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GLUTATHIONE *S*-TRANSFERASE-MEDIATED CHLOROTHALONIL METABOLISM IN LIVER AND GILL SUBCELLULAR FRACTIONS OF CHANNEL CATFISH

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Abstract—Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile) is a broad spectrum fungicide that is a potent acute toxicant to fish. Therefore, the metabolism of chlorothalonil was investigated in liver and gill cytosolic and microsomal fractions from channel catfish (*Ictalurus punctatus*) using HPLC. All fractions catalyzed the metabolism of chlorothalonil to polar metabolites. Chlorothalonil metabolism by cytosolic fractions was reduced markedly when glutathione (GSH) was omitted from the reaction mixtures. The lack of microsomal metabolism in the presence of either NADPH or an NADPH-regenerating system indicated direct glutathione *S*-transferase (GST)-catalyzed conjugation with GSH without prior oxidation by cytochrome P450. Cytosolic and microsomal GSTs from both tissues were also active toward 1-chloro-2,4-dinitrobenzene (CDNB), a commonly employed reference substrate. In summary, channel catfish detoxified chlorothalonil *in vitro* by GST-catalyzed GSH conjugation in the liver and gill. The present report is the first to confirm microsomal GST activity toward CDNB in gill and toward chlorothalonil in liver, and also of gill cytosolic GST activity towards chlorothalonil, in an aquatic species.

Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile, mol. wt = 265.9, Fig. 1) is a broad spectrum fungicide which is used in a variety of agricultural applications, including aerial spraying on potatoes and other crops. Possessing four electrophilic chlorine atoms, chlorothalonil readily undergoes nucleophilic substitution with glutathione and other low molecular weight intracellular thiols in phytopathogenic fungi [1]. Previous studies have indicated that the fungitoxicity of chlorothalonil is due to its ability to inhibit glucose oxidation following depletion of glutathione [1, 2]. Despite its low acute toxicity to rats (LD_{50} = 10 g/kg) [3], chronic dietary exposure of chlorothalonil in that species results in the production of adenomas and carcinomas of the renal tubular epithelium [3].

In contrast to rodents, fish are highly sensitive to the acute toxicity of chlorothalonil. The 96-hr static LC_{50} for channel catfish (*Ictalurus punctatus*) was 52 μg/L§, while that for rainbow trout (*Oncorhynchus mykiss*) is 18 μg/L [4]. The 96-hr flow-through LC_{50} for spotted galaxias (*Galaxias truttaceus*) is 18.9 μg/L while that for golden galaxias (*Galaxias auratus*) is 29.2 μg/L [4]. Furthermore, the toxicity of chlorothalonil to channel catfish was increased 3-fold after depletion of hepatic and gill GSH§‖, indicating a critical role of GSH in protecting against chlorothalonil. Davies [5] showed that the protective effect of GSH in rainbow trout (*O. mykiss*) is due to a decreased amount of chlorothalonil available for protein binding in the liver due to GST-mediated conjugation and excretion. Based on the high correlation of LC_{50} values with liver cytosolic GST activity toward [^{14}C]chlorothalonil in several fish species, it has been suggested that hepatic cytosolic GST induction could be used as a predictor of chlorothalonil toxicity in fish [6]. Cytosolic GST activities have been reported in a variety of tissues in marine and freshwater fish [7–10]. As in mammals [11], fish hepatic GSTs constitute a significant portion of soluble hepatic protein and conjugate a range of electrophilic substrates with GSH, although there is evidence that the range of substrates is less diverse than those catalyzed by rodent liver GSTs [10]. To date, all fish cytosolic GSTs have exhibited some capacity to metabolize 1-chloro-2,4-dinitrobenzene (CDNB), while many others have demonstrated the ability to metabolize epoxides [7, 12].

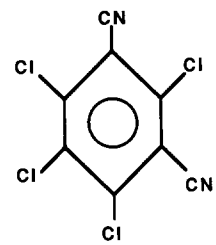

Fig. 1. Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile).

