Iowa State University

March, 1999

Biotransformation of 4-methoxyphenol in rainbow trout (Oncorhynchus mykiss) hepatic microsomes

Steven P. Bradbury
R. Kolanczyk
P. Schmieder
T. Spizzo

Biotransformation of 4-methoxyphenol in rainbow trout (*Oncorhynchus mykiss*) hepatic microsomes

R. Kolanczyk [a],*, P. Schmieder [a], S. Bradbury [a], T. Spizzo [b]

[a] *US Environmental Protection Agency, Duluth, MN 55804, USA*
[b] *Solvay Pharmaceuticals, Baudette, MN 56623, USA*

Abstract

Rainbow trout liver microsomes were used to study the *O*-demethylation and ring hydroxylation of 4-methoxyphenol (4-MP) (4-hydroxyanisole) at 11 and 25°C by directly measuring the production of the primary metabolite hydroquinone (HQ), 4-methoxycatechol (4-MCAT), and additional metabolites. An HPLC method with integrated ultraviolet (UV) and electrochemical detection (ECD) was developed for metabolite identification and quantification at low concentrations. Sample handling with appropriate buffers, solvents, low temperature and light prevented loss of extremely labile metabolites. Saturation kinetics for the production of HQ via *O*-demethylation of 4-MP (0.66–40 mM) was never achieved, with substrate solubility being the limiting factor. The linear rate of HQ formation at 11°C was 22.0 ± 2.2 (coefficient $\pm$ S.E., $r^2 = 0.91$) pmol min^{-1} per mg protein per mM substrate, and at 25°C was 34.0 ± 1.3 ($r^2 = 0.99$) pmol min^{-1} per mg protein per mM substrate. The second major microsomal metabolite 4-MCAT was also identified, with linear rates of ring hydroxylation determined to be 19.0 ± 1.6 ($r^2 = 0.94$) and 17.2 ± 0.5 ($r^2 = 0.99$) pmol min^{-1} per mg protein per mM substrate at 11 and 25°C, respectively. Unlike HQ production, the rate of 4-MCAT production was found to be similar at the two temperatures when linear formation rates were corrected for the effect of temperature on substrate and product solubility at 11°C. Measurement of 'freely dissolved' fraction was essential to the accurate determination of ring hydroxylation and *O*-demethylation reaction rates in rainbow trout microsomes incubated at physiological temperature. Experimental conditions were shown to affect dissolved 4-MP and HQ at 11°C (verified using microdialysis) while not altering substrate and product levels at 25°C. Small but detectable levels of 1,4-benzoquinone were observed in 4-MP microsomal incubations. 1,2,4-Trihydroxybenzene was also detected, with possible routes of production through hydroxylation of HQ or *O*-demethylation of 4-MCAT. A metabolic scheme for bioactivation of 4-MP is proposed and the significance of observed metabolic conversions in rainbow trout microsomes discussed in relation to aquatic toxicity of 4-MP. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: 4-Hydroxyanisole; Bioactivation; Electrochemical detection; Hydrodynammic voltammograms; Metabolism

---

* Corresponding author.

1. Introduction

Biotransformation reactions are generally considered to be a means of chemical detoxification. Phase I oxidation reactions serve to increase chemical polarity and provide adequate substrate for Phase II conjugation reactions, thus facilitating chemical elimination from organisms (Gibson and Skett, 1986; Parkinson, 1996). However, it is also widely recognized that metabolic transformation may result in more toxic chemical forms. While bioactivation has long been associated with increased toxicity in mammalian systems (Mitchell and Horning, 1984; Anders, 1985; Guengerich and Liebler, 1985; Hinson et al., 1994) and some aquatic systems (Ahokas and Pelkonen, 1984; Varanasi and Stein, 1991; Bradbury et al., 1993), little detailed information is available regarding specific bioactivation pathways in aquatic organisms. There is also a general lack of information as to rates of metabolic conversion to potentially more toxic chemical forms, regardless of species.

The measured toxicity of 4-methoxyphenol (4-MP) in an aquatic species has been hypothesized to involve metabolic activation. The 96-h acute toxicity of 4-MP to fathead minnows (Geiger et al., 1985) was investigated as part of a larger effort by the EPA to relate structure of industrial chemicals to biological activity, in this instance acute toxicity. Once chemical structure is correlated with toxicity for chemicals operating through a common mechanism of action, toxicity may be estimated for untested compounds (Bradbury, 1994, 1995). As part of these studies, 4-MP was predicted, based on structure, to be lethal to fathead minnows through the relatively non-specific narcotic mechanism of action (Russom et al., 1997). The potency measured for 4-MP was not inconsistent with what would be expected from chemical narcosis typical of phenolic compounds at acute levels. However, an examination of the dose-response curve suggested that, at lower doses, signs of toxicity were more consistent with formation of reactive, i.e. electrophilic/proelectrophilic, chemical species. It was hypothesized that 4-MP may be undergoing metabolic O-dealkylation and/or hydroxylation to chemical forms which are not only more hydrophilic, but also more reactive such as hydroquinone (HQ), benzoquinone (BQ) and trihydroxybenzene.

Bioactivation of 4-MP (also known as 4-hydroxyanisole or 4-hydroxy-1-methoxybenzene) has been shown to occur in mammalian systems. The depigmenting activity of 4-MP has lead to interest in its use as an antimelanoma drug (Riley, 1985; Pavel et al., 1989). The compound's cytotoxicity toward melanocytes had initially been thought to depend on oxidation of 4-MP by the enzyme tyrosinase. However, recent studies have investigated the role of rat and mouse cytochrome P450-mediated hydroxylations and demethylation in the toxicity of 4-MP (Schiller et al., 1991; Anari et al., 1995).

Thus, a study was undertaken to identify major metabolic products and rates of metabolic conversion of 4-MP in the rainbow trout, an aquatic species commonly used for metabolism studies (Lech, 1974; Franklin et al., 1980; Melancon and Lech, 1984; Buhler, 1995). An objective of this study was to establish the presence of hepatic microsomal O-dealkylation and/or ring-hydroxylation of 4-MP in rainbow trout by identifying metabolites formed in microsomal incubations. Microsomes were exposed to a range of 4-MP concentrations in an attempt to also calculate Michaelis–Menton kinetic constants for the reactions under study. It was the intent to add this information to a growing aquatic database of biotransformation and kinetic information for use in modeling the toxic effects of chemicals. This information is key to a better understanding of xenobiotic metabolism and potential for bioactivation in fish.

2. Methods

2.1. Chemicals

Ammonium acetate, 4-MP, BQ, HQ, methoxyhydroquinone (MHQ), 1,2,4-trihydroxybenzene (THB), 3-methoxycatechol (3-MCAT) and 5-methoxyresorcinol (5-MRES) were obtained from Aldrich Chemical Company (Milwaukee, WI). Reducing equivalents, buffer components, G-6-P dehydrogenase, 7-ethoxyresorufin, and mushroom
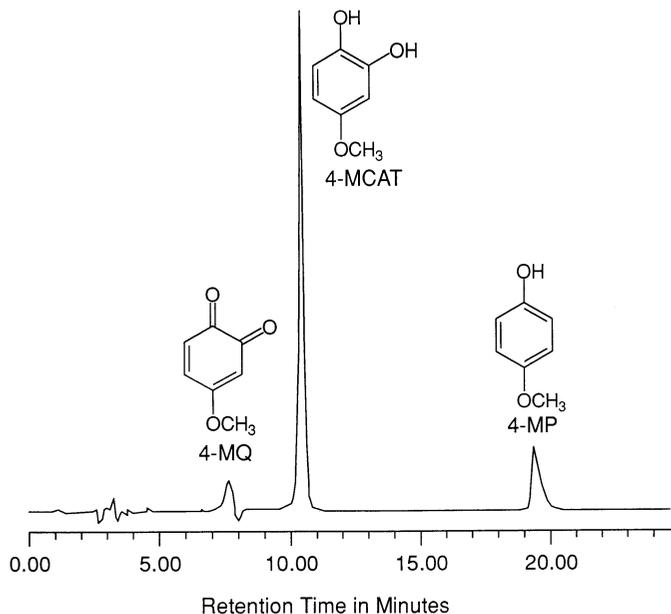
Fig. 1. HPLC-ECD chromatogram ($E^o = +425$ mV [ox]) for determination of 4-MQ (7.7 min) and 4-MCAT (10.5 min) enzymatically synthesized from 4-MP (19.4 min) incubated with mushroom tyrosinase.

tyrosinase were purchased from Sigma (St. Louis, MO). Acetonitrile (ACN) and methanol from Burdick and Jackson (Muskegon, MI) were of analytical grade. Resorufin was obtained from Pierce Chemical Company (Rockford, IL). Disodium ethylenediaminetetraacetate and sodium dithionite were purchased from Fisher Scientific (Eden Prairie, MN).

