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MICROSOMAL AND CYTOSOLIC EPOXIDE HYDROLASE AND GLUTATHIONE *S*-TRANSFERASE ACTIVITIES IN THE GILL, LIVER, AND KIDNEY OF THE RAINBOW TROUT, *SALMO GAIRDNERI*

BASELINE LEVELS AND OPTIMIZATION OF ASSAY CONDITIONS

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Abstract—Microsomal and cytosolic epoxide hydrolase (mEH and cEH respectively) and glutathione *S*-transferase (GST) activities were measured in the liver, kidney, and gills of rainbow trout. Assays were optimized for time, pH, and temperature, using *trans*-stilbene oxide (TSO) and *cis*-stilbene oxide (CSO) as substrates for cEH and mEH, respectively. Optimal pH values for mEH, cEH, and GST were similar to mammalian values (i.e. 8.5, 7.5, and 9). Temperature optima differed between tissues and cell fractions. Specific activity of cEH-TSO was 3–14 times greater than mEH-CSO for all three tissues, and 8–60 times greater on a tissue weight basis. Liver and, to a lesser extent, kidney mEH were active against benzo[*a*]pyrene 4,5-oxide, whereas gill mEH was not active against this substrate. Liver cytosolic GST was active against CSO and 1-chloro-2,4-dinitrobenzene (CDNB) but not TSO, whereas gill and kidney cytosolic GST were active only against CDNB. Liver and kidney microsomal GST were active against CDNB, but no activity was found in gill microsomes. The results are discussed in relation to possible endogenous substrates and uninduced xenobiotic metabolizing capacities of different trout tissues.

Arene and alkene oxides (i.e. epoxides) are important industrial wastes as well as biochemical products of xenobiotic metabolism by the cytochrome P-450-dependent monooxygenase system [1, 2]. Some epoxides are of biological interest because they are capable of alkylating proteins and nucleic acids and, thus, are potential teratogens and carcinogens. The metabolism and clearance of these compounds from the body are aided by the epoxide hydrolases (EH‖; EC 3.3.2.3) and glutathione *S*-transferases (GST; EC 2.5.1.18) [3]. Both enzymes increase water solubility by catalyzing the addition of polar groups: water in the case of EH, and the tri-peptide, glutathione, in the case of GST. Four different epoxide hydrolases have been identified in mammals: microsomal EH (mEH) which hydrates arene oxides and some alkene oxides, cytosolic EH (cEH) which hydrates alkene oxides, cholesterol EH, and leukotriene A_4 hydrolase [4]. *Cis*-Stilbene oxide (CSO) is commonly used as a surrogate substrate for mEH, whereas the corresponding *trans*-isomer (TSO) is commonly used for the analysis of cEH in mice. Both compounds have been used to detect cytosolic GST (cGST) activity [4].

In mammals, certain forms of EH and GST have been used as neoplastic biomarkers [5, 6]. Some species of fish, including the rainbow trout (*Salmo gairdneri*), are very sensitive to known mammalian carcinogens and, therefore, fish models are evolving as important alternatives to mammals in carcinogenesis research [7–9]. Thus, one of our objectives is to investigate the possible use of EH and GST as neoplastic biomarkers in fish. Several authors have reported activities of mEH and cGST in fish liver [10–19], gills [13, 20], and kidney [13, 21]. However, despite the fact that the gills are the organs most exposed to industrial pollutants, and both gill [22] and kidney [23] possess MFO activity, there is little literature concerning branchial or renal EH activity toward arene oxides [13]. Furthermore, neither cEH nor microsomal GST (mGST) has been measured previously in any fish tissue. In this paper we describe the optimal assay conditions for these enzymes in liver, kidney, and gills of rainbow trout, using TSO and CSO, and discuss the relative potential for hepatic, renal and branchial metabolism of alkene and arene oxides.

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‖ Abbreviations: EH, epoxide hydrolases; GST, glutathione *S*-transferases; CSO, *cis*-stilbene oxide; TSO, *trans*-stilbene oxide; TCPO, 1,1,1-trichloropropane-2,3-oxide; CDNB, 1-chloro-2,4-dinitrobenzene; BPO, benzo[*a*]pyrene 4,5-oxide; cEH, cytosolic epoxide hydrolase; mEH, microsomal epoxide hydrolase; cGST cytosolic glutathione *S*-transferase; and mGST, microsomal glutathione *S*-transferase.

