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Rate and capacity of hepatic microsomal ring-hydroxylation of phenol to hydroquinone and catechol in rainbow trout (*Oncorhynchus mykiss*)

Richard C. Kolanczyk *, Patricia K. Schmieder

Mid-Continent Ecology Division, US Environmental Protection Agency, 6201 Congdon Blvd, Duluth, MN 55804, USA

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Abstract

Rainbow trout (*Oncorhynchus mykiss*) liver microsomes were used to study the rate of ring-hydroxylation of phenol at 11 and 25 °C by directly measuring the production of two potentially toxic metabolites, hydroquinone (HQ) and catechol (CAT). An HPLC method with integrated ultraviolet and electrochemical detection was used for metabolite identification and quantification at low (pmol) formation rates found in fish. The Michaelis–Menten saturation kinetics for the production of HQ and CAT over a range of phenol concentrations were determined at trout physiological pH. The apparent Km's for the production of HQ and CAT at 11 °C were 14 ± 1 and 10 ± 1 mM, respectively, with Vmax's of 552 ± 71 and 161 ± 15 pmol/min per mg protein. The kinetic parameters for HQ and CAT at 25 °C were 22 ± 1 and 32 ± 3 mM (Km) and 1752 ± 175 and 940 ± 73 pmol/min per mg protein (Vmax), respectively. The calculated increase in metabolic rate per 10 °C temperature rise (Q_{10}) was 2.28 for HQ and 3.53 for CAT production. These experiments assess the potential for metabolic bioactivation in fish through direct quantification of putative reactive metabolites at the low, but toxicologically significant, chemical concentrations found in aquatic organisms. This work initiates a series of studies to compare activation pathway, rate, and capacity across fish species, providing a basis for development of biologically-based dose response models in diverse species.

Keywords: Phenol; Bioactivation; Electrochemical detection; Rainbow trout; Metabolism; Hydroquinone; Catechol

1. Introduction

Phenol, substituted phenols, and chemicals that can be metabolically converted to phenolic compounds continue to be used extensively in agriculture and industry in the US (Interagency Testing Committee, 1990), with annual production volumes well in excess of one million pounds. The Environmental Protection Agency's Toxics Release Inventory (1990) indicates that each year large quantities of phenolics are released to air, water, and land. Metabolism of phenol is also of substantial toxicological interest. The biotransformation of xenobiotics is generally considered

* Corresponding author. Tel.: +1-218-529-5152; fax: +1-218-529-5003.

E-mail address: kolanczyk.rick@epa.gov (R.C. Kolanczyk).

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to be a means of chemical detoxification. Phase I oxidation reactions for common substrates, such as phenol (Daly et al., 1965; Irons and Sahawata, 1985) serve to increase chemical polarity and provide substrate for Phase II conjugation reactions thus facilitating chemical elimination, as demonstrated for both aquatic and mammalian species (Mulder, 1982; Layiwola et al., 1983; Nagel, 1983). However, it has also been recognized that metabolic transformation may result in more toxic chemical forms. For instance, the conversion of benzene to phenol may facilitate subsequent conversion to reactive quinones such as hydroquinone (HQ) and benzoquinone (BQ) (Medinsky et al., 1995).

Bioactivation reactions which have been studied to a considerable extent in mammals (see Guengerich and Liebler, 1985; Anders, 1985; Hinson et al., 1994 for examples) have also been found to be important in aquatic species (Varanasi and Stein, 1991; Dady et al., 1991; Bradbury et al., 1993; Kolanczyk et al., 1999). Comparisons between mammalian and aquatic metabolic enzyme systems have been made for a few standardized substrates (Gregus et al., 1983; Funari et al., 1987). Yet, there exists relatively little information on pathways and rates of metabolic conversion to potentially more toxic chemical forms, particularly with regard to aquatic species. This lack of information hampers the development of models predictive of chemical toxicity for aquatic species, particularly for compounds likely to be more toxic after metabolic conversion than originally predicted based on parent chemical mode of action (Russom et al., 1997). Additionally, physiologically-based kinetic models used to predict chemical disposition in aquatic species currently work well for chemicals not significantly metabolized (Nichols et al., 1990; Lien et al., 2001), but do not typically predict production and disposition of bioactivated metabolites. Incorporation of metabolism into predictive models requires knowledge of metabolic pathway (products), rates of formation, and characterization of enzymatic capacity.

The metabolic conversion of phenol to quinone metabolites has been studied extensively in mammalian liver microsomes, tissue homogenates, cell suspensions, liver slices, whole organs, and using in vivo exposures (Lunte and Kissinger, 1983; Sahawata and Neal, 1983; Schlosser et al., 1993; Kenyon et al., 1995; Hoffman et al., 1999) with a myriad of analytical, perfusion and microdialysis techniques (Lunte and Kissinger, 1983; Scott et al., 1989; Davies and Lunte, 1995). By comparison, there is relatively little detailed information available regarding potential bioactivation pathways, and associated rates, that can be used to model the disposition of potentially reactive metabolites of phenol in aquatic organisms. Early reports of phenol metabolism in fish centered on the identification of sulfate and glucuronide conjugates with no evidence for HQ formation (Kobayashi and Akitake, 1975; Layiwola and Linnecar, 1981). Indirect evidence for the formation of HQ in phenol exposed fish was shown by the identification of the conjugate HQ-sulfate (Nagel, 1983; Nagel and Urich, 1983; Kasokat et al., 1987). More recently McKim et al. (1999) reported the direct measurement of HQ in the plasma and urine of rainbow trout exposed to phenol in the water. This type of measurement was only recently possible due to the application of sensitive analytical techniques for detection of low but relevant metabolite concentrations. The in vivo data collected can serve as the basis for the validation of fish physiologically-based model for phenol and its' metabolites. However, in vitro metabolic pathway, rate and capacity data is needed to provide independent parameterization of models prior to validation, and for further extension of the modeling approach to many more chemicals of diverse structure.

