Hepatic Clearance of 6 Polycyclic Aromatic Hydrocarbons by Isolated Perfused Trout Livers: Prediction From In Vitro Clearance by Liver S9 Fractions

John W. Nichols,*† Alex D. Hoffman,* Thomas L. ter Laak,‡ and Patrick N. Fitzsimmons*

*U.S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division, Duluth, Minnesota 55804; †Institute for Risk Assessment Sciences, Utrecht University, NL-3508 TD Utrecht, The Netherlands; and ‡KWR Watercycle Research Institute, NL-3433 PE Nieuwegein, The Netherlands

To whom correspondence should be addressed at U.S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division, 6201 Congdon Boulevard, Duluth, MN 55804. Fax: (218) 529-5003. E-mail: nichols.john@epa.gov.

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Isolated perfused trout livers were used to evaluate in vitro-in vivo metabolism extrapolation procedures for fish. In vitro depletion rates for 6 polycyclic aromatic hydrocarbons (PAHs) were measured using liver S9 fractions and extrapolated to the intact tissue. Predicted hepatic clearance (CL,f) values were then compared with values exhibited by intact livers. Binding in liver perfusates was manipulated using bovine serum albumin (BSA) and was characterized by solid-phase microextraction. Additional studies were conducted to develop binding terms (fU; calculated as the ratio of unbound fractions in liver perfusate [fU,PERF] and the S9 system [fU,S9]) used as inputs to a well-stirred liver model. Hepatic clearance values for pyrene and benzo[a]pyrene, predicted by extrapolating in vitro data to the intact tissue, were in good agreement with measured values (< 2-fold difference). This can be partly attributed to the rapid rate at which both compounds were metabolized by S9 fractions, resulting in perfusion-limited clearance. Predicted levels of CL,f for the other PAHs underestimated observed values although these differences were generally small (< 3-fold, except for naphthalene). Setting fU = 1.0 improved clearance predictions at the highest tested BSA concentration (10 mg/ml), suggesting that trout S9 fractions exhibit lower levels of intrinsic activity than the intact tissue or that the full binding assumption (ie, fU = fU,PERF/fU,S9) underestimates the availability of hydrophobic substrates to hepatic metabolizing enzymes. These findings provide qualified support for procedures currently being used to predict metabolism impacts on chemical accumulation by fish based on measured rates of in vitro activity.

Key Words: trout; polycyclic aromatic hydrocarbons; biotransformation; in vitro-in vivo extrapolation; perfused liver; SPME.

Hydrophobic environmental contaminants tend to accumulate in fish and in other aquatic biota, posing a threat to exposed animals and the animals that consume them, including humans. Although standard testing protocols can be used to measure chemical accumulation in fish (OECD, 1996), these methods are time consuming, expensive, and require a substantial number of animals (> 100 per compound). It is critical, therefore, to develop predictive methods for bioaccumulation assessment that are rapid, cost-effective, and minimize the need for whole-animal testing. Ideally, such methods would be based on in silico and/or in vitro approaches (Nichols et al., 2007, 2009a).

A major determinant of whether chemicals accumulate in fish is the extent to which they undergo biotransformation. Fish metabolize chemicals using enzymatic pathways similar to those in mammals (Schlenk et al., 2008). In vitro kinetic data (eg, Ks and VMAX values) have been reported for over 80 compounds and 40 fish species (Fitzsimmons et al., 2007). Other groups have estimated in vivo rates of metabolism by fitting model simulations to observed rates of chemical depuration (de Wolf et al., 1993; Fisk et al., 2000; Gert-Jan de Maagd et al., 1998; Konwick et al., 2006; Sijm et al., 1990; Tolls et al., 2000) or measured levels of accumulation (Arnott et al., 2008a,b). Nevertheless, the paucity of this information remains a major source of uncertainty in bioaccumulation assessments.

Recognizing this deficiency, researchers have begun to use in vitro-in vivo extrapolation procedures to predict biotransformation impacts on chemical accumulation in fish (Cowen-Ellsberry et al., 2008; Dyer et al., 2008; Gomez et al., 2010; Han et al., 2007, 2009). These procedures are based in part on methods pioneered by the pharmaceutical industry for prediction of hepatic clearance (Houston, 1994; Houston and Carlile, 1997; Ito et al., 1998; Obach, 1999). Additional calculations are performed to extrapolate hepatic clearance to a first-order whole-body metabolism rate constant (kMET; 1/h).
which is then used as an input to predictive models of chemical accumulation (Nichols et al., 2006, 2009a). Importantly, these models include log $K_{ow}$-based algorithms that explicitly account for chemical elimination by other routes (e.g., branchial elimination and partitioning to feces; Arnot and Gobas, 2003, 2004). To date, this work has shown that incorporating in vitro metabolism data substantially improves model performance; thus, predicted levels of accumulation are much closer to measured values than predictions obtained assuming no metabolism.

