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Hepatic microsomal desulfuration and dearylation of chlorpyrifos and parathion in fingerling channel catfish: lack of effect from Aroclor 1254

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Abstract

Channel catfish were treated intraperitoneally with 100 mg Aroclor 1254/kg body weight and sacrificed at 96 h to observe the effects of this cytochrome P450 1A (CYP1A) inducer on chlorpyrifos and parathion metabolism. In the initial experiment, hepatic microsomal ethoxyresorufin-*O*-deethylase (EROD) activity of the Aroclor-treated fish was significantly induced but no effects on desulfuration or dearylation of chlorpyrifos or parathion were evident. In the second experiment, Aroclor 1254 did not alter total hepatic microsomal P450s content, but significantly induced hepatic EROD and CYP1A. There were no evident effects to other hepatic CYP isoforms recognized by anti-trout CYP2K1, CYP2M1 and CYP3A27. These experiments indicate that Aroclor 1254 did not induce the P450s responsible for metabolism of the phosphorothionate insecticides. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Parathion; Chlorpyrifos; Channel catfish; P450s; Desulfuration; Dearylation

1. Introduction

Two organophosphorus (OP) insecticides used in agriculture are the phosphorothionates chlorpyrifos (Cp; *O*,*O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothionate) and parathion (Pth; *O*,*O*-diethyl *O*-4-nitrophenyl phosphorothionate). These insecticides can affect fish in natural and culture environments when in close proximity to agricultural fields; channel catfish (*Ictalurus punctatus*) could be exposed to OP insecticides by aerial overspray or run-off. Channel catfish 96-h LC50 values for Cp and Pth are 0.28 and 2.65 mg/l, respectively (Mayer and Ellersieck, 1986).

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Phosphorothionates are relatively poor cholinesterase inhibitors and must undergo activation by cytochromes P450 (P450s) desulfuration to their oxon metabolites, chlorpyrifos-oxon (Cpxn; *O*,*O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphate) and paraoxon (Pxn; *O*,*O*-diethyl *O*-4-nitrophenyl phosphate), to effectively inhibit acetylcholinesterase (AChE), their primary target. A concomitant detoxication reaction of P450s known as dearylation also occurs; the 3,5,6-trichloro-2-pyridinol or 4-nitrophenol is oxidatively cleaved from Cp or Pth, respectively. Presumably, both reactions arise from the same unstable phosphooxythiiran intermediate (Neal and Halpert, 1982). Metabolism pathways are shown in Fig. 1. Relative activation/detoxication activities can substantially influence the toxicity level of the compound. Monooxygenase (MO) activity represents a significant portion of Phase I biotransformation pathways in fish (Melancon and Lech, 1983).

Xenobiotic biotransformation in fish can be significantly affected by exposure to environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs) or polychlorinated biphenyls (PCBs) (Binder et al., 1984; Stegeman and Hahn, 1994). Aquatic environments are commonly contaminated with PCBs and studies have shown that hepatic MO activities in fish can be increased greatly by exposure to these compounds (Lidman et al., 1976; Addison et al., 1978; Elcombe and Lech, 1979).

Inducers of P450s (such as PCBs) can alter both the toxicity of the parent insecticide as well as the time course of esterase inhibition, via the oxon, by increasing the rate of phosphorothionate metabolism. Depending on the isoform profile of P450s and the substrate specificities of the induced isoform(s), induction can lead to either increases in the production of activated metabolites with enhanced toxicity, or increased detoxication with enhanced protection from toxicity (Binder et al., 1984; Stegeman and Hahn, 1994).

Levels as low as 0.2 mg/kg of the PCB mixture Aroclor 1254 have been shown to induce 7-ethoxyresorufin-*O*-deethylase (EROD) activity in rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) hepatic microsomes (Melancon and Lech, 1983). Ankley et al. (1986) reported that Aroclor 1254 increased hepatic microsomal MO activities, including EROD, and established the time-course and dose–response of this compound in channel catfish. Ronis et al. (1992) demonstrated induction of EROD and CYP1A1 proteins in channel catfish hepatic microsomes treated with either daily injections (3 days) of 50 mg/kg Aroclor 1254 or sampled from a PCB-contaminated site.