Although cytosolic GSTs have been purified from a large number of different tissues and species, little is known about the functional properties of microsomal GSTs. Microsomal GSTs play a critical role in the conjugation of hexachloro-1:3-butadiene [13] and mitozantrone [14] in rats. Rodent

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§ Gallagher EP and Di Giulio RT, manuscript in preparation.
‖ Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione *S*-transferases; and GSH, reduced glutathione.

microsomal GSTs are activated by sulfhydryl reagents such as *N*-ethylmaleimide, and thus can be distinguished from cytosolic GSTs [15]. However, unlike cytosolic GSTs, the microsomal forms do not show increased activities after treatment with inducing agents such as phenobarbital, methylcholanthrene, or *trans*-stilbene oxide [15]. The localization of 80% of rat liver microsomal GST in the endoplasmic reticulum suggests a functionality related to the detoxification of intermediates formed during Phase I oxidations [16]. Information regarding the role of microsomal GSTs in xenobiotic metabolism by aquatic species is scarce, consisting of one report of hepatic microsomal GST activity towards CDNB in pike (*Esox lucius*) [17].

The purpose of the present investigation was to examine the ability of channel catfish hepatic and gill cytosolic and microsomal GSTs to metabolize chlorothalonil. We have included the gills in this study since they are the initial site of exposure to waterborne pollutants, and because relatively little is known regarding the role of gill GSTs in xenobiotic metabolism. Finally, activities of catfish liver and gill GSTs towards the reference substrate, CDNB, are reported and discussed in comparison to similar activities in other species to reveal further functional aspects of catfish GSTs.

MATERIALS AND METHODS

Chemicals. Chlorothalonil (99.8% purity) was purchased from ChemService (West Chester, PA). All other chemicals used were of reagent grade and obtained from commercial sources.

Animals. Juvenile channel catfish (1-year-old, 40–80 g) were used in this study to minimize sex-related differences in metabolism. All catfish were obtained from Aquaculture Advisory Service (Raleigh, NC) and maintained at approximately 22° (natural photoperiod) in a 1200-L stainless steel tank with flow-through dechlorinated city water. Fish were fed to satiation every other day with Westco floating catfish food (32% protein; Charlotte, NC). An acclimation period of at least 1 week preceded all experimental use of the animals in order to reduce the effects of stress-related responses resulting from transport.

Preparation of tissue fractions. Catfish were killed by severing the spinal cord. Livers and gills were rapidly excised and washed in ice-cold saline. Gill filaments were trimmed carefully from the gill arches and pooled, and the arches were discarded. Each organ was homogenized with a Brinkmann (Westbury, NY) polytron for 10–15 sec, in 4 vol. (w:v) of ice-cold 100 mM Tris buffer (pH 7.4) containing 1 mM EDTA. Microsomal and cytosolic fractions were isolated by the method of Eriksson *et al.* [18]. Briefly, the homogenates were centrifuged at 2500 *g* (0–4°) for 10 min, and the large particle fraction was discarded. The 2500 *g* supernatants were filtered through a Buchner funnel to remove the floating fatty layer, and centrifuged at 10,000 *g* for 20 min. The cytosolic fraction was isolated from the 10,000 *g* supernatant by centrifugation at 105,000 *g* for 60 min. The microsomal pellets were carefully rinsed in 0.5 M KCl and recentrifuged at 100,000 *g* for 60 min to remove non-microsomal protein [18]. The microsomal pellets were resuspended in 0.1 M Tris buffer (pH 7.4) containing 20% glycerol [19]. Minimal cytosolic contamination of microsomal pellets was demonstrated by low levels of lactate dehydrogenase (LDH) activities (<4% liver cytosol and <3% gill cytosolic LDH) [20]. Protein was measured by the method of Lowry *et al.* [21] using bovine serum albumin as a standard. All tissue fractions were stored at −80° and analyzed within 2 weeks of preparation. Enzyme preparations were thawed only once prior to use in the assays. It has been shown previously that storage under these conditions does not affect GST activities [19].