The current study was undertaken to identify pathway (Phase I oxidative products) and formation rate of enzymatic ring hydroxylation metabolites of phenol in the rainbow trout, a species commonly used for chemical uptake, metabolism, disposition, and toxicity studies (Franklin et al., 1980; Melancon and Lech, 1984; McKim et al., 1987a,b; Buhler, 1995), and for further development of fish physiologically-based toxicokinetic models (Nichols et al., 1990; Lien

et al., 2001). Due to the relatively slow rate of metabolic conversion seen in aquatic species relative to mammals (often 10–100-fold less) the use of mM substrate concentration results in uM to nM formation of product, necessitating the incorporation of highly sensitive analytical detection (Kolanczyk et al., 1999). However, orders of magnitude greater sensitivity of fish to HQ than phenol (DeGraeve et al., 1980) makes relatively low rates of HQ production still toxicologically significant. Study objectives were to (1) identify potentially bioactive Phase 1 metabolites of hepatic microsomal biotransformation of phenol in trout using more sensitive analytical detection than previously applied; (2) characterize the linear formation rate and enzymatic biotransformation capacity for each metabolite formed by utilizing phenol concentrations necessary to achieve enzyme saturation; and (3) determine metabolic product production at physiological temperature relevant for this cold water species, as well as room temperature, to serve as the basis for extrapolation to other species. Additionally, formation of HQ through enzymatic ring hydroxylation determined in the current effort was compared with HQ production via *O*-dealkylation previously measured under identical conditions (Kolanczyk et al., 1999).

2. Materials and methods

2.1. Chemicals

Substrate (phenol) and metabolite standards (HQ, catechol (CAT), BQ) were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). Reducing equivalents, buffer components, G-6-P dehydrogenase and 7-ethoxyresorufin were purchased from Sigma Chemical Co. (St. Louis, MO, and USA). Acetonitrile and methanol from Burdick and Jackson (Muskegon, MI, USA) were of analytical grade. Resorufin was obtained from Pierce Chemical Company (Rockford, IL, USA). Disodium ethylenediaminetetraacetate and sodium dithionite were purchased from Fisher Scientific (Eden Prairie, MN, USA).

2.2. Standard and sample preparation and handling

Standards and samples required special precautions to ensure stability. In all cases, cold solvents, buffers and water were used to dissolve solid compounds. Solutions were kept on ice and protected from the light to avoid degradation of analytes (Kolanczyk et al., 1999). HQ, CAT and BQ standard solutions were prepared daily in acetonitrile:water (1:1).

2.3. Animals

Juvenile rainbow trout (*Oncorhynchus mykiss*) (100–250 g) from Seven Pines Fish Hatchery (Lewis, WI, USA) were held for several weeks in flow-through 815 l tanks with sand filtered Lake Superior water (4 l/min) at 11 °C. Trout were fed commercial Silver Cup trout pellets from Nelson and Sons Inc., (Murray, UT) three times a week at a rate of 1.2% body weight per day, and held under a 16-h light:8-h dark photoperiod.

2.4. Microsomal characterization

Liver microsomes were prepared from fasted rainbow trout as previously described (Kolanczyk et al. 1999). Due to the relatively small size of fish used, and the yield of liver tissue per individual (generally 1–1.5% of total body weight), it was necessary to pool animals to obtain adequate tissue to complete the required incubations. Livers from three trout were combined, without regard to gender or size (100–250 g), for preparation of each of five microsomal samples. Previous investigations in this laboratory have observed no apparent differences in Phase 1 biotransformation of phenol for immature rainbow trout regardless of size and gender (unpublished observations). Isolated microsomes were stored at −80 °C for up to 6 months (Forlin and Andersson, 1985). Each microsomal sample was characterized as to total protein (Bradford, 1976), P450 content per mg microsomal protein as measured by the method of Estabrook et al. (1972) using an extinction coefficient of 0.1 mM/cm and 7-ethoxyresorufin-*O*-deethylase (EROD) activity by a

modified method of Pohl and Fouts (1980) utilizing excitation and emission wavelengths of 530 and 585 nm, respectively. EROD reaction product formed after 10 min at 11 °C was quantified against a resorufin standard curve.