Several studies on desulfuration and dearylation of phosphorothionates by hepatic microsomes have been carried out in mammals (Levi et al., 1988; Forsyth and Chambers, 1989; Chambers et al., 1994); these activities are inducible in rats by phenobarbitol (Ma and Chambers, 1995) and β-naphthoflavone (Chambers et al., 1994). There are reports of phosphorothionate metabolism in fish (Potter and O'Brien, 1964; Murphy, 1966; Benke et al., 1974; Wallace and Dargan, 1987; Boone and Chambers, 1997); however, hepatic microsomal metabolism has only been reported by Boone and Chambers (1997). Desulfuration and dearylation of phosphorothionates in channel catfish hepatic microsomes has not been investigated.

The present study was designed to observe the effects of exposure to environmental PCBs on in vitro hepatic microsomal desulfuration and dearylation of Cp and Pth in fingerling channel catfish. A second experiment was designed to observe microsomal P450s content, isoform composition and protein content.

2. Materials and methods

2.1. Chemicals

Aroclor 1254 was obtained from Chem Service (West Chester, PA, USA). Phosphorothionates were provided by Dr Howard Chambers (Department of Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS, USA). All other biochemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA).

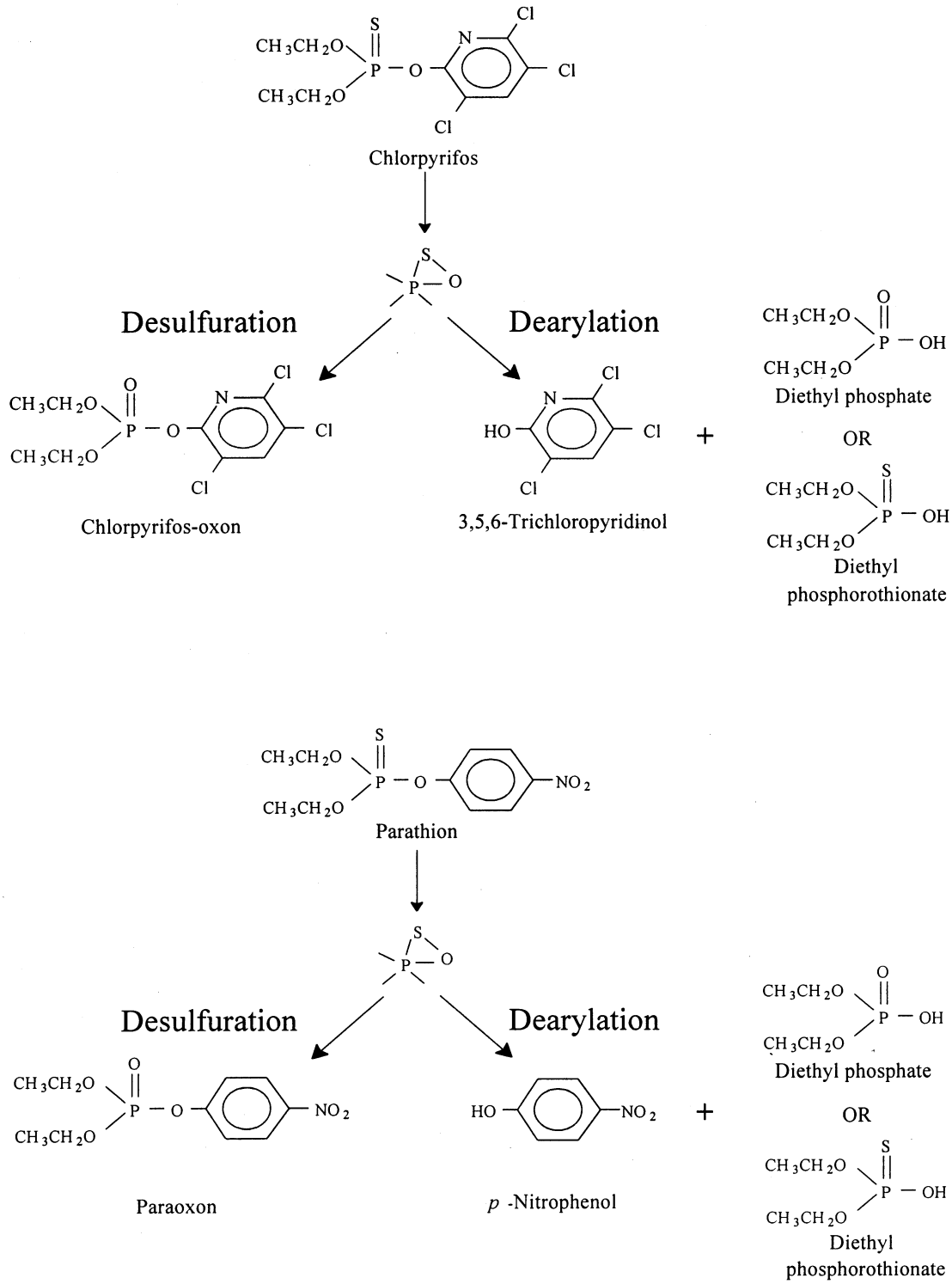

Fig. 1. Metabolism pathways for chlorpyrifos and parathion.