MATERIALS AND METHODS

Animals

Female rainbow trout (N = 6), *S. gairdneri* (Hot Creek strain; 300–400 g), were obtained from the hatchery on the U.C. Davis campus. They were held within a round flow-through chamber at 15°C and

fed a maintenance diet of commercial trout chow (Silver Cup; S.A. Nelson & Sons).

Enzyme preparation

Trout were killed by a blow to the head, and the gills, liver, and kidney were removed. Gill filaments were trimmed from the arches, and the arches were discarded. Each organ was homogenized with a Brinkmann Polytron for 20 sec, in 9 vol. (w:v) of ice-cold 0.25 M sucrose solution containing 1 mM EDTA and 1 mM dithiothreitol (Sigma). Large particles were separated by centrifugation for 30 min at 16,000 *g* in a Sorval RC-B2 at 4°. This solution was then centrifuged for 120 min at 144,000 *g* in a Beckman L5 ultracentrifuge at 4°, and the supernatant and microsomal fractions were separated. The microsomal pellet was washed and resuspended in a volume of the above solution, equal to the original tissue weight. Statham *et al.* [24] showed that this protocol provides removal of contaminating mitochondria, lysosomes and peroxisomes in the first centrifugation, as well as excellent separation of microsomal from soluble proteins in the second centrifugation. Both fractions were either assayed immediately or frozen at −80°.

Enzyme assays

Epoxide hydrolase and glutathione *S*-transferase activities were measured radiometrically using the procedures of Gill *et al.* [25] with [^{3}H]*trans*- (TSO) and *cis*-stilbene oxides (CSO) as substrates. Operationally, we defined cEH as an enzyme found predominantly in the 100,000 *g* supernatant fraction, and mEH as primarily a microsomal enzyme. This generalization is based on extensive studies in mice, but may not be applicable to teleost fishes. Duplicate assays were carried out in 10 × 75 mm glass tubes containing microsomal or cytosolic protein diluted in buffer (1:14, v:v), pH 6.5–10, containing 0.05 M Tris–HCl, 0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol (mEH and cEH assays), or in the same buffer containing 5 mM reduced glutathione (GST assays). This resulted in protein concentrations ranging from about 5–25 μg/ml in the final assay volume. Assays remained linear up to at least 100 μg protein/ml. The pH of assays was adjusted at 25° and allowed to drift with temperature, since the pK_a of protein histidine residues also drifts with temperature [26]. At the extremes of Tris buffer capacity, pH was checked with litmus paper; this was particularly important for GST assays since GSH is an acid. The effect of pH-dependent changes in ionic strength was checked with KCl solutions added to the assays. No effect of ionic strength was found between 10^{-6} and 10^{-2} M. The assay was begun by the addition of 1 μl of 5×10^{-3} M TSO or CSO in ethanol (5×10^{-5} M final concentration) labeled with about 13,000 cpm. At various times the assays were terminated by the addition of 200 μl *n*-dodecane (mEH, cEH) or 1-hexanol (GST). The tubes were vortexed vigorously to extract the parent compound into the organic phase, leaving diols in the aqueous phase. After centrifugation to separate the phases, a 50-μl subsample of the aqueous phase was added to 3 ml ACS (Amersham) and counted in an LKB rack beta counter. The effects of several inhibitors of mammalian mEH and cEH, as well as stilbene oxide metabolites, were also assayed. TCPO (1 μl, 10 M; Sigma), a series of substituted chalcone oxides (1 μl, 2×10^{-2} M in toluene; synthesized in this laboratory) [27], or the meso-. *R,R*-, or *S,S*-1,2-diphenyl-1,2-ethanediol (1 μl, 0.1 M in ethanol; Aldrich) were adeed to the enzyme preparations and incubated at 30° for 10 min, before addition of CSO. Assays were then conducted as above. For comparative purposes, the hydrolysis of benzo[*a*]pyrene oxide (BPO) was determined by the thin-layer method of Jerina *et al.* [28] using [^{3}H]BPO (1 μl, 1×10^{-2} M in dimethyl sulfoxide). GST activity against 1-chloro-2,4-dinitrobenzene (CDNB) was calculated from the change in absorbance at 340 nm using an extinction coefficient of 9.6 mM^{-1} cm^{-1} [29] and a Varian–Cary 219 spectrophotometer. Protein was measured by the micromethod of Bradford [30] using bovine serum albumin as the standard. Enzyme activities were calculated as specific activity (per mg protein) and total activity (per tissue wet weight).