2.5. Microsomal incubations

Incubations of five microsome samples per phenol concentration for metabolite identification and metabolic rate determination were conducted in open microcentrifuge tubes containing the following constituents in a final volume of 500 μl: 25 μl 20 mM $MgCl_2$, 25 μl 10 mM G-6-P, 121 μl 13.5 mM NADP, 40 μl (5 U) G-6-P dehydrogenase, 50 μl microsomes (0.8–0.95 mg/ml protein), and various concentrations of phenol (0.70–80 mM) in 239 ul 0.1 M Trizma–HCl buffer (pH 8.0). Microsomes and cofactors were incubated for 5 min prior to initiation of reaction by addition of substrate. Incubations were conducted for 15 min in a temperature controlled reciprocal shaker at 11 or 25 °C. Cold (4 °C) $ZnSO_4$ 25% (0.05 ml) and $Ba(OH)_2$ saturated (0.05 ml) were added to stop the reaction. Samples were then vortexed, stored on ice for 5 min, and centrifuged 3 min at $18\,200 \times g$. Samples were placed back on ice for 5 min, and centrifuged 3 min at $18\,200 \times g$. Supernatant was transferred to amber HPLC vials equipped with inserts, maintained at 4 °C, and analyzed immediately by HPLC to preserve sample integrity.

2.6. Metabolite identification and quantification

Analysis of microsomal incubation samples was performed on a Beckman System Gold HPLC, equipped with a refrigerated autosampler, diode array UV detector, and a BAS CC-5 dual channel LC-4C electrochemical detector (ECD) (Kolanczyk et al., 1999). Injections (20 μl) were made onto a Shandon Hypersil ODS (C18) 5u 4.6×250 mm column. An isocratic mobile phase (1 ml/min) consisting of 9.6% ACN and 0.1 M sodium acetate (pH 4.2) was employed. The ECD featured a glassy carbon working electrode and Ag/AgCl reference electrode, using hydrodynamic voltammograms to determine optimum oxidation or reduction potentials for each analyte. Optimum ECD potential for detection of HQ was $E° = +0.425$ V [ox], for CAT was $E° = +0.550$ V [ox] and for BQ was $E° = +0.050$ V [red] (range = 10 nA; filter 0.1 Hz). ECD stability and instrument performance were assessed on a daily basis. Dual channel ECD was used for peak identification and quantitation of HQ, CAT and BQ, compared with standards. We have previously determined a detection limit for HQ, BQ and CAT equivalent to 0.02 uM (Kolanczyk et al., 1999). UV-diode array detection was used to quantify levels of phenol, and as additional confirmation of metabolite identification.

2.7. Data analysis

Measurements shown are the mean ± standard error of duplicate or triplicate observations made using each of five microsomal preparations, at each of ten substrate concentrations. Estimates of apparent Km and Vmax (± S.E.) were done in two ways. For each microsomal preparation a saturation curve was fit to ten points, i.e. the mean measured HQ or CAT production at each substrate concentration, resulting in an apparent Km and Vmax estimate for each preparation. A mean Km and Vmax was then calculated from the five individual rate constants, or the 'mean of individuals' (Tables 1 and 2). A second calculation of an apparent Km and Vmax was done by first obtaining the average production of HQ or CAT across all five preparations measured at each of ten phenol concentrations, then fitting a curve to the points from which the 'mean rate across preparations' were obtained (Tables 1 and 2). A non-linear least squares regression program (EZ-Fit™ version 5.03; Perrella Scientific; Amherst, NH, USA) was used to generate parameter estimations from untransformed kinetic data.

The Q_{10}-values of reaction rates were calculated from the equation: $Q_{10} = (K_1/K_2)^{10/(T_1 - T_2)}$ where, K_1 and K_2 are velocity constants corresponding to the temperatures T_1 and T_2.

Statistical comparisons between groups ($n = 9$) were performed using the unpaired t-test at $P \leq 0.05$.

3. Results

The metabolic products identified upon phenol incubation with trout microsomes were the *para*- and *ortho*-ring hydroxylation products, HQ and CAT, respectively. The amount of CAT produced was typically one-half to one-third less than the amount of HQ formed. There was no BQ detected in any of the incubations. Sensitive detection of nanomolar quantities of HQ and CAT produced from mM phenol substrate incubations was achieved using HPLC separation with ECD optimized through the use of hydrodynamic voltammograms. Microsomal assay conditions were optimized for pH, incubation time, cofactor and microsomal protein concentration. Optimum HQ formation occurred at pH 8.0, 74% less was detected at pH 7.4, and 33 and 99% less was seen at pH 8.5 and 6.5, respectively (Fig. 1). CAT formation was also favored at pH 8.0. The pH optima of 8.0 corresponded to the plasma pH for rainbow trout. The rates of HQ and CAT production with the concentration of cofactors utilized, were found to be linear with respect to time up to 20 min. No HQ or CAT formation was detected in absence of phenol or one or more cofactors from the incubation mixture, thus verifying absence of endogenous production of metabolites or compounds that may interfere with detection of compounds of interest.

Table 1
Michaelis–Menten kinetics for ring-hydroxylation in juvenile rainbow trout microsomes over a range of phenol concentrations (1–60 mM) at 11 °C

Preparation	Km (mM)	Vmax (pmol/min per mg)
Hydroquinone		
#1	15 ± 3	480 ± 36
#2	11 ± 4	393 ± 39
#3	12 ± 5	586 ± 79
#4	15 ± 5	492 ± 53
#5	18 ± 7	809 ± 118
Mean of individual rates[a]	14 ± 1	552 ± 71
Mean rate across preparations[b]	15 ± 5	575 ± 65
Catechol		
#1	12 ± 10	144 ± 35
#2	9 ± 6	112 ± 20
#3	10 ± 7	188 ± 36
#4	8 ± 6	198 ± 39
#5	12 ± 5	163 ± 22
Mean of individual rates[a]	10 ± 1	161 ± 15
Mean rate across preparations[b]	12 ± 7	179 ± 30

Each preparation represents the pooled livers from three fish.
[a] Michaelis–Menten constants (mean ± standard error) calculated from five Km and Vmax values resulting from the saturation curve fitting of HQ or CAT production data at each phenol concentration from five individual microsome preparations.
[b] Km and Vmax resulting from the saturation curve fitting of the average HQ or CAT production data at each phenol concentration of five individual microsome preparations.