2.2. Animals and treatments

Sexually immature channel catfish fingerlings were obtained from the College of Veterinary Medicine, Mississippi State University; average weight was 58 and 50 g for the initial and second experiments, respectively; immaturity of this size fingerling prevented accurate sex determination. Fish were fed a commercial catfish feed at the rate of 3% of body weight/day. Fish were held at 30 ± 1°C and a 12-h light/12-h dark light cycle to minimize the reported seasonal variation in P450 activities, liver size, and other enzymes (Chambers and Yarbrough, 1976). Fish were acclimated for 2 weeks in a flow-through environment in 76-l glass aquaria containing 60 l aerated, dechlorinated water (pH 7.6 ± 1; total alkalinity, 75 ± 15 mg/l as $CaCO_3$; chloride, 25 ± 5 mg/l); flow rate was 60 l/h. Initial stocking rate was 24 fish per aquarium with one aquarium per treatment.

For each study, fish were treated with 100 mg Aroclor 1254/kg body weight by intraperitoneal (i.p.) injection, and sacrificed after 96 h. Corn oil was used as the vehicle at the rate of 2 ml/kg; controls were treated with corn oil at the same rate.

2.3. Tissue samples

Fish were quickly decapitated and the liver was surgically removed and rinsed with 0.9% NaCl. Tissue samples were immediately chilled on ice; due to liver size, eight livers were combined for each replication. Microsomal fractions were immediately isolated as described in Forsyth and Chambers (1989), covered with 50 mM Tris–HCl + 150 mM KCl (pH 7.4) buffer and stored at -70 ± 2°C until assayed.

2.4. Phosphorothionate metabolism

Desulfuration was quantified by a modification of the method of Forsyth and Chambers (1989) using 15 mg wet weight equivalent liver microsomes in a final reaction volume of 250 μl. (Previous experiments indicated that bubbling with CO or a mixture of O_2:CO_2 was not necessary for desulfuration or dearylation assays.) Assays were incubated for 5 min (Cp) or 15 min (Pth) in 100 mM Tris–HCl + 5 mM $MgSO_4$ (pH 7.4) buffer at 30°C in the presence or absence of 50 μl NADPH-generating system consisting of 10 mM glucose-6-phosphate, 1 mM $NADP^+$ and 1 U glucose-6-phosphate dehydrogenase. Controls did not contain the NADPH-generating system. Final substrate concentrations were 10 and 50 μM for Cp and Pth. The exogenous source of AChE to trap the oxon as it was produced was the supernatant fraction of an 8.5 mg/ml channel catfish brain homogenate (in 50 mM Tris–HCl, pH 7.4) centrifuged at $17\,500 \times g$ for 1 min. An acetylthiocholine solution was added to yield a final concentration of 875 μM (2 ml final volume) to retard desulfuration. The assay was incubated for an additional 5 min at 30°C and reactions were terminated by the addition of 250 μl of a 1:4 mixture of 0.95% 5,5′-dithiobis-2-nitrobenzoic acid and 5% sodium dodecyl sulfate; absorbance was measured at 412 nm. Standard curves of logit-transformed percent inhibition values for channel catfish brain AChE versus log concentration of Cpxn or Pxn were established for microsomes of each replication; assay results were compared with the appropriate standard curve to quantify the amount of oxon produced during desulfuration.

Dearylation was quantified by a modification of the method described by Chambers et al. (1994). Liver microsomes, 100 mg wet weight equivalent, were incubated in 100 mM Tris–HCl + 5 mM $MgCl_2$ (pH 7.4) buffer at 30°C for 15 min in the presence or absence of 50 μl NADPH-generating system consisting of 100 mM glucose-6-phosphate, 10 mM $NADP^+$ and 1.6 U glucose-6-phosphate dehydrogenase; final volume was 1 ml for Cp and 500 μl for Pth. Controls did not contain the NADPH-generating system. Final substrate concentration was 50 μM. The dearylation products for Cp (3,5,6-trichloropyridinol) or for Pth (4-nitrophenol) were quantified spectrophotometrically at 340 and 405 nm, respectively.