RESULTS

Effects of freezing on enzyme activities

EH and GST activities from each tissue and subcellular fraction were assayed immediately after preparation and after 24, 48 and 72 hr at −80°. There was no significant difference in specific activity between the fresh and the frozen perparations for mEH, cEH or GST. Enzyme activity decreased by about 50% after three freeze–thaw cycles. Enzyme preparations were aliquoted and thawed only one time for optimization of assay conditions.

Epoxide hydrolase

Linearity with time. Enzyme activities were measured after 10, 20, 40 and 60 min at 30°, and pH 7.5 (cEH) or 8.5 (mEH). cEH-TSO was much more active than mEH-CSO (Fig. 1, A–C vs D–F). For each tissue, mEH-CSO and cEH-TSO activities remained linear for at least 40 min (Fig. 1, A–F).

pH Optima. Specific activities were measured over a pH range of 6.5 to 10.0. The cEH of liver, kidney, and gills displayed broad peaks of maximal activity for TSO between pH 7.0 and 7.5 (Fig. 2, A–C). For all tissues the cysolic forms were more active than the microsomal forms (Fig. 2, A–C vs D–F).

Liver mEH-CSO displayed a sharp peak of maximal activity at pH 8.5 (Fig. 2D), similar to that found by Balk *et al.* [12] for pike liver mEH acting on styrene oxide. Although gill (Fig. 2F) and kidney (Fig. 2E) displayed highest activities at about pH 8.5, the peaks were much less distinct than liver.

Temperature optima. Assays were conducted for 10 min at optimal pH (TSO: pH 7.5; CSO: pH 8.5) at 15° (acclimation temperature), 25°, 30°, and 37°. Activity/temperature varied with tissue and fraction. Liver cEH-TSO increased steadily up to 37°, but mEH began to level off at 30° (Fig. 3). Gill and kidney cEH-TSO increased to 30° but reached an asymptote by 37° (Fig. 3A). Gill and kidney mEH-CSO was inactive at 15°, peaked at 30°, and decreased at 37° (Fig. 3B). As with pH and time analyses, cEH-TSO specific activity followed the orger, gills > liver > kidney (Fig. 2, A–C; Fig. 3A),

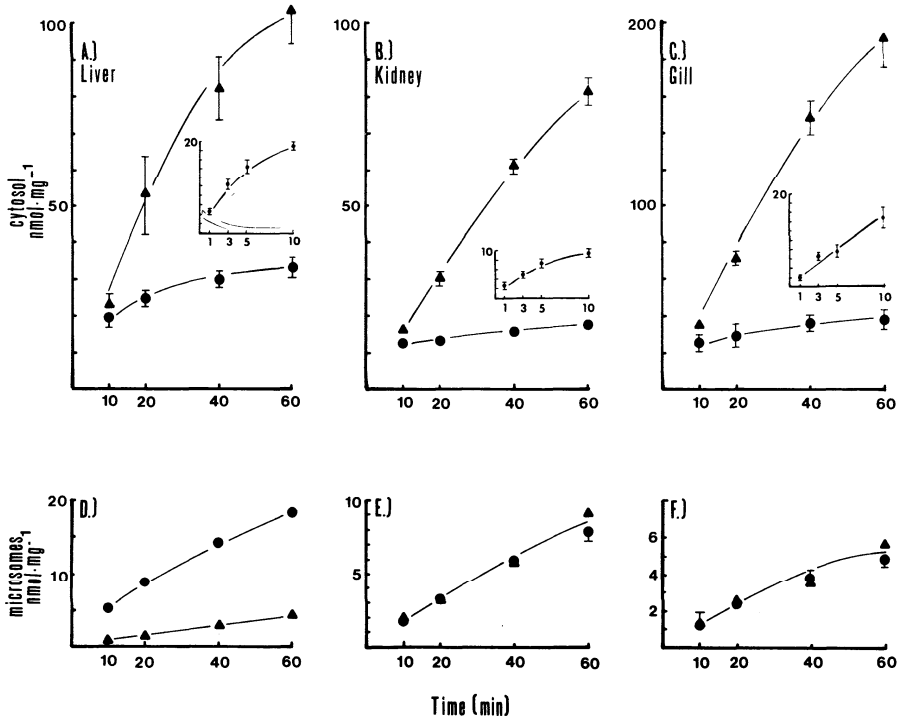

Fig. 1. Time dependence of EH activity using TSO (▲) and CSO (●) as substrates. Points represent means ± SEM of duplicate assays of tissues from three fish. All assays were run at 30°, and pH 7.5 (TSO) or 8.5 (CSO) (A) liver cytosol, (B) kidney cytosol, (C) gill cytosol, (D) liver microsomes, (E) kidney microsomes and (F) gill microsomes. Inserts are early time periods of cEH-CSO activity.