Table 2
The fitted Michaelis–Menten kinetics for ring-hydroxylation in juvenile rainbow trout microsomes over a range of phenol concentrations (1–80 mM) at 25 °C

Preparation	Km (mM)	Vmax (pmol/min per mg)
Hydroquinone		
#1	17 ± 10	1415 ± 293
#2	21 ± 13	1497 ± 357
#3	22 ± 11	1963 ± 389
#4	23 ± 12	1544 ± 315
#5	25 ± 11	2343 ± 429
Mean of individual rates[a]	22 ± 1	1752 ± 175
Mean rate across preparations[b]	21 ± 11	1745 ± 347
Catechol		
#1	29 ± 19	853 ± 236
#2	26 ± 19	720 ± 219
#3	39 ± 19	1131 ± 267
#4	28 ± 17	1062 ± 267
#5	38 ± 16	932 ± 185
Mean of individual rates[a]	32 ± 3	940 ± 73
Mean rate across preparations[b]	31 ± 18	931 ± 227

Each preparation represents the pooled livers from three fish.
[a] Michaelis–Menten constants (mean ± standard error) calculated from five Km and Vmax values resulting from the saturation curve fitting of HQ or CAT production data at each phenol concentration from five individual microsome preparations.
[b] Km and Vmax resulting from the saturation curve fitting of the average HQ or CAT production data at each phenol concentration of five individual microsome preparations.

The concentration of phenol freely dissolved in microsomal incubations at 11 °C was suspect due to previous observations of decreased solubility of another substrate, 4-methoxyphenol, during low temperature incubations (Kolanczyk et al., 1999). The concentration of phenol freely dissolved, determined by acetonitrile addition to microsomes (Kolanczyk et al., 1999), was equal to nominal concentrations at 25 °C, i.e. 1–80 mM, but $27.2 \pm 7.5\%$ less than nominal at 11 °C (data not shown). Phenol concentrations were corrected for this 27% loss prior to calculation of kinetic constants at 11 °C, yielding a range of 0.73–58 mM. No loss of either metabolite, HQ or CAT, was observed during sample processing.

Incubations of trout liver microsomes with phenol (0.73–80 mM) were conducted at the physiologically relevant temperature of 11 °C (Fig. 2) and also at 25 °C (Fig. 3) for direct comparison with other enzyme studies. At 11 °C, nearly linear production of HQ was observed to 20 mM phenol followed by apparent enzyme saturation with measured maximum rates of 350–400 pmol/min per mg protein for most microsome preparations. However, HQ production of 650 pmol/min per mg was measured for one preparation, illustrating the potential for large variation in rates between fish (Fig. 2A). Production of CAT at 11 °C followed a similar trend with a linear increase up to 15 mM phenol, above which a steady rate of 120–140 pmol/min per mg was observed for the majority of microsome preparations (Fig. 2B). As

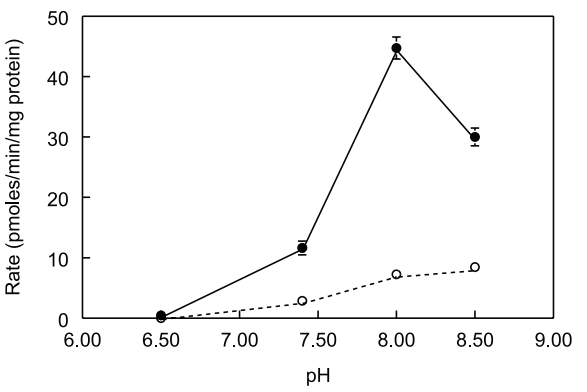

Fig. 1. Effect of pH on the ring-hydroxylation of phenol to HQ (●) and CAT (○).

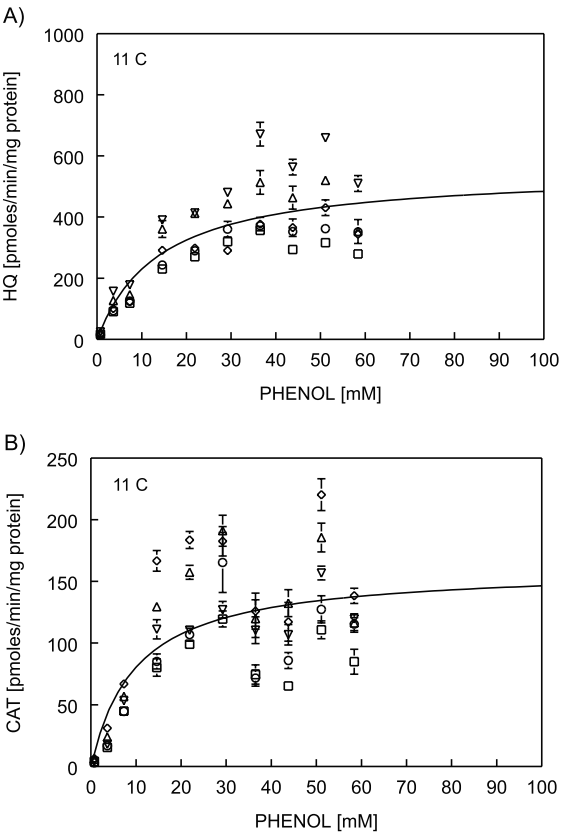

Fig. 2. Rates of simultaneous production of (A) HQ and (B) CAT formation resulting from the incubation of phenol with juvenile rainbow trout microsomes at 11 °C. Each symbol represents the mean ± S.E. for one of five different preparations of microsomes containing three fish livers each. Preparation # 1 (○), #2 (□), #3 (△), #4 (◇), and #5 (▽). Lines represent a curve using average rate constants over five microsome preparations.