2.5. EROD activity

The specific activity of EROD was quantified by a modification of the method of Lake and

Paine (1983). Liver microsomes, 100 mg wet weight equivalent, were warmed in 100 mM Tris–HCl + 5 mM $MgSO_4$ (pH 7.4) buffer at 30°C for 5 min in the presence of 200 μl NADPH-generating system consisting of 10 mM glucose-6-phosphate, 1 mM $NADP^+$ and 10 U glucose-6-phosphate dehydrogenase; the final volume was 1 ml. The reaction was initiated by the addition of 7-ethoxyresorufin (1.5 μM final concentration) and incubated at 30°C for 5 min. Reactions were terminated by the addition of 500 μl 5% $ZnSO_4$ and 500 μl saturated $Ba(OH)_2$ to precipitate proteins. Mixtures were centrifuged for 15 min at 17 500 × *g* and 0–4°C. Resorufin content was determined by mixing 1 ml of the supernatant with 2 ml of 500 mM glycine–NaOH buffer (pH 8.5), and measuring the fluorescence at 582 nm with excitation at 535 nm in a Perkin-Elmer LS 50B luminescence spectrometer.

Table 1
Mean (±S.E.M.) specific activities of hepatic microsomal desulfuration and dearylation of chlorpyrifos and parathion, and EROD in channel catfish fingerlings, 96 h after i.p. injection of 100 mg/kg Aroclor 1254

Parameter	Activity (pmol product formed/min per g wet weight)[a]	
	Control[b]	Aroclor 1254
Desulfuration		
Chlorpyrifos, 10 μM	$436^{X,x} \pm 144$	$691^{X,x} \pm 194$
Chlorpyrifos, 50 μM	$955^{X,x} \pm 261$	$1762^{X,y} \pm 285$
Parathion, 10 μM	$419^{X,x} \pm 37$	$591^{X,x} \pm 120$
Parathion, 50 μM	$502^{X,x} \pm 33$	$851^{X,x} \pm 214$
Dearylation		
Chlorpyrifos, 50 μM	$23\ 473^{X,x} \pm 2758$	$22\ 024^{X,x} \pm 2274$
Parathion, 50 μM	$388^{X,y} \pm 182$	$432^{X,y} \pm 197$
EROD	$667^{X} \pm 209$	$1308^{Y} \pm 115$

[a] Three replications. Means within a row not followed by the same capital letter (X, Y) are significantly different ($P \pm 0.05$) by LSD. Desulfuration or dearylation means within a treatment not followed by the same lower case letter (x, y) are significantly different ($P \pm 0.05$) by LSD.
[b] Vehicle dose, 2 ml/kg corn oil.

2.6. Cytochrome P450

A SLM/Aminco DW2000 split beam spectrophotometer was used to quantify P450s in microsomes by the dithionite-difference spectroscopy method of Matsubara et al. (1976) using an extinction coefficient of 104 mM^{-1} cm^{-1}. The tissue concentration was 200 mg wet weight equivalents/ml.

2.7. Immunological detection

Polyacrylamide gel electrophoresis and Western immunoblotting was performed by the method of Schlenk et al. (1993). Polyclonal antibodies raised in rabbits to rainbow trout P450s CYP1A1, CYP2Kl, CYP2M1 and CYP3A27 were the generous gift of Dr Donald Buhler (Oregon State University), and were utilized because previous studies by Schlenk et al. (1993) and Perkins and Schlenk (1998) indicated the presence of four isoforms recognized by these antibodies. A Bio-RAD GS670 imaging densitometer was used to quantify the optical density units of each band.

2.8. Protein

Protein was quantified for all assays by the method of Lowry et al. (1951). Bovine serum albumin was used as the standard.

2.9. Statistical analysis

Specific activities were analyzed by analysis of variance followed by the least significant difference (LSD) test using SAS on a personal computer. A level of $P < 0.05$ was used to conclude a significant difference among means.