Incubation conditions. The incubation mixtures contained Tris–HCl buffer (0.1 M) with EDTA (1.0 mM), chlorothalonil (4.5 to 13.5 μM) delivered in methanol (2.25% final concentration), and tissue subfraction (0.02 to 0.54 mg protein) in a final volume of 3.0 mL. The apparent pH optimum for GST activities in the various fractions and tissues (8.0 for liver cytosol, 8.5 for liver microsomes, 7.5 for gill cytosol and 8.5 for gill microsomes) was obtained by incubating three pooled tissue fractions at 0.5 pH intervals in the range of pH 6.5 to 8.5, unless otherwise indicated. The reactions, which were initiated by the addition of GSH (1.0 mM) or the appropriate tissue subfraction, were incubated with shaking at 25°. For determination of P450-mediated chlorothalonil metabolism, incubation mixtures contained either 1.0 mM NADPH or an NADPH-regenerating system [0.5 mM NADP, 10 mM glucose-6-phosphate, 1.0 units/mL glucose-6-phosphate dehydrogenase, and 3.0 mM magnesium chloride in 0.1 M Tris–HCl (pH 7.75) containing EDTA (1.0 mM)]. In some experiments, the tissue fractions were inactivated by heating to 80° for 10 min and then were cooled to 4° prior to incubation with chlorothalonil. Aliquots (0.20 mL) were withdrawn from the reactions at various time points and the reaction was terminated by the addition of hexane (0.5 mL). The hexane extracts were vortexed vigorously to extract chlorothalonil into the organic phase. The extraction efficiency of chlorothalonil was 98%, as determined by the HPLC analysis described below.

All incubations were carried out under initial rate conditions where chlorothalonil consumption was a linear function of time and protein concentration. Incubations in the absence of tissue fractions were carried out with each experiment. Non-enzymatic rates of chlorothalonil consumption were subtracted from total rates in the presence of tissue subfractions to give the rate of the enzyme-catalyzed reaction. The data presented are mean values from determinations with at least four different tissue preparations.

HPLC analysis. The change in chlorothalonil concentration was quantified by HPLC analysis of the hexane extracts (225 μL) using a Perkin–Elmer (Norwalk, CT) Series 400 solvent delivery system equipped with a C18 reverse phase column (10 × 25 cm) and an LC-95 UV/Vis detector. The effluent was monitored at 254 nm and peak areas were calculated by an LC-100 integrator. The data processor was used to measure peak areas and retention times. The mobile phase consisted of 20%

Table 1. Metabolism of chlorothalonil by channel catfish liver and gill subcellular fractions*

Fraction	Additions/Omissions GSH	NADPH	N	Chlorothalonil metabolism† (nmol/min/mg protein)
Liver				
Cytosol	+	−	4	388 ± 77
	−	−	3	27 ± 3
Microsomes	+	−	6	51 ± 10
	−	−	3	<1.0
	−	+	1‡	<0.050§
Gill				
Cytosol	+	−	4	115 ± 22
	−	−	4	5.7 ± 0.8
Microsomes	+	−	4	6.9 ± 2.6
	−	−	3	<1.0
	−	+	1‡	<0.050§

* Liver and gill fractions were incubated as described under Materials and Methods in 0.1 M Tris–HCl buffer and 4.5 μM chlorothalonil with either catfish liver cytosol (0.020 to 0.030 mg protein), gill cytosol (0.030 to 0.060 mg protein), liver microsomes (0.05 to 4.6 mg protein) or gill microsomes (0.15 to 5.0 mg protein) in a 3.0-mL reaction mixture. Incubations were carried out up to 1 hr in the presence or absence of chlorothalonil (4.5 μM), GSH (1 mM), and NADPH (1.0 mM) or an NADPH-regenerating system in a final reaction mixture of 3.0 mL. Chlorothalonil concentrations were increased to 4.5 to 13.5 μM in microsomal incubations containing either NADPH or an NADPH-regenerating system due to non-specific substrate binding by increased levels of microsomal proteins. Non-enzymatic rates (without tissue subfraction, or with heat-inactivated subfraction) were subtracted accordingly.

† Results are means ± SEM of N individual tissue preparations.

‡ Microsomal fractions from three to four animals were pooled (N = 1) in these incubation mixtures.

§ Data represent detection limit of assay with NADPH or NADPH-regenerating system and 4.6 mg of microsomal protein.

water and 80% methanol at a flow rate of 1.3 mL/min. Chlorothalonil (retention time of 17 min) was quantitated by comparison to standard curves generated by taking known amounts of chlorothalonil through the identical procedure. The injection volumes did not affect the linearity of detector response.

The GSH conjugates of chlorothalonil were determined by HPLC analysis of trichloroacetic acid-quenched reaction mixtures. Aliquots (200 μL) of the quenched reaction mixtures were chromatographed using a 15-min linear gradient from 0 to 100% methanol using the system described above. Peaks eluting prior to parent (chlorothalonil) were visually inspected and compared to the appearance of polar metabolites in the absence of enzyme.