Putative metabolites of 4-MP that could not be purchased commercially were obtained through enzymatic synthesis. 4-methoxy-1,2-benzoquinone (4-MQ) and 4-methoxycatechol (4-MCAT) were enzymatically synthesized by incubating 4-MP (1.5 mM) with mushroom tyrosinase (15 µg; 4.4 units μg^{-1}) in 100 mM K_2HPO_4 buffer pH 7.5 at room temperature (Menter et al., 1990). The course of this reaction was followed spectrophotometrically with initial appearance of the quinone at the characteristic absorption wavelength of 413 nm. The reaction was also followed using HPLC-ECD ($E^o = +425$ mV [ox]) for determination of 4-MQ, 4-MCAT and 4-MP with retention times of 7.7, 10.5 and 19.4 min, respectively (Fig. 1), (see Section 2.5 for HPLC conditions). Verification of the formation of 4-MQ and 4-MCAT was

done using a Finnegan MAT TSQ-70 HPLC-Mass Spectrometer (LC-MS) utilizing a thermospray interface in the negative ion profile mode. Injections (10 µl) were made onto a Hypersil column (see Section 2.5 for HPLC conditions) with an isocratic mobile phase (1 ml min^{-1}) of 10% acetonitrile and 0.1 M ammonium acetate, pH 4.2. Mass spectral data indicated ions at m/z 199 (M + acetate) and m/z 139 (M-1) for both 4-MCAT and 4-MQ (Fig. 2(A)). It is likely that 4-MQ undergoes a gas phase reduction from m/z 197 to m/z 199 since reduction of quinones via hydrogen migration within the mass spectrometer has previously been observed (Phillips and Bartmess, 1989). This phenomena was supported by analysis of BQ in our laboratory under the same conditions which showed masses consistent with BQ, m/z 167 (M + acetate), and HQ, m/z 169 (M + acetate), for the HPLC retention time associated with BQ (Fig. 2(B)). An intense m/z 168, thought to be M + acetate of the semiquinone (SQ), was also observed. Indications are the gas phase reaction for BQ proceeds at a slower rate than 4-MQ and enables the detector to obtain spectra for BQ, SQ and HQ acetate complex ions.
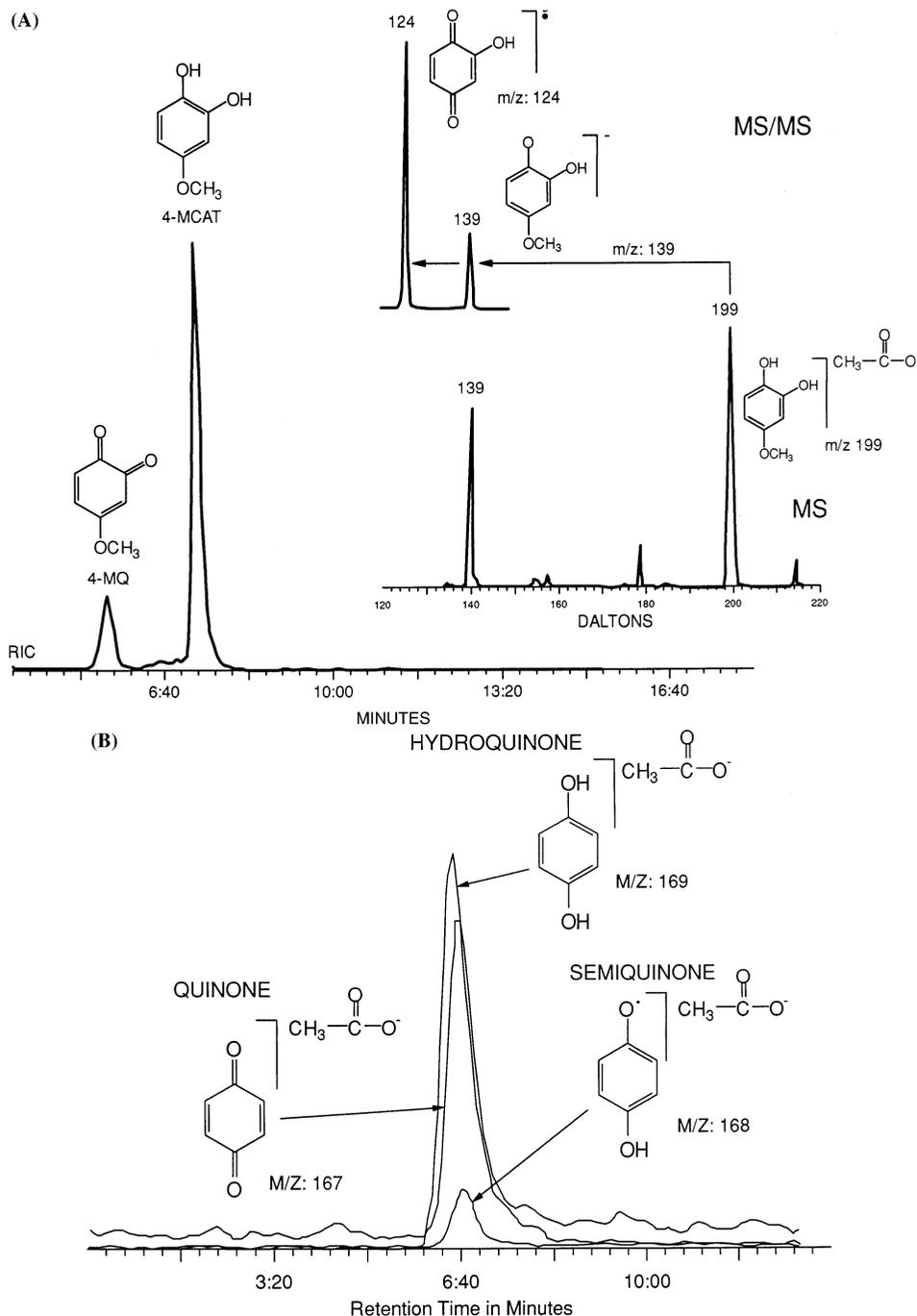
Fig. 2. (A) LC/MS analysis of 4-MCAT and 4-MQ standards produced enzymatically from incubations of 4-MP with tyrosinase. Reconstructed ion chromatogram (RIC) showing separation of 4-MQ and 4-MCAT, with MS analysis indicating ions at m/z 199 (M + acetate) and m/z 139 (M-1) for both chemicals. Subsequent MS/MS analyses of 4-MCAT of both species (shown for 4-MCAT) yielded product ions at m/z 139 (M-1) and m/z 124 (M-1-CH$_3$) from m/z 199. (B) LC/MS analysis of BQ, m/z 167 (M + acetate) exhibiting gas phase reduction to HQ, m/z 169 (M + acetate), and partial reduction to semiquinone, m/z 168 (M + acetate).