3. Results

In the initial experiment (Table 1), no statistically significant differences among the Aroclor-treated fish and the corresponding controls within any of the desulfuration or dearylation assays were found; however, mean specific activities of the Aroclor-treated fish were higher than their

Table 2
Mean (±S.E.M.) quantity or specific activity of channel catfish fingerling hepatic microsomal P450s, CYP1A1, EROD and protein. 96 h after i.p. injection of 100 mg/kg Aroclor 1254

Parameter	Quantity[a]	
	Control[b]	Aroclor 1254
P450s (pmol/g wet weight)	$2490^{X} \pm 57$	$2721^{X} \pm 364$
CYP1A (optical density units)	$1.18^{X} \pm 0.1$	$5.87^{Y} \pm 0.8$
EROD (pmol/min per g wet weight)	$1222^{X} \pm 190$	$1669^{Y} \pm 36$
Protein (mg/g)	$11^{X} \pm 0.3$	$11^{X} \pm 0.4$

[a] Three replications; means within a row not followed by the same capital letter (X, Y) are significantly different ($P \pm 0.05$) by LSD.
[b] Vehicle dose, 2 ml/kg corn oil.

srespective controls. Mean specific activities of EROD were significantly higher (96%) in Aroclor-treated fish compared with controls.

There were no statistical differences between desulfuration specific activities of Cp or Pth at 10 μM substrate concentrations; Cp desulfuration activities at 50 μM were higher than those of Pth, and in the Aroclor-treated fish, this difference was significant. Desulfuration specific activities of Cp at 50 μM substrate concentration was significantly higher than at 10 μM in the Aroclor-treated fish; Cp desulfuration in the controls was also higher at 50 μM compared with 10 μM, although not statistically significant. Desulfuration specific activities of Pth were not significantly different between the 10 and 50 μM substrate concentrations, although the 50 μM concentrations resulted in slightly higher activities. All microsomes significantly dearylated Cp to a greater extent than Pth when assayed at 50 μM substrate concentration. There were no significant differences among protein concentrations (data not shown).

In the second experiment (Table 2), quantification of P450s yielded no significant differences between the controls and the Aroclor-treated fish. The EROD specific activities were significantly higher (37%) among the Aroclor-treated fish than in the controls. Western immunoblot quantification of CYP1A demonstrated significant induction (397%) in the Aroclor-treated fish as compared with the controls. Expression of proteins recognized by the anti-trout antibodies CYP2K1, CYP2M1 and CYP3A27 were not altered by Aroclor treatment (data not shown).

4. Discussion

Approximately 800 P450 genes have been identified throughout phylogeny and nearly 50 have been identified in fish (Nelson homepage, <http://drnelson.utmem.edu/p450db.html>). Because of its use as a biomarker of exposure for compounds such as PAHs, PCBs, chlorinated dioxins, and chlorinated dibenzofurans, most studies of P450s in fish have focused on the CYP1A subfamily (Stegeman, 1993). The classical activities to quantify induction of CYP1A include EROD, ECOD (ethoxycoumarin-O-deethylase), and aryl hydrocarbon hydroxylase (Goksøyr and Förlin, 1992).

Immunoblotting with CYP1A1 antibodies and the EROD assay were used as positive controls to verify induction of CYP1A by Aroclor 1254 in the present study. Desulfuration was assayed at two substrate concentrations because previous work in this laboratory had identified both low and high K_m activities in Sprague–Dawley rat (*Rattus norvegicus*) liver microsomes contributing to the activation of both substrates (Ma and Chambers, 1995). However, it is unknown whether two or more activities displaying different K_m values exist in catfish microsomes. Control desulfuration specific activities were similar for both phosphorothionates at 10 μM substrate concentration, but desulfuration of Cp at 50 μM was approximately two-fold greater than that of Pth. While this indicates that Cp may be more susceptible than Pth to desulfuration at higher concentrations, the high toxicity of Cp might prevent this concentration from being achieved in vivo; 50 μM Cp is equal to 17.5 mg/l and this level would be lethal to channel catfish as a water column concentration. Control dearylation specific activities of Cp were approximately 60-fold greater than that of Pth at 50 μM substrate concentration, implying a far greater detoxication potential for Cp than for Pth. Dearylation specific activities for Cp were

approximately 25-fold greater than desulfuration activities at 50 μM substrate concentration; desulfuration activities of Pth at 50 μM were slightly greater than dearylation activities. These results do not correlate with the acute toxicities of the two compounds.