Spectrophotometric GST assay. For comparative purposes, the spectrophotometric method of Habig *et al.* [22] was used to assay GST activity towards CDNB (1.0 mM) in gill and liver fractions. The reactions were carried out in 0.1 M Tris–HCl at apparent pH optima (data not shown) for the various tissue fractions (liver cytosol pH 7.6, liver microsomes pH 7.5, gill cytosol pH 8.0, gill microsomes pH 7.5).

RESULTS

Chlorothalonil metabolism by microsomal and cytosolic fractions. The results of the GST-mediated conjugation of chorothalonil by liver and gill fractions are presented in Table 1. Catalysis occurred in all four fractions, with the highest rate of metabolism catalyzed by hepatic cytosol. The rate of chlorothalonil metabolism by gill cytosolic fractions was approximately 30% of that catalyzed by hepatic cytosolic fractions. Substrate disappearance was decreased markedly when GSH was omitted from incubation mixtures containing chlorothalonil and cytosolic preparations from either liver or gills. The residual activity in the incubation mixtures not fortified with GSH appeared to be due to a reaction of chlorothalonil with GSH present in the cytosol. Liver microsomes catalyzed the formation of chlorothalonil–GSH conjugates at a rate approximately 7-fold higher than that observed for gill microsomes. Chorothalonil metabolism did not occur after 1 hr of incubation with microsomal preparations lacking GSH in the presence of oxygen and either NADPH or an NADPH-regenerating system (Table 1). Collectively, these results indicate that, under the assay conditions, a GST-catalyzed, GSH-dependent reaction is the dominant pathway for chlorothalonil metabolism in channel catfish liver and gill.

The time course for a typical reaction of the GST-mediated conjugation of chlorothalonil with GSH under assay conditions is shown in Fig. 2. In general, the time course of reactions catalyzed by the gill cytosolic fractions was similar to that observed for liver cytosol (data not shown). Initial velocities were calculated from the first 30 sec of the reactions

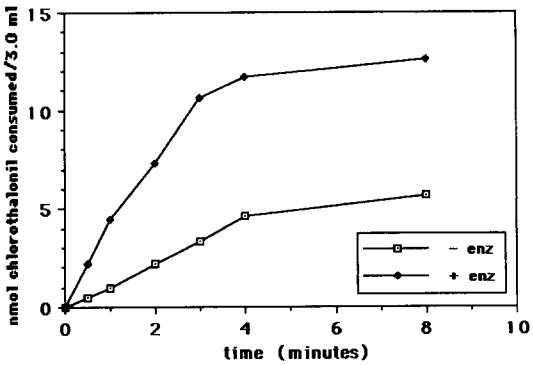

Fig. 2. Time course for the metabolism of chlorothalonil by liver cytosolic fractions. The non-enzymatic rate is included for comparison. Assay conditions are described in Materials and Methods.

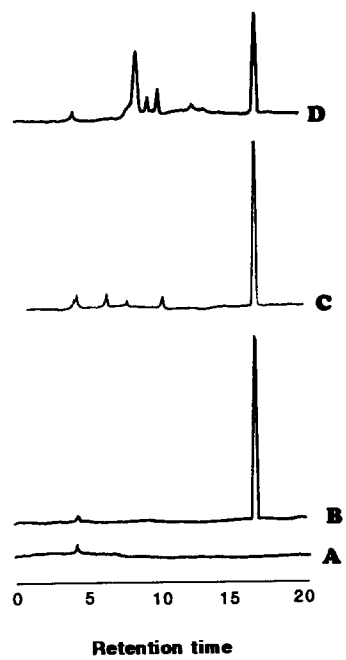

Fig. 3. Representative chromatograms following 10-min incubations of 4.5 μM chlorothalonil in 0.1 M Tris–HCl buffer (pH 8.0), in the presence/absence of 1.0 mM GSH and liver cytosolic fractions (0.025 mg protein). Key: (A) HPLC baseline, (B) chlorothalonil incubated for 10 min in the absence of both GSH and cytosol, (C) appearance of polar metabolites after incubation of chlorothalonil with GSH, and (D) appearance of polar metabolites in complete reaction mixture containing GSH and cytosol.