Notwithstanding these efforts, in vitro-in vivo extrapolations of hepatic metabolism data for fish are limited by several considerations. One concern is the effect of chemical binding on hepatic clearance. Scientists working with mammals routinely account for binding in vivo (in plasma) and in the in vitro metabolizing system when performing these extrapolations for drugs (Austin et al., 2002; Grime and Riley, 2006). Currently, such data for fish in vitro systems (S9 fractions, microsomes, hepatocytes) and blood plasma are extremely limited. On the other hand, there is some question whether binding actually limits hepatic clearance of hydrophobic environmental contaminants by fish. Using a model-based approach, Escher et al. (2011) argued that observed concentrations of some well-metabolized chemicals in fish are much lower than expected, given reported rates of in vitro intrinsic clearance and measured levels of binding (S9 and plasma). Existing extrapolation procedures also assume that the liver is the principal organ of biotransformation. Although this may be true in many cases, limited studies with fish suggest that the gills and gut possess significant metabolic activity as well (Barron et al., 1989; Van Veld et al., 1988).

Recent studies have relied on measured and modeled levels of chemical accumulation in fish to judge the success or failure of metabolism extrapolation procedures. However, if modeled predictions fail to match measured levels of accumulation, there is no way to determine where (the) problem(s) may have occurred. A need exists, therefore, to directly evaluate metabolism extrapolation procedures using research approaches that isolate and address individual issues.

In this study, we used an isolated perfused trout liver model to directly measure hepatic clearance of 6 polycyclic aromatic hydrocarbons (PAHs) with log $K_{ow}$ values ranging from 3.30 to 6.13. Measured clearance values were then compared with values obtained by extrapolating in vitro metabolism data for the same compounds (liver S9 fraction) to the intact tissue. The intent of this approach was to focus on the first part of the overall extrapolation procedure (in vitro assay to the intact liver). As such, these comparisons are not complicated by the possibility of nonhepatic biotransformation or the requirement to scale hepatic clearance to the whole animal. Binding effects on hepatic clearance were examined by changing the amount of bovine serum albumin (BSA) in the liver perfusate. To facilitate these comparisons, we used a solid-phase microextraction (SPME) method to measure free chemical concentrations in perfusate samples (isolated liver studies) and the S9 test system.

MATERIALS AND METHODS

**Chemicals.** Naphthalene (NAPH; CAS no. 91-20-3), fluorene (FLU; 86-73-7), anthracene (ANTH; 120-12-7), phenanthrene (PHEN; 85-01-8), pyrene (PYR; 129-00-0), and benzo[a]pyrene (BAP; 206752-38-7) were purchased from Sigma-Aldrich (St Louis, Missouri). All test chemicals were of reagent grade ($\geq$ 97% purity). Radio-labeled BAP (14C-BAP; $> 97$% pure, 26.6 $\mu$Ci/µmol) was purchased from Sigma-Aldrich and was diluted with unlabeled BAP to a specific activity of 5 $\mu$Ci/µmol. $\beta$-nicotinamide adenine dinucleotide phosphate (NADPH; $> 95$% pure) was purchased from Oriental Yeast Co. (Osaka, Japan). All other reagents and cofactors were purchased from Sigma-Aldrich and were of reagent grade or higher in quality.

**Animals.** Rainbow trout (Oncorhynchus mykiss; Erwin strain) weighing approximately 100 g were obtained from the U.S. Geological Survey Fish and Wildlife Service Laboratory in La Crosse, Wisconsin, and grown up to the desired size (450–600 g). The fish were fed a commercial trout chow (Silver Cup; Nelson and Sons, Murray, Utah) and maintained on a natural (Duluth, Minnesota) photoperiod at 11 ± 1°C. Water used for fish holding was obtained directly from Lake Superior (single pass, sand filtered, and UV treated). Water chemistry characteristics were the following: total hardness, 45–46 mg/l as CaCO$_3$; alkalinity, 41–44 mg/l as CaCO$_3$; pH 7.6–7.8; total ammonia, < 1 mg/l; dissolved oxygen, 85%–100% of saturation.

Sexual maturity influences metabolic activity in fish, particularly as animals approach spawning season (Förlin and Haux, 1990). We therefore evaluated the sexual maturity of donor animals by measuring their gonado-somatic index (GSI), which is the weight of the gonads (ovaries or testes) divided by that of the whole animal. Mean (± SD) GSI values for males and females used to obtain liver S9 fractions were 0.04 (± 0.01) and 0.19 (± 0.02), respectively. Mean GSI values for males and females used for liver perfusion studies averaged 0.04 (± 0.01) and 0.28 (± 0.22), respectively. Changes in GSI that occur as trout grow and develop have been well characterized (Gomez et al., 1999; Le Gac et al., 2001). Based on this information, all of the animals used in this effort appeared to be in very early stages of sexual maturation (stage II/III for females and I/II for males).