MS/MS analysis of m/z 199 (4-MCAT) gave product ions at m/z 139 (M-1) and m/z 124 (M-1-CH$_3$; Fig. 2(A)). Additional LC peaks from the tyrosinase/4-MP reaction had masses at 215 (M + acetate), which would be consistent with a further ring hydroxylation product of 4-MCAT (data not shown).

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 solubilize solid compounds. Solutions were kept on ice and protected from the light. Careful selection of vials, inserts and transfer utensils was made to avoid degradation of analytes induced by trace amounts of acidic or basic materials. HQ and BQ standard solutions were prepared daily in acetonitrile:water (1:1). Trihydroxybenzene proved to be extremely labile in solution; prone to both hydrolysis and oxidation to quinones, therefore a THB stock solution was prepared in acetonitrile with dithiothreitol (50 μM), with aqueous dilutions made just prior to HPLC analysis.

2.3. Animals

Juvenile rainbow trout (*Oncorhynchus mykiss*; 100–300 g) from Seven Pines Fish Hatchery (Lewis, WI) were held for several weeks in flow-through 815-l tanks with sand filtered Lake Superior water (4 l min^{-1}) at 11°C. Trout were fed commercial Silver Cup trout pellets from Nelson and Sons (Murray, UT) three times a week at a rate of 1.2% body weight day^{-1}, and held under a 16-h light:8-h dark photoperiod.

2.4. Microsomal characterization and incubations

Liver microsomes were prepared from fasted rainbow trout which underwent cervical dislocation prior to removal of liver tissue. Livers from three to five trout were homogenized for preparation of each of nine microsomal samples (Dady et al., 1991). Isolated microsomes were stored at −80°C for up to 6 months (Forlin and An-

dersson, 1985). Each microsomal sample was characterized as to total protein and P450 concentrations, and NADPH-cytochrome P450 (*c*)-reductase and 7-ethoxyresorufin-*O*-deethylase (EROD) activities. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin standards and varied from 8.0 to 9.4 mg protein ml^{-1} microsomes. A yield of 18.0 ± 0.8 mg microsomal protein per gram of liver was obtained over the nine microsome sets prepared. Rainbow trout microsomes contained 0.63 ± 0.10 nmol P450 (mg microsomal protein)$^{-1}$ as measured by the method of Estabrook et al. (1972) using an extinction coefficient of 0.1 mM^{-1} cm^{-1}. Microsomal NADPH-cytochrome P450 (*c*)-reductase activity varied from 39.0 to 48.2 cytochrome-c reduced min^{-1} per mg microsomal protein by the method of Phillips and Langdon (1962) at 11°C using an extinction coefficient of 0.021 cm^2 nmol^{-1}. EROD activity ranged from 10.8–46.2 pmol min^{-1} per mg microsomal protein as determined 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.

Incubations of microsomes with 4-MP (0.66–40 mM) for metabolite identification and metabolic rate determinations were conducted in open 1.5 ml microcentrifuge tubes at 11 or 25°C, containing the following constituents in a final volume of 500 μl 0.1 M Trizma–HCl buffer (pH 8.0); 20 mM MgCl$_2$; 10 mM G-6-P; 13.5 mM NADP; (5 Units) G-6-P dehydrogenase; and microsomes (50 μl, 0.8–0.95 mg ml protein^{-1}). 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 water bath shaker at 11 or 25°C. Cold (4°C) acetonitrile (0.5 ml) was added to stop the reaction. Samples were then vortexed, stored on ice for 3 min, and centrifuged 1–2 min at $14000 \times g$. Supernatant was transferred to amber HPLC vials equipped with inserts, maintained at 4°C, and analyzed immediately by HPLC to preserve sample integrity.

Experiments were conducted to ensure the observed metabolite production was enzymatically driven. Microsomes were incubated with 4-MP in absence of one or more of the cofactors ($MgCl_2$, G-6-P, G-6-P dehydrogenase, NADP) and no metabolites (HQ or 4-MCAT) were detected. Incubation of cofactors with 4-MP produced no metabolites. Microsomes, cofactors, and microsomes plus cofactors were incubated in absence of 4-MP, again with no detectable levels of HQ or 4-MCAT observed, thus verifying absence of endogenous production of metabolites or compounds that may interfere with detection of compounds of interest.

The concentration of 4-MP freely dissolved in microsomal incubations at 11°C was suspect due to presence of turbidity in incubation samples. Therefore, efforts were undertaken to measure 4-MP in solution at 11 and 25°C. To quantify dissolved substrate concentration at each temperature, ACN at 11 or 25°C was added to a series of incubation samples followed by immediate HPLC analysis. In addition, 4-MP concentration at each incubation temperature was measured via microdialysis (McKim et al., 1993; Gunaratna and Kissinger, 1997). A CMA-20 straight microdialysis probe from Carnegie Medicin (Acton, MA) with a 10-mm membrane and molecular weight cut-off of 20000 was prepared as described previously (McKim et al., 1993). Recovery calibration of the microdialysis probe was conducted in a stirred 2-ml GC-vial containing known concentrations of HQ and 4-MP in 100 mM Tris–HCl pH 8.0 with cofactors utilized in microsomal incubations except for G-6-P dehydrogenase. Dialysis recoveries, 29% and 48% for HQ and 4-MP, respectively, were calculated by HPLC. Probes were routinely calibrated prior to, and following, each use. Incubations for measurement of free substrate concentrations were performed with microsomes plus all cofactors in a final volume of 1.8 ml. The concentration of 4-MP freely dissolved, determined by microdialysis or ACN addition, was equal to nominal concentrations at 25°C ($102 \pm 3\%$ of nominal), but $34 \pm 2\%$ less than nominal at 11°C (data not shown). 4-MP concentrations were corrected for this 34% loss prior to kinetic determinations at 11°C.

In addition, experiments conducted with 4-MP (11°C) in the presence of microsomal protein and other cofactors but in the presence or absence of G-6-P dehydrogenase were used to confirm loss of 4-MP only in presence of the dehydrogenase. Experiments similar to those confirming 4-MP losses with low temperature and G-6-P dehydrogenase were used to investigate losses of HQ and 4-MCAT under the incubation and analysis conditions described previously, i.e. processing of samples in 4°C ACN with observed losses of $30 \pm 7\%$ and $29 \pm 7\%$, respectively at 4 and 11°C; again verified with microdialysis (data not shown). Rates of HQ and 4-MCAT production were corrected for this loss.

2.5. Metabolite identification and quantification

Analysis of microsomal incubation samples and standards was performed on a Shandon Hypersil 5 μ 4.6×250 mm C18 RP-HPLC column (Alltech Associates, Deerfield, IL) and a System Gold HPLC (Beckman, Fullerton, CA) equipped with a refrigerated autosampler (Beckman model 507), diode array UV detector (Beckman model 168), and a dual channel electrochemical detector (ECD; model CC-5, LC-4C, BAS, Lafayette, IN), comprised of a glassy carbon working electrode and a Ag/AgCl reference electrode, configured in parallel. The injection volume was 20 μl. An isocratic mobile phase (1 ml min^{-1}) of 9.6% acetonitrile and 0.1 M sodium acetate (pH 4.2) was used.