catalyzed by cytosolic fractions because marked substrate depletion occurred after 2 min. Excess substrate depletion did not occur as rapidly for liver microsomal fractions and was not a problem in reactions catalyzed by gill microsomes. Therefore, later time points were considered and longer incubations were employed for quantification of initial rate velocities for these fractions. As illustrated in Fig. 3, the loss of substrate in the reaction mixtures incubated with hepatic cytosol was accompanied by an increase in multiple polar metabolites which elute prior to the parent compound. In general, similar patterns in metabolite production were observed in reaction mixtures containing other tissue fractions (chromatograms not shown). However, there was no attempt to identify or quantitate polar metabolites of cholorothalonil in this study.

pH dependence. The pH-dependent activity profiles for channel catfish liver cytosolic and microsomal GSTs toward chlorothalonil are shown in panels A and B of Fig. 4. Peak activity for the cytosolic GSTs occurred at pH 8.0, whereas peak microsomal GST activity occurred at pH 8.5. For reference, non-enzymatic activity (chemical reaction) is displayed in Fig. 4E, reflecting the formation of the glutathione anion as pH increases. The substantial non-enzymatic rate for conjugation of chlorothalonil with GSH at pH > 8.0 represents a direct reflection of the ability of GSH to bind with chlorothalonil at multiple sites [6]. Gill cytosolic GST activity peaked sharply at pH 7.5 (Fig. 4C), whereas microsomal GST activity displayed a broad peak with maximal activity at pH 8.5 (Fig. 4D).

Spectrophotometric GST assay. Cytosolic and microsomal fractions were also assayed spectrophotometrically regarding their respective abilities to metabolize CDNB (Table 2). As with chlorothalonil, GST-catalyzed conjugation of CDNB with GSH occurred in all four fractions in the order: liver cytosol > gill cytosol > liver microsomes > gill microsomes. In addition, the rate of CDNB metabolism exceeded that for chlorothalonil in both tissues and fractions.

DISCUSSION

Although the exact mechanism of chlorothalonil toxicity to fish is unknown, covalent binding and inactivation of mitochondrial respiratory chain proteins may account for at least part of its toxicity [4]. This hypothesis is also supported by a report demonstrating that short-term *in vivo* exposure to chlorothalonil reduces lamellar diffusion capacity and increases ventilatory frequency in rainbow trout [23]. Uptake of chlorothalonil from the water by fish is rapid, and levels in the liver are initially enhanced due to protein binding [4]. The confirmation of biliary chlorothalonil–GSH conjugates in the bile of rainbow trout provides supporting evidence for the protective role of GSTs, which limit the amount of chlorothalonil available for protein binding [5].

Unlike CDNB, chlorothalonil does not undergo a spectral shift upon conjugation with glutathione [6], thus precluding a spectrophotometric assay. The HPLC assay developed here represents a fairly straightforward and rapid method to assess the metabolism of chlorothalonil under *in vitro* conditions in situations when radiolabeled compound may not be available. Assay conditions for GST-mediated chlorothalonil metabolism are limited by the rapid non-enzymatic reaction rate and by the low water solubility (2.25 μM) of chlorothalonil. The latter necessitates the use of low cytosolic protein concentrations (<50 μg/mL) in order to avoid excess substrate depletion. Higher protein concentrations