**Preparation and characterization of liver S9 fractions.** Pooled liver S9 fractions from 5 male and 6 female trout were prepared using procedures described by Johanning et al. (2012). Animals sampled for this effort ranged from 470 to 540 g. Individual fish were killed with an overdose of 1 mg/ml of ice-cold buffer (Hanks’ balanced salt solution, phenol red/CaCl$_2$/MgSO$_4$ free, cat. no. H6648; supplemented with 2.3 mM EDTA and 10 mM HEPES, pH 7.8) through the hepatic portal vein. Each liver was weighed, minced in 2 volumes (wt/vol) of homogenization buffer, and homogenized using 4–5 strokes of a Potter-Elvehjem mortar and pestle. The homogenization buffer (pH 7.8) consisted of 50 mM Tris, 150 mM KCl, 2 mM EDTA, 1 mM dithiothreitol, and 250 mM sucrose. Homogenates from fish of each gender were pooled and centrifuged at 13 000 x g (4°C) for 20 min. Aliquots (0.5 ml) of the S9 fraction were then flash frozen in liquid nitrogen and stored at −80°C until used.

To assess the activity of liver S9 fractions, we performed a set of characterization assays using well-known substrates for cytochrome P450 1A (CYP1A), UDP-glucuronyltransferase (UGT), and glutathione-S-transferase (GST). CYP1A activity was characterized by measuring rates of ethoxyresorufin-O-deethylation (EROD) and BAP ring hydroxylase (aryl hydrocarbon hydroxylase; AHH). EROD activity was characterized by measuring rates of ethoxyresorufin-O-deethylase using a microplate reader (BioTek Instruments, Winooski, Vermont; Ex. 550 nm, Em. 580 nm) and quantified by comparison to resorufin standards. AHH activity was measured according to Collett et al. (1986). Incubations (0.5 ml) consisted of 1 mg/ml S9 protein, 1 mM NADPH, and 5 mM ethoxyresorufin in 100 mM potassium phosphate buffer. Resorufin was measured by fluorescence on a microplate reader (BioTek Instruments, Winooski, Vermont; Ex. 550 nm, Em. 580 nm) and quantified by comparison to resorufin standards. AHH activity was measured according to Collett et al. (1986). Incubations (0.5 ml) consisted of 5 mM MgCl$_2$, 2 mM NADPH, 0.5 mg/ml S9 protein, and 80 mM $^{14}$C-BAP in 50 mM Tris buffer. Polar metabolites were quantified by liquid scintillation (Packard.
HEPATIC CLEARANCE PREDICTION IN TROUT

2550; Perkin-Elmer, Waltham, Massachusetts). Radioactivity was measured in control samples containing denatured (boiled) S9 to correct for any radioactivity that had partitioned to the aqueous phase but was unrelated to metabolite production.

UGT activity was measured as described by Castrén and Oikari (1983). Incubations (200 µl) were conducted in 500 mM potassium phosphate buffer containing 1 mg/ml S9 protein, 12.7 µM amaminic, 10 mM EDTA, 7 mM UDPGA, and 100 mM p-nitrophenol (p-NP). Substrate (p-NP) was measured by absorbance (BioTek Instruments; 400 nm) and quantified by comparison to a set of p-NP standards. Loss of p-NP in control samples (denatured S9 or active S9 without UDPGA) was determined to correct for nonenzymatic losses.

GST activity was measured using the method of Habig et al. (1974). Incubation mixtures (1.4 ml) consisted of 2 mM reduced glutathione (GSH) and 2 mM 1-chloro-2,4-dinitrobenzene in 100 mM potassium phosphate buffer. Reactions were initiated by adding S9 protein (0.1 mg/ml), and absorbance was monitored for 5 min at 340 nm (Varian 300, Agilent Technologies Inc., Santa Clara, California). Control samples without S9 protein were analyzed simultaneously to determine nonenzymatic changes in absorbance. Enzyme activity was calculated using a molar absorption coefficient of 9.6 mM⁻¹ cm⁻¹.

All characterization assays were performed under saturating substrate conditions at the physiological temperature (11°C) and pH (7.8) for trout. Substrates were added using a small volume of acetone as a carrier solvent. The resulting concentration of acetone in the incubation mixtures was 1% (vol/vol) in all cases. Protein concentration in the S9 fraction was measured using Peterson’s modification of the Lowry method (Sigma technical bulletin TP0300). Total CYP content was measured by the method of Omura and Sato (1964) using a molar absorption coefficient of 91 mM⁻¹ cm⁻¹.