with HQ, at least one microsomal preparation produced almost double the usual CAT rate.

Production of HQ and CAT at 25 °C followed a similar pattern to that observed at the lower temperature when considering relative rates, with HQ > CAT, and inter-microsome variability (Fig. 3A and B). However, much greater absolute rates were measured with a usual rate of 1200 pmol HQ per min per mg going up to 1800 pmol HQ per min per mg for one preparation, and a usual rate of 600 pmol CAT per min per mg extending as high as 900 pmol CAT per min per mg for one set.

The Michaelis–Menten saturation kinetics for the production of HQ and CAT via ring-hydroxylation were evaluated. Estimates of apparent Km and Vmax ($\pm$S.E.) were done in two ways to demonstrate not only average rates but also the wide variation measurable among fish. At 11 °C the apparent Km for phenol hydroxylation to HQ was 14 ± 1 and to CAT was 10 ± 1 mM, with corresponding Vmax values of 552 ± 71 and 161 ± 15 pmol/min per mg protein, respectively, calculated as the 'mean of individual' preparations (Table 1). The resulting kinetic constants calculated as the 'mean across preparations' were: $\text{Km} = 15 \pm 5$ mM for HQ, and 12 ± 7 mM for CAT, with $\text{Vmax} = 575 \pm 65$ pmol HQ per min per mg protein and 179 ± 30 pmol CAT per min per mg protein. While either method for calculation of mean Km and Vmax seems adequate, determination of rate constants for individual microsomes provides additional information. While, Km did not appear to be different across preparations, the highest Vmax for HQ production at 11 °C (809 pmol/min per mg protein), measured for preparation # 5 (Table 1), was twice that of the lowest (393 pmol/min per mg protein), measured for preparation # 2.

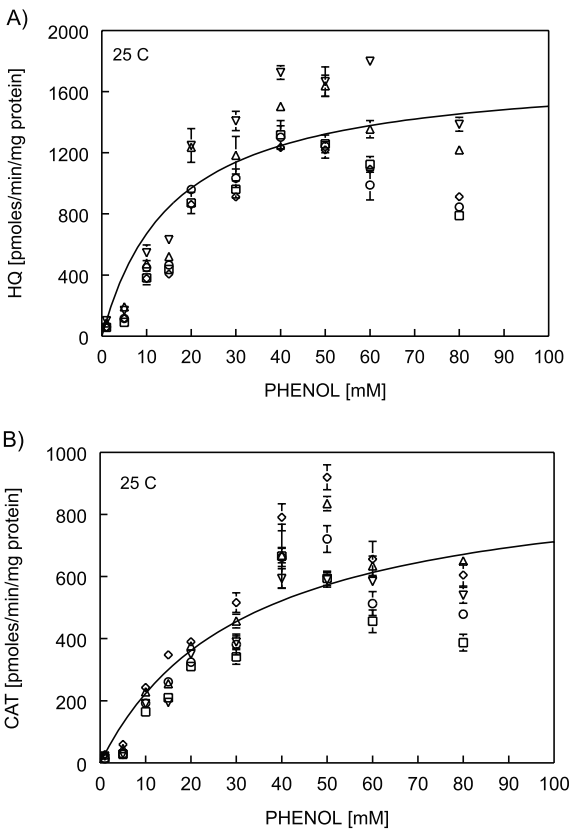

Fig. 3. Rates of simultaneous production of (A) HQ and (B) CAT formation resulting from the incubation of phenol with juvenile rainbow trout microsomes at 25 °C. Each symbol represents the mean $\pm$ S.E. for one of five different preparations of microsomes containing three fish livers each. Preparation # 1 (○), # 2 (□), # 3 (△), # 4 (◇), and # 5 (▽). Lines represent a curve using average rate constants over five microsome preparations.