Hydrodynamic curves were constructed to determine optimum oxidation or reduction potentials for each analyte (Fig. 3). Repeated analysis of standard (or incubation sample for identification of 10.5 min peak) was conducted to measure area response at various potential settings. Response area derived from the variable electrode was normalized with respect to area generated from a constant reference potential on the second electrode. Optimum ECD potential for detection of HQ and 4-MCAT was $E^{\circ} = +0.425$ V [ox] and for detection of
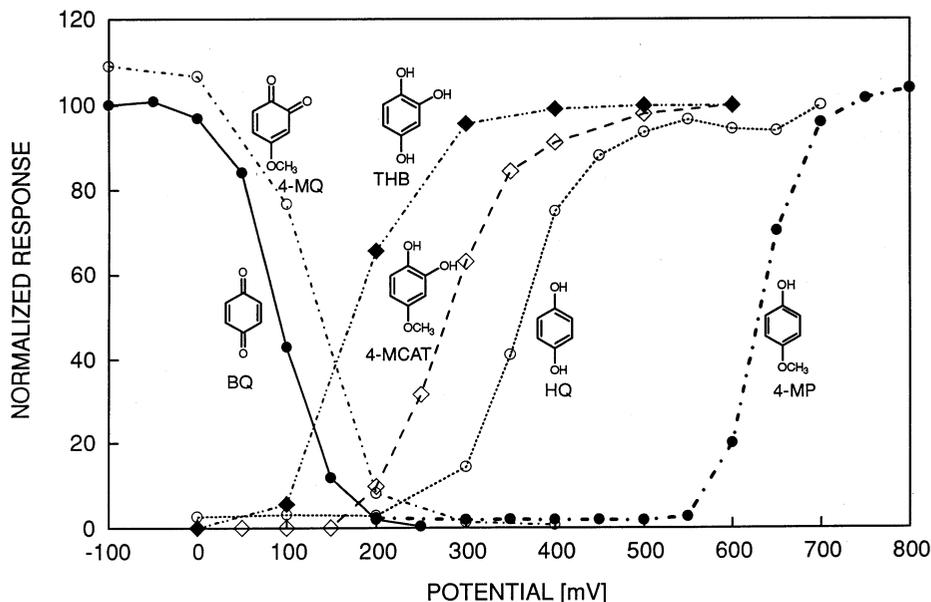
Fig. 3. Hydrodynamic voltammograms of 4-MP and its proposed oxidized and reduced metabolites, BQ, 4-MQ, THB, 4-MCAT and HQ, used to determine optimal potentials for electrochemical detection.

BQ was $E^\circ = +0.050$ 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 compared with standards (confirmed by LC/MS) and for HQ, 4-MCAT and BQ quantitation. UV-diode array detection was used to quantify levels of 4-MP, and as additional confirmation of metabolite identification. Detection of THB, with a BAS single channel ECD with a glassy carbon working electrode set to $E^\circ = +0.175$ V [ox], was performed as above, with the exception of a mobile phase consisting of 0.05 M sodium acetate (pH 4.2). Measurement of HQ in microsomal samples was based on a standard curve. In the absence of a purified 4-MCAT standard, concentrations of 4-MCAT were estimated by comparing observed ECD peak areas with HQ standard curves based on the finding that at an oxidation potential of $+0.425$ V the molar response ratios of HQ, 3-MCAT and MHQ were all equivalent. HQ and 4-MCAT peak areas were corrected for 30% loss during sample preparation, as described previously.

3. Results

Qualitative examination of HPLC-ECD chromatograms of microsomal reaction mixtures (Fig. 4) indicated that, in addition to anticipated peaks for reaction product HQ (4.5 min) and substrate 4-MP (19.5 min), a relatively large peak was present at 10.5 min. The compound associated with this retention time was subsequently determined to be 4-MCAT, as follows. Initially a hydrodynamic curve ($E^\circ = +200 - +350$ mV) for the compound eluting at 10.5 min was constructed utilizing a typical microsomal incubation sample, which indicated the unknown analyte to be in the reduced state (Fig. 5). Consequently, the unidentified metabolite was postulated to be a product of ring hydroxylation in absence of demethylation. Due to the lack of commercially available 4-MCAT and 4-methoxyresorcinol standards, hydrodynamic curves were generated for the structural isomers 3-methoxycatechol (3-MCAT; $E^\circ = +300 - +500$ mV), 5-methoxyresorcinol (5-MRES; $E^\circ = +800 - +1000$ mV), and methoxyhydroquinone (MHQ; $E^\circ = +150 - +400$ mV) and compared with that of the unknown metabo-
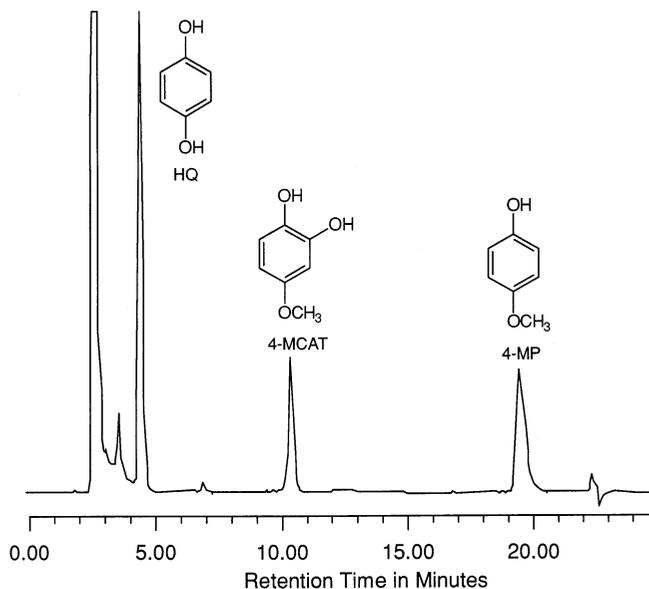
Fig. 4. A representative HPLC-ECD chromatogram of rainbow trout liver microsomes and cofactors incubated with 4-MP for 15 min at 25°C. HQ (4.6 min) and 4-MP (19.4 min) were identified upon comparison with standards. The peak at 10.5 min was identified as 4-MCAT (see text and Fig. 2). Similar chromatograms were obtained from preparations incubated at 11°C.

lite (Fig. 5). Evidence supported a structure having both *ortho* and *para*, but not *meta*, ring substitution patterns based on similarity of the voltammogram for the unknown structure to those of *ortho*- and *para*-hydroxylated MHQ and 3-MCAT. Enzymatically generated 4-MCAT (Section 2) was analyzed by HPLC-ECD (Fig. 1), LC/MS and MS/MS (Fig. 2(A)) and produced a direct retention time match to the microsomal reaction peak at 10.5 min. Hydrodynamic curves generated from 4-MCAT standard and the 10.5 min peak from microsomal incubations proved to be identical.

Microsomal assay conditions for identification and quantification of 4-MP metabolites were optimized for pH, incubation time, cofactor and microsomal protein concentration, with optimum HQ formation at pH 8.0, 25% less at pH 7.5, and 67% and 80% less at pH 8.5 and 6.5, respectively. With the concentration of cofactors utilized, the rate of HQ production was found to be linear with respect to time up to 20 min.