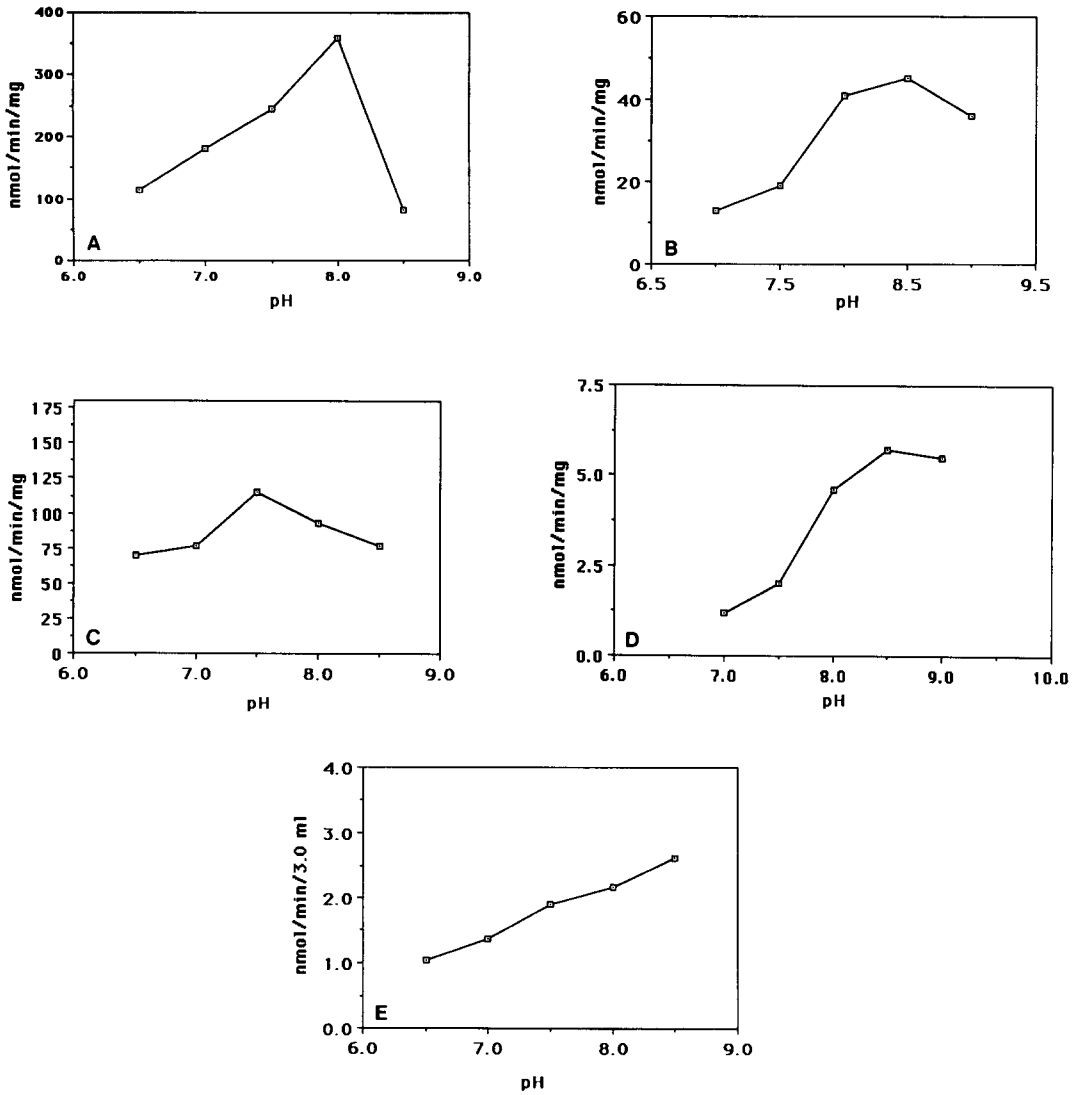

Fig. 4. Effect of pH on glutathione *S*-transferase activity using chlorothalonil as substrate. Each point represents three pooled tissue fractions, with assays conducted in duplicate. Experimental conditions are the same as for Table 1. Key: (A) liver cytosol, (B) liver microsomes, (C) gill cytosol, (D) gill microsomes, and (E) non-enzymatic rate.

Table 2. Activities of glutathione *S*-transferase towards 1-chloro-2,4-dinitrobenzene in channel catfish liver and gill subcellular fractions*

Tissue	Fraction	N	Activity† (nmol/min/mg protein)
Liver	Cytosol	4	1311 ± 120
	Microsomes	4	174 ± 38
Gill	Cytosol	4	711 ± 139
	Microsomes	4	25 ± 1

* Activities were determined by the spectrophotometric assay of Habig *et al.* [22].

† Values are means ± SEM of N animals.

(0.166 to 1.53 mg/mL) were used in microsomal incubations (minus GSH) in the presence of oxygen and an NADPH-regenerating system, since neither substrate disappearance nor a significant non-enzymatic reaction occurred under those conditions. However, precise determination of substrate loss in the presence of high microsomal protein concentrations (>1 mg/mL) was hampered by binding of chlorothalonil to microsomal proteins (data not shown). Accordingly, the limit of assay sensitivity in the microsomal reactions (minus GSH) was 0.05 nmol/min/mg protein.