Similar variations in reaction rates among microsomal preparations were noted at 25 °C. The Km, calculated as 'mean of individuals' preparations were 22 ± 1 mM (HQ) and 32 ± 3 mM (CAT); the Vmax was 1752 ± 175 pmol HQ per min per mg protein and 940 ± 73 pmol CAT per min per mg protein (Table 2). As was noted with the lower temperature, constants calculated as 'mean across preparations' were similar to those calculated as 'mean of individuals', with a Km of 21 mM for HQ and 31 mM for CAT, and Vmax of 1745 pmol HQ per min per mg protein and 931 pmol CAT per min per mg protein, respectively (Table 2). Interestingly, the apparent Km estimated for CAT at 25 °C was greater than the corresponding Km for the HQ reaction at the same temperature, a trend not observed when metabolism was measured at the physiological temperature for trout of 11 °C. Despite the lower apparent enzyme affinity, the maximal rate of CAT production increased more than expected in comparison to rate increases with temperature noted for HQ production. This was best demonstrated by comparing calculated rate changes with a 10 °C increase in temperature (i.e. Q_{10}). The Q_{10} for maximal production of HQ was 2.28 and for CAT was 3.53, which were shown to be statistically different ($P < 0.0001$) over the five preparations of microsomes. The *K*m, 14 ± 3 mM (mean $\pm$ S.D.), for the formation of HQ at 11 °C was shown to be statistically different ($P = 0.0036$) than that at 25 °C, 22 ± 3 mM. A temperature effect ($P = 0.0002$) was observed on the Vmax for the formation of HQ at 11 °C (552 ± 159 pmol/min per mg) and 25 °C (1752 ± 392 pmol/min per mg). Statistical difference was also

observed for CAT formation Km and Vmax ($P < 0.0001$) across temperatures between 11 °C (10 ± 2 mM; 161 ± 35 pmol/min per mg) and 25 °C (32 ± 6 mM; 940 ± 164 pmol/min per mg). As was observed at 11 °C, the greatest capacity for HQ production at 25 °C was measured with microsomal preparation #5, which was almost double that of the lowest capacities measured for #1 and #2.

To better evaluate the differences in microsomal preparations that might explain noted differences in metabolism, comparisons were made of individual fish sex, body weight and liver weight for the three fish from which livers were pooled for each microsomal preparation. This information is presented in Table 3 along with protein, P450 content, and EROD activities. Each preparation, with the exception of #3, contained hepatocellular protein from both male and female fish. The ratio of liver weight to body weight, or hepatosomatic index (HST), was < 1.3 for all fish in the three preparations yielding the lower apparent maximal rates of HQ production at either temperature, #1, #2, #4. Both preparations #3 and #5 included two fish with HSI > 1.3, and one trout in each with a HSI of 1.6. The HSI can be used as an indication of enlarging liver compared with body weight as typically seen in fish such as trout as they seasonally approach sexual maturity (Forlin and Haux, 1990). The two fish with HSI of 1.6 were also the largest of the entire data set in terms of body weight. The measured EROD activities, an indicator of metabolic *O*-dealkylation, were also elevated for these preparations, as were the apparent Vmax for HQ production at both 11 and 25 °C (Tables 1 and 2), although P450 protein did not appear elevated in preparation #5.

4. Discussion

The microsomal biotransformation of phenol was measured in the present study for the purpose of obtaining specific metabolic rate information needed for refinement of physiologically-based kinetic models for fish, and to obtain metabolic pathway information needed for development and validation of metabolism simulation models. A better understanding of how metabolic pathway, linear rate, and maximal capacity vary with chemical structure is needed for more accurate prediction of the potential for metabolic activation, with associated increases in toxicity, as well as for accurate prediction of metabolic detoxification leading to decreased accumulation and reduced toxicity. While many aquatic species have been characterized as to content of multiple P450 isoforms (Buhler, 1995), relatively less is known about rate of conversion of the wide variety of chemical substrates aquatic species are potentially exposed to, with the exception of a few well characterized 'standard' substrates. Specific pathway and rate information is needed to account for not only parent chemical, but also disposition of metabolites in physiologically-based toxicokinetic models for fish, and ultimately for development of linked physiologically-based kinetic models with toxicodynamic models that would allow estimation of a chemicals' increased toxic potential through accurate prediction of metabolic bioactivation rate.

HQ was the major metabolite produced by hepatic microsomal biotransformation of phenol in trout. CAT was also found to be a significant ring-hydroxylation product of phenol, although produced in lesser amounts. Microsomal metabolism of phenol measured in rodent species resulted in HQ:CAT product ratios of 20:1 in rats (Sahawata and Neal, 1983) and about 15:1 in both rats and mice (Schlosser et al., 1993). This study allowed the first estimation of HQ:CAT product ratios for trout, which were 3.5:1 at 11 °C and 2:1 at 25 °C. These results indicate there is greater relative *ortho*-hydroxylation rate in fish compared with that observed in rodents, although absolute rates of metabolism are lower in aquatic species. At physiological temperatures the rate of microsomal production of HQ from 1 mM phenol was 7020 pmol/min per mg in rats (Sahawata and Neal, 1983), about 350 times more than in trout at 19 pmol/min per mg. While biotransformation of mM concentrations of phenol results in nM and pM amounts of metabolite in rats and fish, respectively, consideration must be made as to the toxicity of metabolites relative

Table 3
Characterization of rainbow trout microsomes

Microsome preparation	Sex	Body weight (g)	Liver weight (g)	HIS[a] (%)	Microsomal protein (mg/g liver)	P450 Protein (nmols/mg)	EROD (pmol/min per mg)
#1	Male	190	2.38	1.3	17.8 ± 0.8	0.50 ± 0.04	5.9 ± 1.4
	Male	160	1.91	1.2			
	Female	144	1.44	1.0			
#2	Male	161	1.91	1.2	18.3 ± 0.2	0.49 ± 0.00	5.7 ± 1.2
	Male	154	1.83	1.2			
	Female	143	1.78	1.2			
#3	Male	121	1.79	1.5	18.6 ± 0.2	0.58 ± 0.00	13.0 ± 7.0
	Male	105	1.38	1.3			
	Male	252	4.02	1.6			
#4	Male	191	2.25	1.2	20.7 ± 0.4	0.52 ± 0.05	4.6 ± 0.6
	Female	142	1.40	1.0			
	Female	201	2.59	1.3			
#5	Female	214	3.46	1.6	16.9 ± 0.1	0.47 ± 0.06	10.6 ± 5.7
	Male	150	2.14	1.4			
	Male	144	1.72	1.2			

Livers from three fish were combined for each microsomal preparation. Mean ± S.D.
[a] Hepato somatic index.

to substrate. Specifically, HQ with a 96 h LC_{50} for rainbow trout reported as 0.097 mg/l was shown to be about 100 times more toxic than phenol with an LC_{50} of 8.9 mg/l (DeGraeve et al., 1980).