In an effort to quantify the rate of 4-MP metabolic biotransformation, incubations of trout liver microsomes with 4-MP concentrations (0.66–40 mM) were conducted at the physiologically relevant temperature of 11°C, as well as 25°C. Saturation kinetics for the production of HQ via microsomal O-demethylation over a range of 4-MP concentrations was never achieved. The rate of HQ formation was linear up to 26 mM 4-MP, the measured concentrations of 4-MP freely dissolved when 40 mM 4-MP is added to microsomal incubations at 11°C (Fig. 6(A)). A linear rate of HQ formation was also determined at 25°C where, upon the addition of 40 mM 4-MP, a dissolved concentration of 40 mM was achieved (Fig. 6(B)). Substrate concentrations greater than those reported in Fig. 6(A) exceeded solubility at 11°C (as detailed in Section 2). The rate of O-dealkylation, determined as the slope of the linear regression of HQ production versus 4-MP concentration (Fig. 6(A)), was 22.0 ± 2.2 ($\pm$ S.E., ($r^2 = 0.91$) pmol min^{-1} per mg protein per mM substrate at 11°C. Linear rate of HQ formation at 25°C (Fig. 6(B)) was 34.0 ± 1.3 ($r^2 = 0.99$) pmol min^{-1} per mg protein per mM substrate. Maximum rates of HQ production upon addition of 40 mM 4-MP were 606 ± 67 and 1399 ± 86 pmol min^{-1} per mg protein, deter-
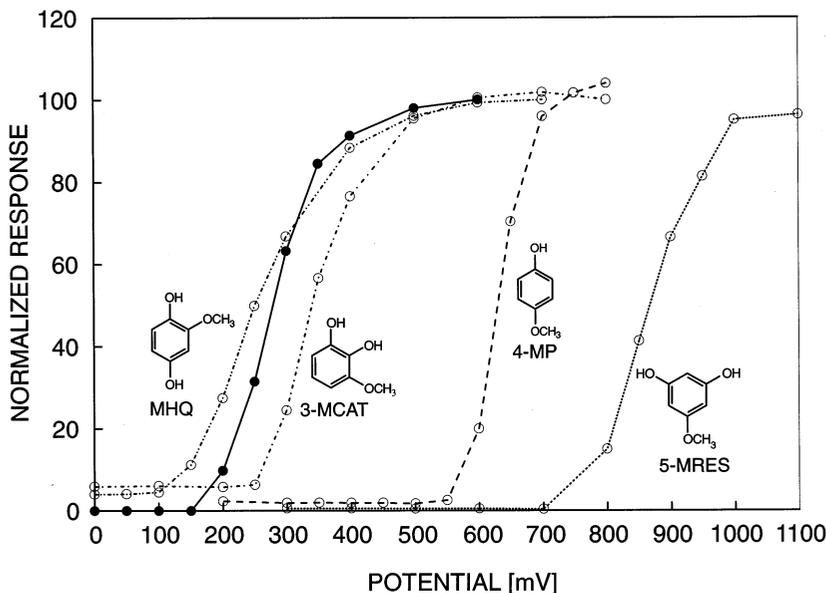
Fig. 5. Hydrodynamic voltammogram of the metabolite at 10.5 min (●) subsequently identified to be 4-MCAT (see Figs. 1 and 4 and text), resulting from microsomal incubation of 4-MP. Voltammograms of commercially available methoxyhydroquinone (MHQ), 3-methoxycatechol (3-MCAT), 4-MP and 5-methoxyresorcinol (5-MRES) are also shown.

mined at 11 and 25°C, respectively. These numbers are however somewhat misleading due to the difference in dissolved 4-MP concentration in each incubation. Linear formation rates were also estimated for the appearance of 4-MCAT at 11 and 25°C (Fig. 6(A and B)). The linear rate of 4-MP ring hydroxylation to 4-MCAT was determined to be 19.0 ± 1.6 ($r^2 = 0.94$) pmol min^{-1} per mg protein per mM substrate at 11°C and 17.2 ± 0.5 ($r^2 = 0.99$) pmol min^{-1} per mg protein per mM substrate at 25°C, even though the maximal rates upon addition of 40 mM 4-MP to microsomes at 11 and 25°C were 465 ± 50 and 691 ± 27 pmol min^{-1} per mg protein. Interestingly, the rate of 4-MCAT production per mM dissolved substrate was essentially the same at the two temperatures.

Double reciprocal plot of substrate versus velocity is shown in Fig. 7 for the production of HQ and 4-MCAT at 11°C and is representative of data collected at 25°C. Regression statistics applied to this data show the y-intercept not significantly different from zero. This confirms the linearity of the metabolic rates for concentrations of 4-MP tested in this system, and precludes the

calculation of K_m and V_{max} for these reactions.

Relatively low, but detectable, levels of BQ were found in the incubations (Fig. 6(A and B)). While Fig. 6 indicates the presence of BQ, it is also possible that 4-MQ may have been produced, based on similar retention times and hydrodynamic voltammograms for BQ and 4-MQ (Fig. 3). Quantification and estimation of the rate of formation of BQ (or 4-MQ) was difficult, likely due to the presence of high concentrations of reducing equivalents in the microsomal system, as well as the high reactivity of BQ (Comporti, 1989; O'Brien, 1991; Monks et al., 1992).

Detectable levels of THB were also discovered at higher concentrations of 4-MP at 11 and 25°C. The route for THB production is uncertain; however, possible routes include hydroxylation of hydroquinone or O-demethylation of 4-MCAT.

4. Discussion

A proposed metabolic map for 4-MP, based on assumptions of O-demethylation and ring hydroxylation reactions, is shown in Fig. 8. Because it
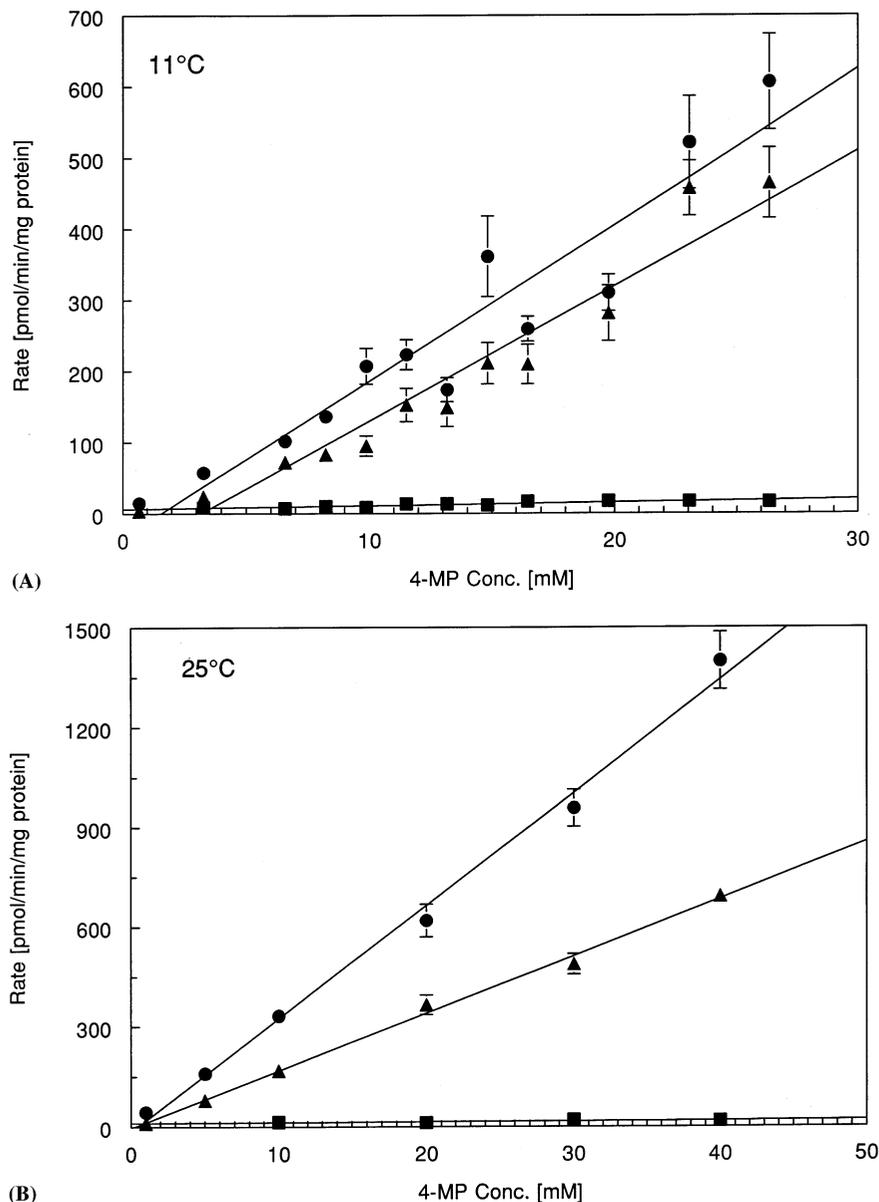
(A)

(B)

