the enzyme was not substrate limited. There were no differences between cytosol and microsomes from control and treated fish (fig 3.8; 3.9). Specific activities were expressed in terms of percentage activity that corresponds to the ratio of hydration for control and treated fish.
Figure 3.8. Activity of mEH using CSO as substrate in trout fed control diet for 12 weeks and diet containing dieldrin for 9 weeks. Treated fish were fed dieldrin for an additional 3 weeks because they showed signs of toxicity. Value (nmol/min/mg protein) for fish fed dieldrin was divided by the mean value of control and specific activity was in percentage control activity. Values are mean ± S.E.
Figure 3.9. Activity for cEH using TSO as substrate in trout fed control diet for 12 weeks and diet containing dieldrin for 9 weeks. Treated fish were fed control diet for an additional 3 weeks because they showed signs of toxicity. Value (nmol/min/mg protein) for fish fed dieldrin was divided by the mean value of fish fed control diet and specific activity was expressed in percentage control activity. Values are mean ± S.E.
3.5 Concentration of dieldrin in liver rainbow trout

The concentrations of dieldrin residues were analyzed by GC/ECD. Mean recoveries (n = 4) were 84%. Fish received an estimated dose of 0.324 ug/g body weight/day. Liver weight was between 0.5 and 1 g. Concentrations in the liver were significantly higher at week 9, which was the time at which fish showed signs of toxicity. The results for liver conformed to the predicted pattern. The concentration of dieldrin in liver increased with treatment time. Liver dieldrin concentration decreased markedly after the organochlorine was removed from the diet (3.10). No dieldrin was detected in livers of fish fed control diet.
Figure 3.10. Concentration of dieldrin in fish livers fed dieldrin and control diet for 3 to 9 weeks. Fish received an estimated dose of 0.324 ug/g/day for 3 to 9 weeks. After week 9, fish fed dieldrin were fed a control diet for an additional 3 weeks because they showed signs of toxicity. Dieldrin concentration increased at week 9 but decreased markedly after dieldrin was removed from the diet. No dieldrin was detected in control fish livers. Values are means ± S.E.
Dieldrin pretreatment of rainbow trout stimulated biliary excretion of polar metabolites of a subsequent dose of [\(^{14}\text{C}\)]dieldrin [11]. This was not explained by changes in total body lipid or by induction of CYP. Previous bioaccumulation studies in rainbow trout fed dieldrin showed decreased assimilation efficiency during chronic exposure through diet and water [10]. In this present study, we tested the hypothesis that the dieldrin stimulated biliary excretion of [\(^{14}\text{C}\)]dieldrin observed in rainbow trout was related to induction of mEH. Epoxide hydrolase catalyzes the hydrolysis of dieldrin to its corresponding vicinal 6, 7 trans-aldrindihydrodiol. Rainbow trout received via diet an estimated dose of 0.324 \(\mu\)g of dieldrin/g body weight and a 4\% growth ration for 3 to 9 weeks. At week 9, dieldrin-fed fish were fed control diet and a reduced 2\% maintenance ration for an additional 3 weeks because they showed signs of toxicity. Body weight decreased and mortality increased slightly among fish fed-dieldrin at 9 weeks. At week 12, the body weight between control and treated fish was not significantly different.

Epoxide hydrolase activities for microsomes and cytosol were assayed with a radiometric assay. Parent compound epoxide and metabolite diol were differentially partitioned after vigorous vortexing and centrifugation of the incubation mixture. Assays were run at room temperature and at optimal pH. Epoxide hydrolase activities were not significantly different in cytosol or microsomes from control and dieldrin-fed fish. The activity of cHE or mEH in fish after dietary exposure to dieldrin was not previously studied. The results of this study showed that increased biliary excretion of a
challenge dose of $[^{14}C]$dieldrin was not explained by induction of cEH or mEH. Previous work demonstrated after 10 and 12 weeks of dieldrin pretreatment, distribution of a single dose of $[^{14}C]$dieldrin significantly increased in bile by 500% and liver by 200% in rainbow trout [10]. It was hypothesized the rainbow trout adapted to the organochlorine dieldrin [11]. In a subsequent study, immunoquantification of CYP isozymes in isolated microsomes showed no differences between control and dieldrin-fed fish [12]. In vitro metabolism of various substrates for major CYP isozymes was not different in hepatic microsomes and dieldrin-fed trout [12]. Epoxides were metabolized both by glutathione transferase and epoxide hydrolase [15]. Trans and cis-stilbene oxides, respective specific substrates for cEH and mEH, were also good substrates for GSH transferase [21]. We depleted glutathione in microsomes and cytosol by dialysis. Analysis by high pressure liquid chromatography after dialysis showed levels of glutathione were efficiently reduced in microsomes by 60% and cytosol by 80%. The percentage of depletion for cytosol is in agreement with the literature since the best success was achieved by dialysis of the cytosol when GSH transferase is high [22]. We avoided the use of inhibitors. $[^{3}H]$diol metabolites were differentially extracted with 1-hexanol and counted by LSC. This indicated no mercapturates in the final aqueous phase. The mEH activity for rainbow trout was 3 to 4-fold higher than previously reported [22]. Differences in specific activity were reported between 26 strains of mice [16]. Specific activities for rainbow trout in this study were expressed in percentage control activity by dividing specific activity for each treated group to the mean activity of control group. Enzyme activities for mEH and cEH were almost the same. One-way ANOVA and two-way ANOVA analyses showed no differences among treated groups,
p-value = 0.19, but there was a significant effect for time, p-value < 0.05 at the 95% confidence level. Concentration of dieldrin residues in liver rainbow trout fed control diet and dieldrin was analyzed by GC/ECD. The concentration of dieldrin in liver was time dependent. Previous bioaccumulation studies available were for whole animals rather than specific organs [10]. Data on accumulation in liver were not available. Highest concentrations were at week 9 when fish showed signs of toxicity. However, the concentration dropped markedly after dieldrin was removed from the diet. Therefore, liver eliminated dieldrin rather rapidly, consistent with increased of biliary excretion of the organochlorine after 9 or more weeks of exposure [10,11]. Increased biliary excretion of subsequent doses of [³H]DMBA, and [¹⁴C]BaP after chronic exposure to dieldrin was also demonstrated [23,24]. Therefore, dieldrin pretreatment stimulated biliary excretion of lipophilic xenobiotics with different structures was proposed as a general phenomenon involved in hepatic disposition of lipophilic xenobiotics [12]. Hepatic cytosol from dieldrin-fed rainbow trout bound about 2-fold more [³H] DMBA than that from control fish [23]. Increased binding capacity and intracellular trafficking perhaps partially explained alteration of disposition and increased biliary excretion of a subsequent dose of radiolabeled [¹⁴C]dieldrin or [¹⁴C]BP [24]. Recently, a role for a putative binding protein aggregate that contained heat shock protein HSP90 was proposed in intracellular trafficking of BP in rainbow trout liver [25]. Earlier studies with CYP isozymes [11,12] and current results with cEH and mEH suggested intracellular trafficking was potentially rate limiting for hepatic elimination of lipophilic xenobiotics in dieldrin pretreated rainbow trout.
BIBLIOGRAPHY