Oxidation of HQ to BQ in vivo results in a metabolite thought to be responsible for macromolecular binding and increased toxicity in mammalian systems (Schlosser et al., 1990). Secondary metabolism of dihydroxybenzenes to quinones after trout microsomal biotransformation of 4-methoxyphenol to HQ and 4-methoxycatechol was presumed upon measurement of either BQ or 4-methoxybenzoquinone (MBQ) (Kolanczyk, et al., 1999). Definitive identification of either BQ or MBQ was not possible due to co-elution of the two compounds under the chromatographic conditions. However, BQ was not detected in the present study of phenol biotransformation. This was likely due to the presence of high concentrations of reducing equivalents in the microsomal system, as well as the high reactivity of BQ (O'Brien, 1991). To substantiate this hypothesis, experiments were performed in which various concentrations of HQ and BQ were added to the incubation matrix of microsomes and cofactor regenerating system. Following a 15 min incubation, greater than 90% of added HQ was recovered; however, the added BQ was not directly detectable but measured as an observed 10–30% conversion to HQ.

The ranges of phenol concentrations used in the present investigation were necessary to achieve enzyme saturation at the temperatures under investigation, thus allowing the calculation of kinetic constants under standardized conditions. In fact, the highest mM phenol concentrations used were purposefully incorporated to determine if a point was reached at which enzymatic rate was adversely affected due to apparent protein toxicity/denaturation or temperature dependent solubility effects, thus assuring that lower phenol concentrations were not adversely impacting metabolic rate determinations. The in vitro concentrations utilized typically exceeded phenol concentrations administered by inhalation, skin absorption, or ip injection in aquatic in vivo studies (Nagel, 1983; Layiwola and Linnecar, 1981; McKim et al., 1999; Kasokat et al., 1987; Nagel and Urich, 1983). However, in vitro work with subcellular fractions have often utilized similar mM concentrations (Schlenk and Buhler 1991; Dady et al., 1991; Soldano et al., 1992; Kolanczyk et al., 1999). The apparent Km and Vmax determined for HQ and CAT formation are necessary inputs for subsequent development of models to predict phenol metabolism in vivo, and will be compared with HQ production measured during phenol inhalation exposures (McKim et al., 1999) and in situ liver microdialysis delivery exposures (unpublished results).

The current study applied a sensitive analytical method (HPLC/ECD) to achieve an approximately 1000-fold increase in sensitivity over conventional UV or colorimetric detection methods, allowing the direct quantification of HQ and CAT at low, yet toxicologically significant concentrations observed in fish, thus allowing the calculation of rate constants for each individual oxidative metabolite. The direct measurement of HQ was only possible following the development of these sensitive analytical methods due to the small quantities produced by fish. Early studies of phenol metabolism in fish detected sulfate and glucuronide conjugates of both phenol and HQ (Kobayashi and Akitake, 1975; Layiwola and Linnecar, 1981; Nagel, 1983; Nagel and Urich, 1983; Kasokat, et al., 1987), but did not allow direct measurement of the major metabolite. Using the technique applied to the current work, McKim et al. (1999) reported the presence of HQ in the urine and plasma of rainbow trout exposed aqueously to phenol. Microsomal incubations with phenol resulted in the measurement of uM concentrations of HQ which were comparable to the measured in vivo concentration of HQ in the blood and urine of rainbow trout (McKim et al., 1999). The present study extends the work of McKim et al. (1999) through direct measurement of metabolic rate and estimates of apparent Km and Vmax of HQ and CAT from phenol in trout at physiological temperature. Availability of this type of information allows prediction of metabolite formation needed for independent parameterization of kinetic models (Clewell and Andersen, 1985; Schlosser et al., 1993; Medinsky, 1995).

The linear rate of microsomal ring-hydroxylation of phenol to CAT (17 ± 5 pmol/min per mg per mM substrate) at 25 °C was similar to the conversion of 4-methoxyphenol to 4-methoxycatechol (17 ± 1 pmol/min per mg per mM substrate) observed by Kolanczyk et al. (1999). However, at 11 °C the rate of *ortho*-hydroxylation of phenol (7 ± 1 pmol/min per mg per mM substrate), was significantly less than the formation of 4-methoxycatechol (19 ± 3 pmol/min per mg per mM substrate) from 4-methoxyphenol (Kolanczyk et al., 1999). Further analysis of the linear rate of HQ formation from *para*-hydroxylation of phenol at 11 °C (22 ± 6 pmol/min per mg per mM substrate) and 25 °C (45 ± 17 pmol/min per mg per mM substrate) was nearly the same as that from the *O*-dealkylation of 4-MP (22 ± 2 pmol/min per mg per mM substrate and 34 ± 1 pmol/min per mg per mM substrate, respectively), (Kolanczyk et al., 1999). Determination of the rate constants through direct measurements of low pmol formation rates allows comparison of specific rate data not previously obtainable in aquatic species, as well as comparisons of formation rates of similar products (i.e. HQ) derived from different enzymatic processes (*O*-dealkylation vs. ring-hydroxylation). This type of information is essential to the development of accurate predictive models of biotransformation and toxicity in aquatic species.