Fig. 6. (A) Rate of simultaneous production at 11°C of HQ (●), 4-MCAT (▲), and BQ or 4-MQ (■) after 15 min incubation of rainbow trout liver microsomes with measured 4-MP concentrations of 0.66-26.4 mM. Each point reflects the mean ± S.E. of five microsomal preparations. (B) Rate of simultaneous production at 25°C of HQ (●), 4-MCAT (▲), and BQ or 4-MQ (■), after 15-min microsomal incubations with measured 4-MP concentrations of 1.0–40 mM. Each point reflects the mean ± S.E. of nine microsomal preparations.

was postulated that *O*-demethylation would be the predominant reaction, initial efforts focused on the detection of HQ. Hydroquinone was found to be the primary metabolite of 4-MP in trout microsomes. Subsequent investigation determined

the significant secondary metabolite to be 4-MCAT. BQ and THB were also detected in microsomal incubations. Unlike previous studies in which *O*-demethylation of substituted aromatic compounds was quantified by colorimetric mea-
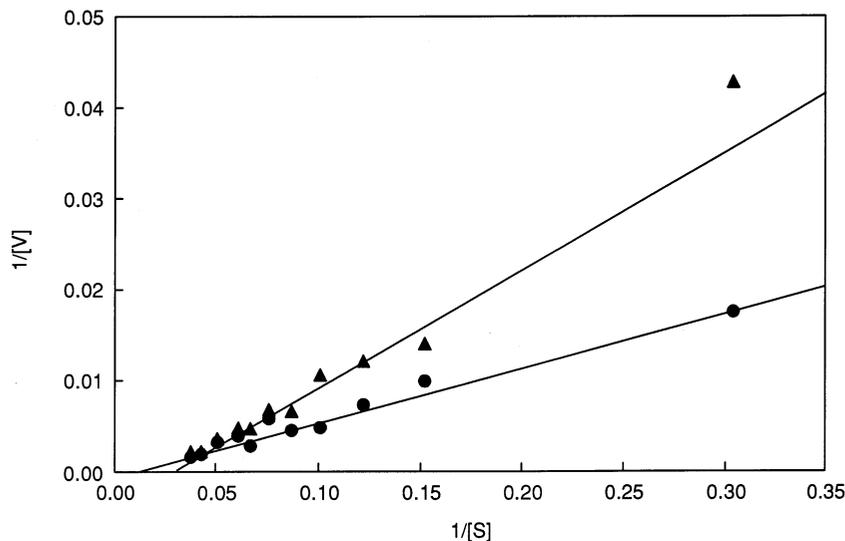
Fig. 7. Double reciprocal plot of 4-MP substrate concentrations and velocity showing linear rate of production of HQ (●) and 4-MCAT (▲) at 11°C. HQ regression statistics for lines fit to the data: $r^2 = 0.995$, y-intercept $= 0.0013 \pm 0.0014$; 4-MCAT regression statistics: $r^2 = 0.998$, y-intercept $= -0.0091 \pm 0.0036$.

surement of formaldehyde (Nash, 1953; Cheeseman, 1984; Yang et al., 1985; Schiller et al., 1991) the rate of 4-MP conversion to HQ was determined by direct quantification using HPLC combined with ECD. Due to the approximately thousand-fold increase in sensitivity over UV or colorimetric detection methods, the use of HPLC-ECD allowed the direct measurement of HQ, 4-MCAT, BQ and THB, at pmol levels.

Rate of HQ production upon addition of 5 mM 4-MP (with only 3.3 mM 4-MP in solution at 11°C) was 0.057 ± 0.006 and 0.160 ± 0.010 nmol min^{-1} per mg protein, at 11 and 25°C, respectively. These observed rates are similar to rates of formaldehyde production from rat (0.1 ± 0.1 nmol min^{-1} per mg; Cheeseman, 1984) and mouse liver microsomes (0.1 ± 0.2 nmol min^{-1} per mg; Schiller et al., 1991) incubated with 5 mM 4-MP at 37°C. The high standard deviations noted from the mammalian studies are a reflection of measurements made near detection limits for formaldehyde assays. The apparent similarity in 4-MP metabolism between trout, rat and mouse microsomes is somewhat surprising when considering trout microsomes have been shown to *O*-dealkylate 7-ethoxyresorufin at an order of

magnitude slower rate compared with rat and mouse microsomes (Franklin et al., 1980) and fish metabolizing enzymes have been thought to generally have lower activity than similar enzyme systems in mammals (Goksoyr et al., 1987).

The proposed pathway for metabolic oxidation of 4-MP in trout microsomes (Fig. 8) is qualitatively similar to the one shown for mice (Schiller et al., 1991), although differences in the relative amounts of metabolites produced are apparent. Schiller et al. (1991) found 4-MCAT to be the primary metabolite although HQ was also detected. Sulfate and glucuronide conjugates of 4-MCAT, and lower levels of unconjugated 4-MCAT, have also been identified as the main human urinary metabolite of 4-MP along with additional *O*-methyl derivatives (Pavel et al., 1989). However, the metabolic scheme proposed by Anari et al. (1995) for 4-MP metabolism in rat hepatocytes, determined with HPLC using electrochemical detection, is similar to that reported here with trout microsomes. Anari et al. (1995) reported *O*-demethylation to HQ and subsequently BQ, as well as ring hydroxylation resulting in formation of 4-MCAT and 4-MQ. Discrepancies in findings between trout, rat and
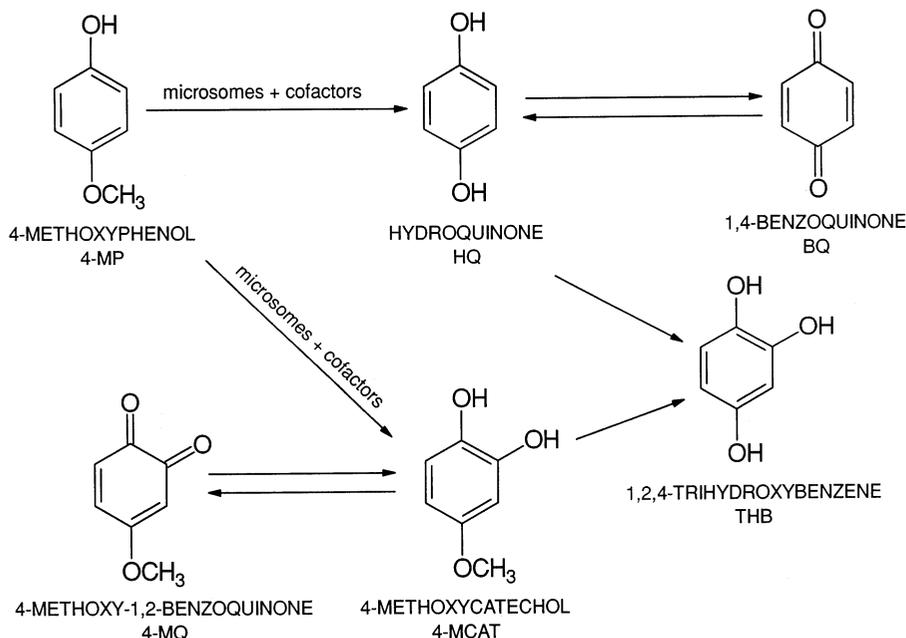
Fig. 8. Proposed metabolic scheme for metabolism of 4-MP by rainbow trout microsomes via *O*-demethylation to HQ, BQ and THB, and by ring hydroxylation to 4-MCAT, and subsequently 4-MQ.

mouse studies likely reflect differences in analytical methods (UV, colorimetric or HPLC-ECD) and experimental systems (microsomes, isolated cells, or whole organism). It is important to note that the more reactive metabolites would not be expected to be detected in large amounts in urine. In addition, the balance between the production of BQ and HQ from 4-MP has also been shown to be affected by the presence of hydroperoxides that enhance the P450 peroxygenase system and reduce monooxygenase contribution. Anari et al. (1995) found BQ to be the primary metabolite formed when isolated rat hepatocytes were exposed to both 4-MP and *t*-butylhydroperoxide, whereas HQ was the main metabolite formed in the absence of the organic hydroperoxide.