Measurement of in vitro intrinsic clearance. In vitro metabolism of the 6 PAHs was characterized at 11°C using a substrate depletion approach. Incubations were conducted in 200 µl reaction mixtures consisting of 100 mM potassium phosphate buffer (pH 7.8), 1.0 mg/ml S9 protein, 12.7 µM amaminic, and cofactors necessary for phase I and II metabolism of the PAH substrates: 2 mM NADPH, 2 mM UDPGA, 0.5 mM GSH, and 0.1 mM adenosine 3'-phosphate 5-phosphosulfate (PAPS). Reactions were initiated by adding substrate (acetone carrier; 1% vol/vol final concentration) and terminated at predetermined times with 600 µl of ice-cold acetonitrile. The samples were then mixed for 20 s and centrifuged at 3000 × g (4°C) for 5 min to precipitate protein. Supernatants were transferred to 2-ml GC vials and stored at −80°C before use.

Inactivation of pyrene metabolism in trout liver S9 fractions. Dots—heat denatured controls; open triangles—24 h at 25°C, minus cofactors; filled squares—20 µM α-naphthoflavone; filled triangles—24 h at 25°C; open squares—minus cofactors; open circles—no treatment. All points represent the mean of 5 independent measurements. Comparable results were obtained using benzo[a]pyrene as the metabolic substrate.

however, these treatments reduced activity to a level comparable to that of denatured controls.

Based on these findings, all subsequent binding studies were performed at 11°C using pooled S9 fractions inactivated by time (24 h at 25°C) and the absence of cofactors. Six replicate determinations were made for each compound at concentrations designed to match those used in S9 metabolism experiments and liver perfusion studies.

Isolated perfused liver studies. Trout livers were isolated and perfused using methods described by Nichols et al. (2009b). The clearing solution consisted of Hanks’ balanced salt solution (phenol red/CaCl₂/MgSO₄ free, cat. no. H6648) supplemented with 10 mM HEPES, 5 mM sodium pyruvate, and 60 mM EDTA. Control perfusate was prepared by dissolving 90 g modified Hanks’ balanced salts (phenol red/NaHCO₃ free, cat. no. H1387) in 10 l of purified water (Millipore, Billerica, Massachusetts). To this we added HEPES (10 mM), NaHCO₃ (4.38 mM), glucose (5.5 mM), and 147.0 ml 1N NaOH. The solution was vigorously gassed for 24 h with a 0.5% CO₂/99.5% O₂ gas mixture using an air stone. Variable amounts of BSA (fraction V; cat. no. A9418) were then added (see below), followed by gentle stirring and continued equilibration with the gas mixture introduced to the reservoir headspace. Hepatic clearance studies were performed using control perfusates to which test chemicals had been added. Test chemicals in acetone (0.002%–0.02% vol/vol final solvent concentration) were spiked into the perfusate and allowed to equilibrate for 24 h before use.

Fish were euthanized with an overdose of MS 222 (300 mg/l, buffered with 900 mg/l NaHCO₃). Livers were cannulated in situ and perfused with ice-cold clearing solution (8.0 ml/min) for 5 min. Perfusion was then switched to ice-cold control perfusate, again at 8.0 ml/min. If, after 5 min, the liver was not completely cleared of blood, it was discarded.

Hepatic veins were cannulated with Renasil tubing (Braintree Scientific, Braintree, Massachusetts), sized to fit the opening of each vessel. The tubings (usually 2) were secured with Nexaband topical tissue adhesive (Abbott Laboratories, North Chicago, Illinois). The cannulated liver was then dissected from the gastrointestinal tract with the gallbladder intact, weighed, and transferred to the perfusion apparatus. Control and experimental perfusates were maintained at 11 ± 0.3°C in water-jacketed reservoirs, and the entire perfusion apparatus was kept in a temperature-controlled enclosure (Recvo model 3004, Thermo Fisher Scientific, Asheville, North Carolina). All experiments were

FIG. 1. Inactivation of pyrene metabolism in trout liver S9 fractions. Dots—heat denatured controls; open triangles—24 h at 25°C, minus cofactors; filled squares—20 µM α-naphthoflavone; filled triangles—24 h at 25°C; open squares—minus cofactors; open circles—no treatment. All points represent the mean of 5 independent measurements. Comparable results were obtained using benzo[a]pyrene as the metabolic substrate.
performed at a constant flow rate of 8.9 ml/min/kg-body weight, which is equal to the estimated total hepatic blood flow rate for rainbow trout at 11°C (Nichols et al., 1990). Perfusion pressure, perfusion rate, and temperature were continuously recorded using a physiological monitoring system (model 5000, Modular Instruments, Malvern, Pennsylvania). Perfusate samples afferent and efferent to the liver were analyzed using a multiprofile blood analysis system (model ABL800 FLEX, Radiometer America, Westlake, Ohio) to determine pH, PO$_2$ (nm Hg), PCO$_2$ (nm Hg), and concentrations (mM) of Na$^+$, K$^+$, Ca$^{2+}$, D-glucose, and L-lactate. These measurements were made at 37.0±0.15°C. Measured PO$_2$ values were then used to calculate oxygen consumption (VO$_2$; μmol/h/g-tissue) by the liver at 11°C. Equations used to perform these calculations were given by Nichols et al. (2009b).