Boone and Chambers (1997) demonstrated that Cp desulfuration and dearylation specific activities were approximately two-fold greater than Pth using 50 μM concentrations of each substrate in mosquitofish (*Gambusia affinis*) hepatic microsomes; no inducers were used in that study. Comparison of their work with the 50 μM substrate concentration controls of the present study demonstrates that mosquitofish desulfuration was greater (approximately nine- and seven-fold, respectively) for Cp and Pth than for channel catfish; dearylation of Cp was greater (four-fold) in channel catfish, while dearylation of Pth was greater (eight-fold) in mosquitofish. In the present study, the substantially higher control dearylation specific activities of Cp compared both with Pth dearylation and with Cp desulfuration suggests that Cp should have a lower acute toxicity than Pth.

Acute toxicity of the parent insecticides to fish reflects AChE sensitivity to oxon inhibition (Straus and Chambers, 1995; Boone and Chambers, 1996); therefore, metabolism of the insecticides may be less important in influencing the final acute toxicity levels in fish than it is in mammals. Detoxication esterases, which are not present in large concentrations in channel catfish (Straus and Chambers, 1995) as they are in rats, may protect rats from these compounds far more effectively than fish.

Chambers et al. (1994) reported male rat microsomal desulfuration specific activities at 50 μM substrate concentration for Cp and Pth to be 6.7 and 50.9 nmol/min per g wet weight, while dearylation at 50 μM activities were 29.1 and 13.8 nmol/min per g wet weight, respectively. Comparing results from their study with the present study, rat desulfuration specific activities for Cp and Pth were approximately seven- and 101-fold higher than in channel catfish, respectively. Dearylation specific activities in rat and channel catfish microsomes were similar for Cp, but were 36-fold higher in rat than in channel catfish for Pth. Assay incubation temperatures for the rat and the channel catfish were 37 and 30°C (as a representative environmental temperature), respectively, and comparisons should be made with caution. The generally higher rates of desulfuration and dearylation in the rat may be due in part to the adaptation to a higher physiological operating temperature. The absence of flavine-containing monooxygenase (FMO) activity may also account for any differences; however, Cp and Pth are poor FMO substrates. Schlenk et al. (1993) reported that FMO activity did not appear to be present in liver microsomes from channel catfish or that they rapidly degraded during tissue homogenization.

In the initial study, EROD was induced in the Aroclor-treated fish, while microsomal protein was not (data not shown); induction of Cp and Pth desulfuration or dearylation specific activities was not evident. Significant induction of hepatic EROD and CYP1A were observed in the Aroclor-treated fish of the second study; however, expression of other hepatic P450 isoforms was not altered. These results indicate that treatment with 100 mg Aroclor 1254/kg does not induce the P450 isoform(s) most responsible for desulfuration or dearylation, and that the isoform(s) responsible for these activities are probably not CYP1A.

Previous studies by Schlenk et al. (1993) and Perkins and Schlenk (1998) indicated the presence of four isoforms recognized by antibodies raised against rainbow trout hepatic P450s (CYP1A1, CYP2K1, CYP2M1 and CYP3A27). Although treatment with Aroclor did not alter profiles of the proteins recognized by these antibodies, there is a possibility that other P450 isoforms, which were not recognized by the anti-trout antibodies in channel catfish, may catalyze the desulfuration or dearylation of these compounds.

The EROD specific activity of the control treatment from the study of Ankley et al. (1986) was 6.7 pmol/min per mg protein; a study by Ankley and Blazer (1988) showed that EROD specific activities varied from 10.6 to19.5 according to diet. Another study by Ankley et al. (1989) reported EROD specific activities that were four-fold higher than previous studies in their laboratory, and indicated that the higher values

were probably due to differences in the purity of resorufin used as a standard for calibration of activity. Ronis et al. (1992) demonstrated control EROD specific activities to be 57 pmol/mg per min. Factors such as channel catfish strain differences or laboratory technique may also affect results of the EROD assay.

In summary, this is the first report establishing specific activities of hepatic microsomal desulfuration and dearylation in channel catfish. These specific activities do not reflect the acute toxicity levels of Cp and Pth, and demonstrate that levels of Aroclor 1254, which induce EROD or CYP1A1, do not significantly induce desulfuration or dearylation. The variability in the present studies ($n = 3$ was chosen due to logistics) was high in contrast to laboratory rats; this should be addressed in future studies by using a larger sample size. Future studies are needed to evaluate known inducers/inhibitors of P450s as potential regulators of phosphorothionate desulfuration or dearylation specific activities; such evaluations could possibly lead to a better understanding of OP biotransformation and toxicity. Sexual dimorphism within a catfish population on the specific activities of desulfuration and/or dearylation should also be examined, as well as other sexual or seasonal differences in enzyme activity.

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