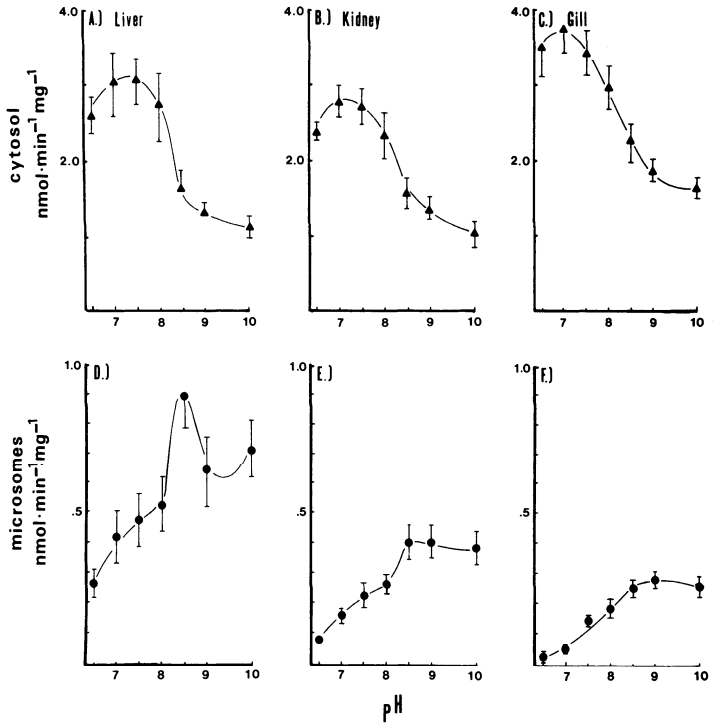

Fig. 2. Effect of pH on EH activity using TSO (▲) and CSO (●) as substrates. Points represent means ± SEM of duplicate assays of tissues from six fish. All assays were run at 30°. (A) liver cytosol, (B) kidney cytosol, (C) gill cytosol, (D) liver microsomes, (E) kidney microsomes, and (F) gill microsomes.

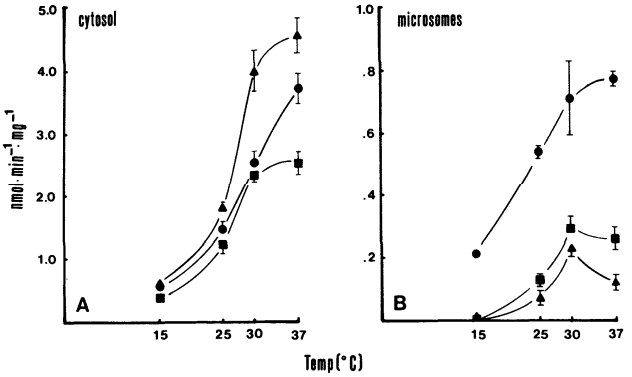

Fig. 3. Effect of temperature on (A) cytosolic EH using TSO at pH 7.5, and (B) microsomal EH using CSO at pH 8.5, in liver (●), kidney (■), and gills (▲). Points represent means ± SEM of duplicate assays of tissues from three fish. No activity was found for gill or kidney mEH-CSO at 15°.

and mEH-CSO followed the order, liver > kidney > gills (Fig. 1, D–F; Fig. 3B).

Alternative substrates and inhibitors. Although CSO is generally used to measure mEH, and TSO to measure cEH, cytosolic and microsomal fractions displayed some activity with the alternative substrate. Microsomes from gill and kidney hydrolyzed TSO at the same rate as CSO, but liver TSO activity was much lower than CSO activity (Fig. 1, D–F). Although cEH from all three tissues hydrolyzed CSO at about the same initial rate as TSO, an asymptote was reached within about 5 min (Fig. 1, A–C, inserts). Little additional hydrolysis was found after 60 min (Fig. 1, A–C). Addition of the meso, R,R, and S,S diols resulted in only about 20% inhibition of cEH-CSO activity. Furthermore, preincubation of cEH with CSO had no effect on TSO hydrolysis.