Trout livers were perfused using one of 3 experimental designs. In the first set of experiments, livers were perfused for 8 h with solutions containing 10mg/ml BSA and a fixed concentration of test chemical (N = 2 livers perfused with each PAH; 12 livers total). Measured PAH concentrations in the liver perfusates were (μM; mean ± SD) the following: NAP—2.29±0.03; FLU—0.86±0.02; ANTH—0.14±0.04; PHEN—1.06±0.31; PYR—0.24±0.02; BAP—0.12±0.03. In a second set of experiments, livers were perfused initially with solutions containing 10mg/ml BSA (N = 3 livers perfused with each PAH; 18 livers total). After 4 h, the livers were switched to a solution containing 1 mg/ml BSA. Total PAH concentrations were again maintained at a constant level. Measured concentrations were (μM; mean ± SD) the following: NAP—2.29±0.17; FLU—0.81±0.24; ANTH—0.14±0.04; PHEN—1.14±0.16; PYR—0.27±0.03; BAP—0.11±0.01. In a third set of experiments, chemical concentrations in the perfusate were increased in a stepwise manner at 1.5h intervals (4 total concentrations; N = 3 livers perfused with PHEN, PYR, or BAP). These experiments were performed using a solution containing 1 mg/ml BSA, and the 2 intermediate PAH concentrations were designed to bracket measured concentrations from the second set of experiments. Measured total concentrations (μM; mean ± SD) ranged from 0.03±0.004 to 5.24±0.06 for PHEN, 0.016±0.003 to 1.42±0.07 for PYR, and 0.007±0.001 to 0.96±0.04 for BAP.

Assessment of chemical binding. Free concentrations of each PAH in liver perfusates and S9 samples were determined by SPME (Heringa and Hermens, 2003; Vaes et al., 1996). Sampling was conducted using glass fibers (Poly Micro Industries, Phoenix, Arizona; 1–1.5 cm long, 430 μm diameter) coated with poly(dimethylsiloxane) (PDMS; 35 μm thickness). Perfusate samples (6 ml) collected during the course of an experiment were expelled into 7 ml glass scintillation vials. Fibers were added, and the samples were incubated at 11°C using a Stuart roller mixer (Bibby Scientific Ltd., Staffordshire, UK). For S9 binding studies, 50 ml of solution containing 1 mg/ml S9 protein was spiked with test chemical and stirred for 1 h. Subsamples (6 ml) were pipetted into 7 ml glass scintillation vials. A fiber was then added, and the solution was incubated at 11°C on the roller mixer. Fibers were removed after a prescribed time period and extracted with 1 ml of ice-cold acetonitrile. Samples were vortexed for 20 s, refrigerated at 4°C for 1 h, vortexed a second time for 20 s, and centrifuged for 4 min at 3000 × g. A 400 μl subsample of the supernatant was then transferred to a 2-ml GC vial containing a glass micro insert. Sample extracts (water, perfusate, and S9 fractions as well as SPME fiber extracts) were analyzed by HPLC-FLD. Samples were injected using a Beckman Model 507 refrigerated auto sampler onto a Beckman Model 126 HPLC system (Beckman Instruments, Fullerton, California) equipped with a Model 168 diode array detector and Waters Model 2475 multi-wavelength fluorescence detector (Waters, Milford, Pennsylvania). Chromatography was performed using a Hypersil Green PAH, 5 μm, 2.1×100 mm column (Thermo Scientific, Bellefonte, Pennsylvania) at an isocratic flow rate of 0.5 ml/min. Solvent A consisted of 90% deionized water and 10% acetonitrile. Solvent B consisted of 10% deionized water and 90% acetonitrile. A pump ratio of 75% B was most commonly used although minor changes were made to take into account log K$_{ow}$ differences among the analytes. Quantification was performed at the following excitation/emission wavelengths (nm): NAP—280/340; FLU—280/340; ANTH—250/390; PHEN—250/390; PYR—237/385; BAP—255/420. Identification and quantification of all compounds was based on reproducible retention times of standards and samples. Recoveries of PAHs spiked into liver perfusate and S9 fractions were consistently greater than 95% and were not used to correct measured values. The “free” or “unbound” fraction of each chemical in the perfusate (f$_{unb,PAH}$; unitless) or S9 system (f$_{unb,S9}$; unitless) was calculated as the ratio of the free concentration to the total concentration.