The rate of metabolism of 4-MP to HQ and 4-MCAT in this study was found to be linear through the concentration ranges tested, with maximum concentration dictated by solubility limitations in the experimental system, especially at 11°C. Similar solubility limitations may be encountered in blood as well as in intracellular compartments and should be considered when

utilizing in vitro metabolism studies for predicting in vivo transformations. The inability to achieve saturation prohibited the calculation of K_m and V_{max} for the reactions in question.

Temperature effects on enzyme activity can be described by Q_{10}, with a Q_{10} of 2 representing a doubling of enzymatic activity with a 10°C temperature rise. Values of Q_{10} about 2–2.5 have been observed for many metabolic reactions (Koivusaari and Andersson, 1984; Dady et al., 1991). The linear rate of 4-MP *O*-dealkylation to HQ at 11°C in the present study was 65% of that observed at 25°C, resulting in a Q_{10} value of 1.3. This is relatively small in comparison with some Q_{10} values, although perhaps consistent with the observations of Somero (1978) and Koivusaari and Andersson (1984) that Q_{10} of the reaction at unsaturated substrate concentration will be less than observed under saturating substrate concentration. Interestingly, for the ring hydroxylation of 4-MP to 4-MCAT, the rate per mmol substrate was essentially the same regardless of temperature. The apparent temperature sensitivity of HQ production yet an apparent insensitivity of the

4-MCAT reaction may indicate the possible involvement of different P450 isozymes in each reaction, if indeed either of the reactions reported in this study are subsequently proven to be P450 mediated processes. Snegaroff and Bach (1990) suggested different temperature optima for different isozymes may contribute to observed differences in temperature maxima of EROD and λ' ethoxycoumarin-O-deethylase ECOD activity in trout microsomes. In addition, Schlenk et al. (1997) observed a lack of correspondence between methoxychlor hydroxylation and demethylation reactions, and suggested regiospecific catalysis by multiple isoforms may be occurring in catfish.

Evidence exists in aquatic organisms to suggest 4-MP may be bioactivated in vivo. Experimental data with fathead minnows exposed to 4-MP in 96 h tests (Geiger et al., 1985) during which behavior was also monitored, suggests deviation from a previously predicted non-specific narcosis mode of action. Based solely on the chemical structure of 4-MP and its octanol/water partition coefficient, 4-MP is predicted to act through a relatively non-reactive mode of toxic action in fathead minnows (Russom et al., 1997). Dose-response profile obtained for the acute toxicity of this chemical, however, showed greater toxicity at low dose levels than would be predicted to occur based on the presumption of no bioactivation and all toxicity attributable to unmetabolized 4-MP. Additionally, an analysis of the observed behavior during the acute toxicity test revealed convulsive and hyperactive behavior consistent with a fish behavior syndrome associated with electrophilic/ proelectrophilic (i.e. reactive) chemical toxicity (Russom et al., 1997). The observed behavior was inconsistent with behavior syndromes indicated by depressed locomotor activity with little response to outside stimuli, that would be expected from less potent and less reactive, i.e. narcosis, modes of action. These observations led to the hypothesis that, at least at chemical concentrations below the LC_{50}, 4-MP was being metabolically activated to a more toxic chemical form. Results of the present study support this hypothesis. 4-Methoxyphenol was found to be metabolized in trout liver microsomes to hydroquinones and quinones (Fig. 8) capable of interacting with cellular nucleophiles (O'Brien, 1991; Monks et al., 1992; Anari et al., 1995), which could account for increased toxicity. Hydroquinone-glutathione (HQ-GSH) and 4-MCAT-GSH conjugates, in addition to HQ and BQ, have been characterized in rat microsomes exposed to 4-MP in the presence of GSH, supporting a possible arylation/GSH depletion mechanism of toxicity (Anari et al., 1995). The production of 4-MCAT and potential interconversion to 4-MQ, a cytotoxic quinone, also appears to increase toxic potential over that expected from the parent phenol (Riley, 1985). Cooksey et al. (1987) also presented evidence attributing cytotoxicity of 4-MQ to addition reactions with protein and non-protein sulfhydryl groups. Therefore, the metabolism of 4-MP to HQ, 4-MCAT, and BQ and/or 4-MQ as shown in this study, supports the potential for bioactivation of 4-MP to reactive chemical forms in fish.

5. Conclusions

In this study, 4-MP was found to be metabolized in trout microsomes primarily to HQ and 4-MCAT, the results of O-demethylation and ring hydroxylation reactions, respectively. Additional metabolites, identified as BQ (and/or 4-MQ) as well as THB, were present in small but detectable quantities. A metabolic pathway for metabolism of 4-MP in rainbow trout was proposed following confirmation of metabolite identity (utilizing hydrodynamic voltammograms generated by HPLC with electrochemical detection, enzymatic synthesis, and mass spectral identification) as well as quantification of formation rates. The measurable 'free' amounts of the primary metabolites, HQ and 4-MCAT (10^2 pmol min^{-1} per mg protein), were ten times greater than measurable BQ and THB quantities (10^1 pmol min^{-1} per mg protein). This may be a reflection of their formation subsequent to HQ or 4-MCAT, or may be due to their labile nature and potential high reactivity with macromolecules present, or both. Further work will be needed to verify the presence and significance of the proposed pathways in vivo, however, the identification of these species as 4-MP metabolites supports the hypothesis that bioacti-

vation of 4-MP in fish species may be responsible for observed dose-response relationships in whole organism studies.

The methods utilized in the present study, and associated results serve as a basis for further investigations into toxic mechanisms resulting from metabolic bioactivation. Saturation kinetics were not achieved for metabolites produced either by *O*-demethylation or ring hydroxylation, due to limited substrate solubility at low temperatures. These results emphasize the importance of determining substrate and product solubilities within the reaction mixture at relevant physiological temperature. The importance of special handling procedures for labile metabolites, as well as the development and application of new and sensitive analytical techniques for the quantification of metabolites at picomolar levels was demonstrated. Also, the extent to which reactive metabolites may bind to components of in vitro reaction mixtures and go undetected was discussed. The present work again emphasized the need to study metabolism at physiologically relevant temperatures and to accurately quantify freely dissolved substrate and metabolite, especially when conducting studies at temperatures that are significantly lower than that at which chemical solubility is frequently determined. Methods developed, as well as metabolic rates determined in vitro, serve as a basis for identification and quantification of potentially reactive metabolites in aquatic animals in vivo.

Acknowledgements

References

Ahokas, J.T., Pelkonen, O., 1984. Metabolic activation of polycyclic hydrocarbons by fish liver cytochrome P-450. Mar. Environ. Res. 14, 59–69.

Anari, M.R., Kahn, S., Liu, Z.C., O'Brien, P.J., 1995. Cytochrome P-450 peroxidase/peroxygenase mediated xenobiotic metabolic activation and cytotoxicity in isolated hepatocytes. Chem. Res. Toxicol. 8, 997–1004.

Anders, M.W. (Ed.), 1985. Bioactivation of Foreign Compounds. Academic Press, Orlando, FL.

Bradbury, S.P., Dady, J.M., Fitzsimmons, P.N., Voit, M.M., Hammermeister, D.E., Erickson, R.J., 1993. Toxicokinetics and metabolism of aniline and 4-chloroaniline in medaka (*Oryzias latipes*). Toxicol. Appl. Pharmacol. 118, 205–214.

Bradbury, S.P., 1994. Predicting modes of action from chemical structure: An overview. SAR QSAR Environ. Res. 2, 89–104.

Bradbury, S.P., 1995. Quantitative structure-activity relationships and ecological risk assessment: an overview of predictive aquatic toxicology research. Toxicol. Lett. 79, 229–237.

Buhler, D.R., 1995. Cytochrome P450 expression in rainbow trout: an overview. In: Arinc, E., Schenkman, J.B., Hodgson, E. (Eds.), NATOASI Series. Molecular Aspects of Oxidative Drug Metabolizing Enzymes, Springer-Verlag, New York, Vol. H 90. pp. 159–180.