Microsomes from liver formed diols of BPO at a rate of 3 nmol/min/mg protein, and kidney at 1 nmol/min/mg protein, but no activity was found in gill microsomes. No activity against BPO was found in the cytosol from liver, kidney, or gill.

The classical inhibitor of mammalian mEH, TCPO, inhibited 66% of trout liver mEH-CSO activity at 0.1 M. We also tested a series of substituted chalcone oxide derivatives which have been shown to be highly specific inhibitors of mammalian cEH. Trout gill cEH-TSO was inhibited 86% by 1×10^{-4} M 4-methoxy chalcone oxide.

Glutathione S-*transferase*

No GST activity against CSO or TSO was found in the cytosolic fraction of either kidney or gills, but cytosol from both tissues conjugated CDNB (Table 1). cGST activity was found in the liver against CSO and CDNB, but not TSO (Table 1). GST-CDNB activity was found in the microsomal fraction of liver and kidney, but not gill; no mGST-CSO or mGST-TSO activity was found (Table 1).

Liver cGST-CSO activity at pH 8.5 and 30° was linear with time up to 40 min, but reached an asymptote by 60 min (Fig. 4A). Non-enzymatic conjugation of CSO also increased linearly with time and accounted for about 42% of the total activity at any time (Fig. 4A).

Table 1. Specific activities, and activities per gram tissue wet weight, for cytosolic and microsomal epoxide hydrolase and glutathione *S*-transferase at 30°, pH 7.5 (TSO) or 8.5 (CSO, CDNB)

	Specific activity (nmol/min/mg protein)	Activity (nmol/min/g)
cEH-TSO		
Liver	3.04 ± 0.34	138.7 ± 7.6
Kidney	2.78 ± 0.21	159.5 ± 16.0
Gill	3.76 ± 0.34	98.2 ± 8.5
mEH-CSO		
Liver	0.889 ± 0.10	8.7 ± 1.9
Kidney	0.402 ± 0.06	3.3 ± 1.0
Gill	0.278 ± 0.03	6.8 ± 1.3
cGST-CSO		
Liver	0.605 ± 0.01	2.7 ± 0.2
Kidney	ND*	ND
Gill	ND	ND
cGT-CDNB		
Liver	348 ± 17	15,879 ± 555
Kidney	122 ± 4	6,999 ± 272
Gill	160 ± 36	4,180 ± 646
mGST-CDNB		
Liver	69 ± 19	673 ± 152
Kidney	14 ± 2	113 ± 17
Gill	ND	ND

Values are means ± SEM, N = 6.
* Not detectable.

Liver cGST-CSO exhibited one plateau at about pH 7.5 and another plateau and peak activity at pH 9.0 (Fig. 4B). Non-enzymatic conjugation of CSO increased linearly from pH 6.5 to 10 in the presence of glutathione (Fig. 4B).

cGST-CSO activity increased linearly with temperature from 15° to 30°, and increased more steeply to 37° (Fig. 4C). Non-enzymatic conjugation did not increase with temperature (Fig. 4C).

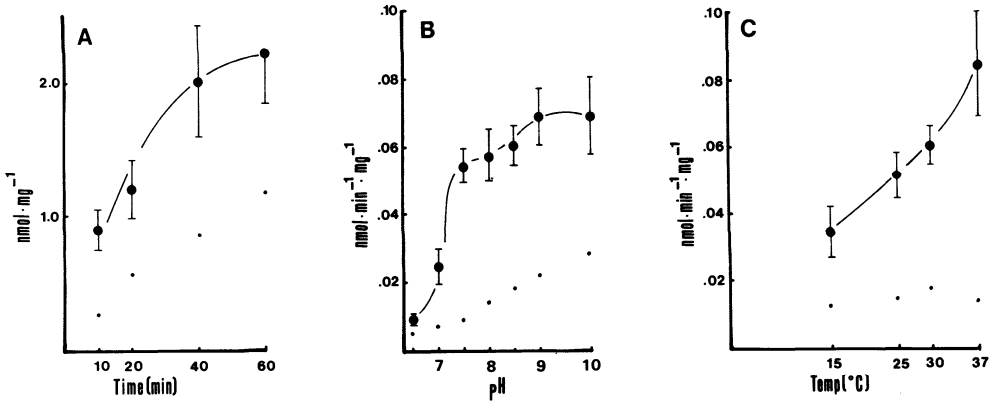

Fig. 4. Liver cytosolic GST activity using CSO (●) as substrate. Points represent means ± SEM of duplicate assays of tissues from three fish. Non-enzymatic hydrolysis (·) has been subtracted from means (A) Time dependence. All assays were run at 30°, at optimal pH. (B) Effect of pH on liver cytosolic GST activity. All assays were run at 30°. (C) Effect of temperature on GST activity. All assays were run at pH 8.5.