Analysis of hepatic clearance data. Total concentrations of test chemicals in perfusate samples afferent (PAH$_{aff}$) and efferent (PAH$_{eff}$) to the liver were used to calculate the rate of substrate disappearance (V$_{PAH}$), hepatic extraction fraction (E$_{PAH}$), and hepatic clearance (CL$_{PAH}$).

\[
V_{PAH} = \frac{(PAH_{aff} - PAH_{eff})}{E_{PAH}} \text{ perfusion rate (2)}
\]

\[
E_{PAH} = \frac{PAH_{aff} - PAH_{eff}}{PAH_{aff}} \text{ perfusion rate (3)}
\]

\[
CL_{PAH} = \frac{V_{PAH}}{PAH_{aff}} \text{ perfusion rate (4)}
\]

Extrapolation of in vitro intrinsic clearance to hepatic clearance by isolated perfused livers. Measured CL$_{IN_VITRO,INT}$ values were extrapolated to the intact liver using a scaling factor (L$_{sc}$) that represents the S9 protein content of liver tissue. The value of L$_{sc}$ (163 mg/g liver) was set equal to that determined previously for trout liver by Nichols et al. (2013) based on measured levels of CYP and glucose-6-phosphatase activity in crude tissue homogenates and S9 fractions. The resulting intrinsic clearance estimate (CL$_{INT}$; ml/h/g) was then used as an input to a well-stirred liver model that accounts for potential rate limitations imposed by the rate of chemical delivery to the tissue (Rowland et al., 1973; Wilkinson and Shand, 1975).
Here the term \( Q_n \) (ml/h/g liver) refers to the measured perfusion rate of individual livers. The binding term \( f_b \) was initially set equal to the ratio of unbound chemical fractions in liver perfusates (\( f_u,\text{PDMS} \)) and in the S9 system (\( f_u,\text{S9} \)) used to measure metabolism (Austin et al., 2002; Grime and Riley, 2006). Additional extrapolations were performed by setting \( f_b = 1.0 \), assuming that chemical concentrations available to metabolizing enzymes in vitro and in the perfused liver are effectively the same.

**Statistical analysis.** Statistical analyses were performed on untransformed CL\( _U \) values. This approach treats each measured clearance as an independent observation and assumes that clearance values (which are normalized for differences in chemical concentration) exhibit homogeneity of variance across different treatments. Clearance values measured in the first set of experiments (constant BSA and chemical concentration) were evaluated using linear regression methods to determine whether there were significant changes over time. The null hypothesis was that clearance does not change (i.e., the slope of the data plotted vs time is zero). In the second set of experiments, each BSA concentration was considered to be a different treatment, and differences in mean clearance were compared using a two-sample t test. Clearance values for individual livers measured during the concentration dependence studies were evaluated using single-factor ANOVA, assuming that each chemical concentration is an independent treatment. The null hypothesis was that there were no differences in clearance among the 4 tested concentrations. Pairwise comparisons among different tested concentrations were performed using the Newman-Keuls multiple range test. All statistical tests were conducted using a significance level of .05.

**RESULTS**

**In Vitro Metabolism by Liver S9 Fractions**

The protein contents of pooled S9 fractions from male and female trout were 24.5 ± 0.6 and 25.8 ± 0.4 mg/ml, respectively. The measured EROD activity for males (7.7 ± 0.4 pmol/min/mg protein) was nearly identical to that for females (7.3 pmol/min/mg protein). Measured levels of GST activity also did not differ between the sexes (479 000 ± 24 000 and 515 000 ± 20 000 pmol/min/mg protein, respectively). The pooled S9 fraction from males exhibited a higher level of AHH activity than that measured for females (50.0 ± 1.4 and 44.1 ± 1.0 pmol/min/mg protein, respectively). For UGT, this pattern was reversed (684 ± 73 pmole/min/mg protein for males and 854 ± 34 pmol/min/mg protein for females). In either case, however, these differences were small (< 25%).

**In vitro** clearance data for the 6 PAHs are reported in Table 1. All compounds exhibited first-order depletion kinetics, and all except NAPH were extensively metabolized within 90 min (> 50% loss of starting substrate). In contrast, NAPH was metabolized at a much slower rate requiring that assays be run for 3 h to achieve an acceptable (approximately 15%) level of depletion. Moreover, denatured samples containing NAPH also exhibited first-order depletion kinetics, presumably due to volatilization. For NAPH, therefore, the metabolism rate constant was determined by subtracting the slope of the fitted line for denatured material from that of active samples. Measured CL\( _{\text{IN VITRO,INT}} \) rates for the 6 compounds varied by 2 orders of magnitude. However, values determined from replicate incubations for each compound exhibited a high degree of precision (CV from 2.8% to 14.3%). Substrate depletion curves for 3 PAHs, selected to illustrate the large differences in clearance rate, are shown in Figure 2.

Measured rates of PAH metabolism by S9 fractions from male and female trout were almost identical. These data were, therefore, combined when extrapolating in vitro clearance values to the intact liver. The rank order of PAH metabolism rate by liver S9 fractions was BAP > PYR >> PHEN > ANTH > FLU > NAPH. When plotted against chemical log \( K_{\text{PDMS}} \), log-transformed CL\( _{\text{IN VITRO,INT}} \) values exhibited a highly significant positive correlation (log CL\( _{\text{IN VITRO,INT}} \) = 0.82 log \( K_{\text{PDMS}} \) − 3.69; \( r^2 = .82 \)).