The present study provides Michaelis–Menten rate constants at 11 and 25 °C for future species comparison and extrapolation. The two temperatures provide a basis for rate comparison to other 'cold-water' species (brook trout, lake trout) typically run at 11 °C (work in progress at this laboratory) and 'warm-water' species (medaka, catfish, etc.) typically run at 25 °C. In the past, much in vitro fish metabolic rate data was collected at 25 °C to maximize enzymatic rates. While not directly applicable to this study, the collection of rate data at 25 °C will allow comparison to be made across reaction types, as well as species. Rates determined at physiological temperature, 11 °C, are also important for in vitro to in vivo extrapolation. Measurements made at 37 °C are not appropriate for cold water fish species with a high potential for protein denaturation to affect measured rates. Therefore, a lower temperature was used to allow calculation of Q_{10} values, which could be compared with those for mammalian species.

Temperature effects on enzyme activity can be described by Q_{10}, with a Q_{10} of two representing a doubling of enzymatic activity with a 10 °C temperature rise. Values of Q_{10} around 2–2.5 have been observed for *O*-dealkylation, ring-hydroxylation, and UDP-glucuronyltransferase (Koivusaari and Andersson, 1984) and *N*-hydroxylation (Dady et al., 1991) in rainbow trout. The Q_{10} calculated using Vmax for production of HQ was 2.3 and for CAT was 3.5, indicating a greater effect of temperature on *ortho*-hydroxylation. The increase in apparent Km with increasing temperature for both HQ and CAT production reported here is consistent with previous observations (Koivusaari and Andersson, 1984; Somero, 1978). The biotransformation of phenol to HQ has been measured in mice at 37 °C with reported Km and Vmax of 0.38 mM and 7100 pmol/min per mg protein, respectively (Lunte and Kissinger, 1983). A Q_{10} based on trout HQ production rate at 11 °C and mouse HQ production at 37 °C would be 2.7, close to frequently observed Q_{10} values of 2–2.5 perhaps suggesting similarity in maximum capacity of enzymes responsible for *para*-hydroxylation of phenol to HQ between these species.

However, the Km measured at the physiological temperature for each species is much lower in mice than trout (ca. a 40-fold difference), indicating mice enzymes exhibit a greater affinity for the substrate than trout. Lunte and Kissinger (1983) also observed formation of CAT from phenol in mouse microsomes but did not quantify its occurrence.

It should be noted that the data presented here showed some apparent deviation from Michaelis–Menten kinetics, especially at lower phenol concentration at 25 °C (Fig. 3A and B). These deviations from the curve fit to the data (Fig. 3A and B) could be explained by either an enzyme lag effect or quantification uncertainties at relatively low HQ and CAT concentrations near their detection limits. Even though there are apparent deviations from Michaelis–Menten kinetics, the Km and Vmax parameters are good descriptors com-

monly used for approximating maximal reaction rates. Therefore, these statistics are still deemed appropriate for comparison of species and other substrates as well as in vitro to in vivo extrapolation. To justify any use of a new descriptor other than constants used to describe Michaelis–Menten type kinetics would require a more rigorous data set than available here.

The current study, and that of Kolanczyk et al. (1999), provide pathway and rate data for metabolic production of HQ in rainbow trout from two different enzymatic reaction types, ring-hydroxylation and *O*-dealkylation. Trout cells have also been shown to exhibit biochemical responses indicative of quinone toxicity that are qualitatively similar to those reported for mammalian cells (Tapper et al., 2000), although direct comparison of concentration–response profiles are somewhat complicated by differences in cellular media and physiological temperatures, and paucity of measured exposure concentrations. Therefore, pathways and rates of metabolic conversions resulting in production of HQ and quinone species are important to quantify in fish, as well as in mammals, when attempting to understand and predict reactive chemical toxicities (Russom et al., 1997). Mammalian physiologically-based pharmacokinetic models incorporate in vitro metabolism and bioactivation to predict chemical distribution and forcast toxicity (Clewell and Andersen, 1985; Medinsky, 1995) and it is hoped similar parameters may be used successfully in fish models. Collection of metabolic rate data in aquatic species, like that presented in the current study and Kolanczyk et al. (1999), is essential for the advancement of toxicokinetic and metabolic simulation models, as well as the prediction of susceptibility of aquatic organisms to bioactivated compounds.

The methods utilized in the present study, and associated results serve as a basis for comparison of metabolic bioactivation reactions across species. The study provided pathway, rate, and capacity information for the biotransformation of phenol to reactive quinone precursors, HQ and CAT, in rainbow trout at physiologically relevant temperatures. The application of new and sensitive analytical techniques to the direct quantification of metabolites at low picomolar concentrations present in aquatic species was demonstrated. The availability of this type of information is essential to the development of accurate models predictive of increased toxicity due to metabolic activation.

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