DISCUSSION

The pH optima for TSO hydrolysis by cytosol (i.e 7.5) was different from that for CSO by microsomes (8.5), indicating that, as in mammals [31], at least two distinct epoxide hydrolyzing enzymes are found in different cellular compartments of rainbow trout tissues. Furthermore, trout mEH and cEH were similar to mammalian enzymes in regard to inhibition. TCPO, a commonly used inhibitor of mEH in mammals [4], inhibited mEH-CSO activity in trout, and 4-methoxy chalcone oxide, a potent inhibitor of mammalian cEH [27], also inhibited trout cEH-TSO. However, in contrast to mammalian studies of liver [31] and lung [32] in which mEH activity is generally greater than cEH, the liver, kidney, and gills of trout had much greater specific activity toward TSO and CSO. Gill cEH activity was about twice that of either liver or kidney, and was also greater than mouse liver assayed under similar conditions [31]. The endogenous substrate for cEH is not known in either fish or mammals, but rat cEH exhibits very high turnover rates with arachidonic acid epoxides [33]. In this respect, it is interesting to note that trout gill had the highest cEH-TSO activity, and that crude homogenates of trout gill produce hydroxy epoxides of arachidonic and decosaxhexaenoic acid *in vitro* [34]. Thus, cEH may possibly play a role in the physiological control of branchial ionoregulation in the trout, since some eicosanoids have been shown to increase short-circuit current in isolated opercular membranes of *Fundulus heteroclitus* [35].

Few studies have compared the abilities of hepatic with extrahepatic tissues to metabolize either arene or alkene oxides [13], despite the fact that the gill is the first organ to contact water-borne contaminants, and the kidney receives the bulk of the post-branchial blood flow. Cytosolic fractions from liver, gill, and kidney all failed to hydrolyze BPO and were inhibited within about 5 min when incubated with CSO. Thus, cEH probably does not play a significant role in arene oxide metabolism in any organ system of trout. On the other hand, liver microsomes were active against both CSO and BPO, and the rate of BPO hydrolysis by trout liver microsomes, 3 nmol/min/mg protein, was similar to that found for hepatic microsomes of other fish species [13]. However, no activity against BPO was found with gill microsomes, despite the fact that they hydrolyzed CSO at about one-third the rate of the liver. These differences in the ability of mEH to hydrolyze CSO and BPO suggest that hepatic mEH may be different from that found in the gill. Further support comes from the temperature–activity plots. Hepatic mEH-CSO activity increased with temperature from 15° up to 37°, whereas gill was inactive at 15° and decreased from 30° to 37°. Evidence was also found which suggests that the kidney may contain a mixture of isozymes which are found predominantly in either gill or liver. Although kidney mEH hydrolyzed BPO, mEH-CSO was inactive at 15° and decreased at 37°, as was found for gill.

In the presence of glutathione, cGST from liver, kidney and gills was active against CDNB, as was mGST from liver and kidney, but not gills. This again suggests that kidney contains a mixture of hepatic and branchial enzymatic features. mGST was not active against CSO or TSO in any tissue, but cGST was active against CSO in the liver. This supports previous reports from trout [16, 20, 21] that only the liver is active against epoxy substrates, although it must be emphasized that only CSO and 1,2-epoxy-3-(nitrophenoxy)propane have been tried in this species. On the other hand, little skate (*Raja erinacea*) liver, kidney, gill, spleen, and testis cGST conjugates BPO, styrene 7,8-oxide, and octene 1,2-oxide [13]. Although trout cGST-CDNB was about as active as that found in rats [16], cGST-CSO activity was only about 1.8% that found in the mouse [36].

In conclusion, the gill appears to be well suited for the metabolism of alkene, but not arene oxides, as measured by the surrogate substrates TSO and CSO. As in mammals, the trout liver is well suited to metabolize both types of substrates, but is unique in

its capacity for arene oxide metabolism by both mEH and cGST. The kidney appears to have intermediate alkene and arene oxide metabolizing capacities. Relative to mammals, trout appear to be equal or better suited for alkene oxide metabolism, but they are not well suited for arene oxide metabolism. This latter point may, in part, help explain their apparent sensitivity to carcinogenesis [7–9].

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