**PDMS-to-Water (\( K_{\text{PDMS}} \)) Partition Coefficients**

Log-transformed \( K_{\text{PDMS}} \) values for the 6 PAHs increased linearly with chemical log \( K_{\text{OW}} \), and in each case, values determined at the 2 tested concentrations were in good agreement (< 10% difference among means, excepting BAP that was tested at only one concentration). The data were, therefore, combined to develop a single fitted relationship: log \( K_{\text{PDMS}} \) = 0.80 log

**TABLE 1**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Males(^a)</th>
<th>Females(^a)</th>
<th>All(^b)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Substrate Concentration (( \mu )M)</td>
<td>CL( _{\text{IN VITRO,INT}} ) (ml/h/mg protein)</td>
<td>Substrate Concentration (( \mu )M)</td>
</tr>
<tr>
<td>Naphthalene</td>
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<td>Benzo[a]pyrene</td>
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<td>16.33 ± 1.67</td>
<td>0.17 ± 0.01</td>
</tr>
</tbody>
</table>

\( ^a \) Data are given as the mean ± SD of values for 3 independent experiments conducted for each chemical and pooled S9 sample.

\( ^b \) Mean ± SD of all values for both genders (\( N = 6 \)).
$K_{ow} + 0.25$ ($r^2 = .97$). Similar relationships have been given for the same compounds by previous authors (DiFilippo and Eganhouse, 2010).

**Chemical Binding in Liver Perfusates ($f_{u,perf}$) and S9 Fractions ($f_{u,S9}$)**

Measured $K_{PDMS}$ values for each compound were used to calculate free chemical concentrations in liver perfusates and S9 fractions according to equation 1. Free concentrations in S9 fractions decreased with increasing chemical log $K_{ow}$ (Fig. 3). A plot of log $K_{ow}$ versus the log of the bound/free ratio [expressed as $(1 - f_{u,S9})/f_{u,S9}$] yielded the linear relationship: log $(1 - f_{u,S9})/f_{u,S9} = 1.16 \log K_{ow} - 3.83$ ($r^2 = .99$).

Chemical binding in trout liver perfusates also increased with chemical log $K_{ow}$ (Fig. 4); however, correlation coefficients for these fitted relationships were relatively low ($r^2 = .54$ and .49 for the 1 and 10 mg/ml BSA solutions, respectively). Moreover, the proportional difference in binding between each solution varied among chemicals. Thus, free fractions at 1 mg/ml BSA ranged from 5.1 (FLU) to 10.3 (NAPH) times greater than those determined at 10 mg/ml BSA.

**Performance of Isolated Perfused Livers**

Donor fish weights, isolated liver weights, fish gender, and liver perfusion rates are provided elsewhere (Supplementary Table I). This table also gives the pH, PO$_2$, PCO$_2$, and HCO$_3^−$ concentration in perfusate samples afferent to each group of livers. Previous studies of livers perfused in the manner described here showed that physiological performance could be maintained for up to 10 h (Nichols et al., 2009b). In this study, all livers were perfused for 8 h. Physiological performance was preserved throughout this period, as indicated by stable VO$_2$ and vascular resistance and negligible leakage of K$^+$ (data not shown).

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**FIG. 2.** Log$_{10}$-transformed substrate depletion profiles for active (closed circles) and inactive (open circles) rainbow trout liver S9 fractions from representative incubations with pyrene (A), phenanthrene (B), and naphthalene (C). Each data point is the mean $\pm$ SD of duplicate determinations. Solid lines represent linear fits to the data. Metabolism rate constants ($k$) were determined from the slope of each depletion curve. That given for naphthalene takes into account nonmetabolic losses from inactive control samples.

**FIG. 3.** Binding of 6 polycyclic aromatic hydrocarbons in liver S9 fractions. Data are plotted as the log of the bound-to-free ratio [(1 − $f_{u,S9}$)$f_{u,S9}$] versus log $K_{ow}$. Each point represents the mean (± SD) of 6 independent determinations. The best-fit line that describes this relationship is: log $(1 - f_{u,S9})/f_{u,S9} = 1.16 \log K_{ow} - 3.84$ ($r^2 = .99$).
Hepatic Clearance by Isolated Perfused Livers

Isolated livers were initially perfused with solutions containing a fixed (10 mg/ml) concentration of BSA and 1 of 6 PAHs. Representative data for PYR and FLU are shown in Figure 5. Measured clearance values for different livers exposed to the same compound tended to be in good agreement. For 5 of 12 livers examined, hepatic clearance did not change over time (ie, the fitted slope was not significantly different from zero). In 2 cases, clearance decreased over time (significant, negative slope), whereas in 5 instances, clearance actually increased (significant, positive slope). Using measured clearance values at 1 and 8 h, we calculated percentage-wise changes in activity, referenced to the 1 h value. For livers exhibiting a significant decrease in clearance, the mean (± SD) change over the course of an experiment was 24 ± 13%, whereas for livers exhibiting an increase in activity, the mean change was 24 ± 15%. Given the lack of a consistent pattern with respect to changes in hepatic clearance and the modest nature of observed changes, we concluded that there was no need to account for possible time-dependent changes in liver performance during subsequent experimental efforts.