Cheeseman, K.H., 1984. 4-Hydroxyanisole and the rat liver microsomal drug metabolizing enzymes. In: Riley, P.A. (Ed.), Hydroxyanisole. Recent Advances in Antimelanoma Therapy. IRL Press, Oxford, pp. 183–189.

Cooksey, C.J., Land, E.J., Riley, P.A., Sarna, T., Truscott, T.G., 1987. On the interaction of anisyl-3,4-semiquinone with oxygen. Free Rad. Res. Comms. 4, 131–138.

Comporti, M., 1989. Three models of free radical-induced cell injury. Chem. Biol. Interact. 7, 1–56.

Dady, J.M., Bradbury, S.P., Hoffman, A.D., Voit, M.M., Olson, D.L., 1991. Hepatic microsomal *N*-hydroxylation of aniline and 4-chloroaniline by rainbow trout (*Onchorhyncus mykiss*). Xenobiotica 21, 1605–1620.

Estabrook, R.W., Peterson, J., Baron, J., Hildebrandt, A.G., 1972. The spectrophotometric measurement of turbid suspensions of cytochromes associated with drug metabolism. Methods Pharmacol. 2, 303–350.

Forlin, L., Andersson, T., 1985. Storage conditions of rainbow trout liver cytochrome P-450 and conjugating enzymes. Comp. Biochem. Physiol. 80B, 569–572.

Franklin, R.B., Elcombe, C.R., Vodicnik, M.J., Lech, J.J., 1980. Comparative aspects of the disposition and metabolism of xenobiotics in fish and mammals. Fed. Proc. 39, 3144–3149.

Geiger, D.L., Northcott, C.E., Call, D.J., Brooke, L.T. (Eds.), 1985. Acute Toxicities of Organic Chemicals to Fathead Minnows (*Pimephales promelas*), vol. 2. Center for Lake Superior Environmental Studies, University of Wisconsin, Superior, WI, pp. 167–168.

Gibson, G.G., Skett, P. (Eds.), 1986. Pathways of drug metabolism. In: Introduction to Drug Metabolism. Chapman and Hall, New York, pp. 1–37.

Goksoyr, A., Andersson, T., Hansson, T., Klungsoyr, J., Zhang, Y., Forlin, L., 1987. Species characteristics of the hepatic xenobiotic and steroid biotransformation systems of two teleost fish, atlantic cod (*Gadus morhua*) and rainbow trout (*Salmo gairdneri*). Toxicol. Appl. Pharmacol. 89, 347–360.

Guengerich, F.P., Liebler, D.C., 1985. Enzymatic activation of chemicals to toxic metabolites. CRC Crit. Rev. Toxicol. 14, 259–307.

Gunaratna, C., Kissinger, P.T., 1997. Application of microdialysis to study the in vitro metabolism of drugs in liver microsomes. J. Pharm. Biomed. Anal. 16, 239–248.

Hinson, J.A., Pumford, N.R., Nelson, S.D., 1994. The role of metabolic activation in drug toxicity. Drug Metab. Rev. 26, 395–412.

Koivusaari, U., Andersson, T., 1984. Partial temperature compensation of hepatic biotransformation enzymes in juvenile rainbow trout (*Salmo gairdneri*) during the warming of water in spring. Comp. Biochem. Physiol. 78B, 223–226.

Lech, J.J., 1974. Glucuronide formation in rainbow trout: effect of salicylamide on the acute toxicity, conjugation and excretion of 3-trifluoromethyl-4-nitrophenol. Biochem. Pharmacol. 23, 2403–2410.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

McKim, J.M. Jr., McKim, J.M. Sr., Naumann, S., Hammermeister, D.E., Hoffman, A.D., Klaassen, C.D., 1993. In vivo microdialysis sampling of phenol and phenyl glucuronide in the blood of unanesthetized rainbow trout: implications for toxicokinetic studies. Fundam. Appl. Toxicol. 20, 190–198.

Melancon, M.J., Lech, J.J., 1984. Metabolism of [^{14}C]2-methylnaphthalene by rainbow trout (*Salmo gairdneri*) in vivo. Comp. Biochem. Physiol. 79C, 331–336.

Menter, J.M., Townsel, M.E., Moore, C.L., Williamson, G.D., Soteres, B.J., Fisher, M.S., Willis, I., 1990. Melanin accelerates the tyrosinase-catalyzed oxygenation of *p*-hydroxyanisole (MMEH). Pigment Cell Res. 3, 90–97.

Mitchell, J.R., Horning, M.G., 1984. Drug Metabolism and Drug Toxicity. Raven Press, New York.

Monks, T.J., Hanzlik, R.P., Cohen, G.M., Ross, D., Graham, D.G., 1992. Contemporary issues in toxicology: quinone chemistry and toxicity. Toxicol. Appl. Pharmacol. 112, 2–16.

Nash, T., 1953. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochem. J. 55, 416–421.

O'Brien, P.J., 1991. Molecular mechanisms of quinone cytotoxicity-review. Chem.-Biol. Interact. 80, 1–41.

Parkinson, A., 1996. Biotransformation of xenobiotics. In: Klaassen, C.D. (Ed.), Casarett and Doull's Toxicology, The Basic Science of Poisons, 5th ed. McGraw-Hill, St Louis, MO, pp. 113–186.

Pavel, S., Holden, J.L., Riley, P.A., 1989. Metabolism of 4-hydroxyanisole: identification of major urinary excretory products. Pigment Cell Res. 2, 421–426.

Phillips, L.R., Bartmess, J.E., 1989. Electrochemically assisted fast atom bombardment negative ion mass spectrometry of quinones. Biomed. Environ. Mass Spectrom. 18, 878–883.

Phillips, A.H., Langdon, R.G., 1962. Hepatic triphosphopyridine nucleotide-cytochrome c reductase: isolation, characterization, and kinetic studies. J. Biol. Chem. 237, 2652–2660.

Pohl, R.J., Fouts, J.R., 1980. A rapid method for assaying the metabolism of 7-ethoxyresorufin by microsomal subcellular fractions. Anal. Biochem. 107, 150–155.

Riley, P.A., 1985. Radicals and melanomas. Phil. Trans. R. Soc. Lond. B 311, 679–689.

Russom, C.L., Bradbury, S.P., Broderius, S.J., Hammermeister, D.E., Drummond, R.A., 1997. Predicting modes of toxic action from chemical structure: acute toxicity in the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 16, 948–967.

Schiller, C.D., Gescher, A., Jheeta, P., 1991. Mechanism of toxicity of the antimelanoma drug 4-hydroxyanisole in mouse hepatocytes. Eur. J. Cancer 27, 1017–1022.

Schlenk, D., Stresser, D.M., McCants, J.C., Nimrod, A.C., Benson, W.H., 1997. Influence of B-naphthoflavone and methoxychlor pretreatment on the biotransformation and estrogenic activity of methoxychlor in channel catfish (*Ictalurus punctatus*). Toxicol. Appl. Pharmacol. 145, 349–356.

Snegaroff, J., Bach, J., 1990. The effects of temperature on the basal activity of cytochrome P450 in rainbow trout (*Salmo gairdneri*). Comp. Biochem. Physiol. 95B, 515–519.

Somero, G.N., 1978. Temperature adaptation of enzymes: biological optimization through structure-function compromises. A. Rev. Ecol. Syst. 9, 1–29.

Varanasi, U., Stein, J.E., 1991. Disposition of xenobiotic chemicals and metabolites in marine organisms. Environ. Health Perspect. 90, 93–100.

Yang, C.S., Tu, Y.Y., Koop, D.R., Coon, M.J., 1985. Metabolism of nitrosamines by purified rabbit liver cytochrome P450 isozymes. Cancer Res. 45, 1140–1145.