The time course for a typical reaction involving chlorothalonil metabolism by hepatic cytosol (Fig. 2) was similar to the same reaction reported by Davies [6] for rainbow trout. The lack of cytosolic catalysis in the absence of GSH, together with the

lack of effect by NADPH on the rate of microsomal metabolism, suggests direct GST-mediated catalysis without prior activation by cytochrome P450. Since chlorothalonil contains several electrophilic carbon atoms, it is an excellent subtrate for nucleophilic attack by GSH. The different pH activity profiles for cytosolic and microsomal GST activities (Fig. 4) suggest distinct GSTs in those two subcellular compartments. To our knowledge, the present study is the only report of gill microsomal GST activity in a fish species. Fish microsomal GSTs, as opposed to those of rodents, cannot be distinguished from cytosolic GSTs via activation by sulfhydryl reagents such as *N*-ethylmaleimide [15], thus raising the possibility that some of the microsomal activity observed in this study was due to adsorbed cytosolic GST. However, the level of cytosolic contamination of the microsomal pellets (<4% cytosolic LDH) would not account for total GST activity observed in both gill and liver microsomes towards either CDNB or chlorothalonil. In addition, the different pH optima observed in both tissues for microsomal and cytosolic GSTs towards CDNB and chlorothalonil strongly suggest the presence of different proteins.

The rate of gill cytosolic GST activity towards CDNB was higher than previously reported for other aquatic species [7, 8]. Lauren *et al.* [24] reported that gill cytosolic GSTs of rainbow trout incubated at 25° are active toward CDNB but not *cis*-stilbene oxide or its corresponding *trans*-isomer. In that study, no GST activity toward CDNB, *cis*-stilbene oxide or *trans*-stilbene oxide was observed in trout gill microsomal preparations. The authors concluded that trout gills were well equipped to metabolize alkene, but not arene, oxides. In general, substrate specificities for aquatic animal GSTs are not as broad as those in rodents [10, 25]. Nimmo [8] reported that trout gill GSTs have a more narrow substrate specificity, as well as a different isoenzyme pattern, than liver GSTs. Presently, we are unaware of reports regarding substrate specificities of individual GST isoforms in the channel catfish.

The present report of GST activities toward both CDNB and chlorothalonil in channel catfish gill cytosolic and microsomal fractions has important consequences for the ability of this species to cope with environmental contaminants. For fish, the primary uptake route of waterborne pollutants is through the gill. As a result, the gill, more so than the liver, may be exposed continuously to toxic compounds. Furthermore, the detoxification ability of the gill can have a profound effect on the level of a toxicant reaching other organs. For example, chlorothalonil accumulates over 1000-fold in the trout gill and remains elevated after the fish are transferred to toxicant-free water [4]. Miller *et al.* [26] demonstrated that cytochrome P450E can be strongly induced in the gill of scup (*Stenotomus chrysops*) and rainbow trout by β-naphthoflavone. Immunohistochemical analysis of gills from both species revealed that the P450E is induced primarily in the pillar cells of the secondary lamellae. Since cytochrome P450 is responsible for the activation of many compounds to electrophilic intermediates that are subsequently conjugated with GSH, the finding of gill microsomal GST activity toward CDNB and chlorothalonil is not surprising.

The rate of GST catalysis of GSH–CDNB conjugation reported here in channel catfish hepatic cytosol is about four times higher than that reported for rainbow trout by Lauren *et al.* [24] under similar assay conditions. Channel catfish hepatic microsomal GSTs conjugate CDNB at a rate similar to that reported by Morgenstern *et al.* [17] for pike, but over 2.5-fold more than the same activity reported by Lauren *et al.* [24] for rainbow trout. In addition, Lauren *et al.* did not detect gill microsomal GST activity toward CDNB in rainbow trout. It is possible that channel catfish may be at an advantage over the rainbow trout in detoxifying certain electrophilic compounds or reactive intermediates. However, more detailed enzymatic and *in vivo* studies are required to verify this hypothesis.

In summary, liver and gill fractions of the channel catfish are well equipped to detoxify chlorothalonil and CDNB. To our knowledge, this is the first report of gill microsomal GST activity in a teleost species. Since enzyme-mediated GSH conjugation is an important pathway in the detoxication of reactive intermediates produced during Phase I oxidation of many procarcinogens, channel catfish may be at an advantage when exposed to these agents in the wild. Published reports of tumors in channel catfish taken from polluted sites are virtually non-existent, especially when compared to those involving other ictalurids such as the brown bullhead (*Ictalurus nebulosus*). Whether these observations reflect behavioral, biochemical, or physiological differences remains to be established. Comparative studies concerning the metabolism of electrophilic compounds in ictalurids may shed light on the nature of these differences.

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