In the second set of experiments, free concentrations of test chemicals in liver perfusates were manipulated by changing the amount of BSA in solution. This change in BSA concentration has a small impact on osmolarity of the perfusate; however, previous studies have shown that the physiological performance of livers perfused at 1 or 10 mg/ml BSA is essentially identical (Nichols et al., 2009b). Changes in chemical binding associated with the change in BSA concentration are expressed in Table 2 as the ratio $f_{\text{U,PERF},10 \text{g/ml BSA}}/f_{\text{U,PERF},1 \text{g/ml BSA}}$. A 10-fold decrease in the amount of BSA added to solution resulted in a 5.7-fold to 10.3-fold increase in $f_{\text{U,PERF}}$, depending on the test chemical.

The effect of changing BSA content on $\text{CL}_{\text{H}}$ was evaluated statistically by examining data for individual livers. Mean $\text{CL}_{\text{H}}$ values for each chemical and BSA treatment were then averaged to characterize fold changes in clearance for each set of 3 livers. A decrease in perfusate BSA content from 10 to 1 mg/ml resulted in a significant increase in $\text{CL}_{\text{H}}$ for all livers exposed to NAPH, FLU, ANTH, PHEN, and BAP. In each case, however, the fold increase in $\text{CL}_{\text{H}}$ was substantially less than the fold increase in $f_{\text{U,PERF}}$ (Figure 6 and Table 2). The largest increase in $\text{CL}_{\text{H}}$ (3.1-fold) was observed for NAPH and was associated with a 10.3-fold increase in $f_{\text{U,PERF}}$. Increases in $\text{CL}_{\text{H}}$ of FLU, ANTH, PHEN, and BAP ranged from 1.2- to 1.6-fold. Surprisingly, the decrease in BSA content resulted in a small but significant decrease in $\text{CL}_{\text{H}}$ of PYR in all 3 livers tested.

One possible explanation for these findings is that metabolism pathways responsible for PAH clearance were close to saturation under both test conditions (1 and 10 mg/ml BSA). If this was true, an increase in $f_{\text{U,PERF}}$ would not be expected to result in increased $\text{CL}_{\text{H}}$. To address this possibility, we conducted concentration dependence studies using PHEN, PYR, and BAP as test compounds. Representative data from this set of experiments are shown in Figure 7. Significant differences

FIG. 4. Binding of 6 polycyclic aromatic hydrocarbons in perfusates used for isolated perfused liver studies. Data are plotted as the log of the bound-to-free ratio $[(1 - f_{\text{U,PERF}})/f_{\text{U,PERF}}]$ versus log $K_{\text{OW}}$. Each point represents the mean (± SD) of 6 independent determinations. Dots—perfusate containing 1 mg/ml BSA. Open circles—perfusate containing 10 mg/ml BSA. The best-fit lines that describe these relationships are (for the 1 and 10 mg/ml BSA solutions, respectively) the following: log $(1 - f_{\text{U,PERF}})/f_{\text{U,PERF}} = 0.32 \log K_{\text{OW}} - 0.12$ ($r^2 = .54$), and log $(1 - f_{\text{U,PERF}})/f_{\text{U,PERF}} = 0.32 \log K_{\text{OW}} + 0.69$ ($r^2 = .49$). Abbreviation: BSA, bovine serum albumin.

FIG. 5. Clearance of PYR and FLU by trout livers perfused with a solution containing 10 mg/ml bovine serum albumin. Linear regressions were used to test the null hypothesis that clearance does not change over time (ie, the slope does not differ significantly from zero). This hypothesis was accepted for 1 liver exposed to PYR (dots) and 1 liver exposed to FLU (solid squares). A significant, positive slope was evident for 1 liver exposed to PYR (open circles). A significant, negative slope was observed for 1 liver exposed to FLU (open squares). Abbreviations: FLU, fluorene; PAH, polycyclic aromatic hydrocarbon; PYR, pyrene.

shown). The pH of perfusate efferent to the liver was consistently lower than that of the afferent solution, and this difference was generally accompanied by a small increase in PCO$_2$. A summary of physiological data for 39 livers evaluated in this study is given elsewhere (Supplementary Table II).
Overall, therefore, although CLH appeared to decline some-
experiment. All summary data are given as the mean ± SD.

<table>
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<th>Chemical</th>
<th>$f_{U,PERF,10 mg/ml BSA}^{a}$</th>
<th>$f_{U,PERF,1 mg/ml BSA}^{a}$</th>
<th>1 mg/ml BSA</th>
<th>10 mg/ml BSA</th>
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<tr>
<td>Fluorene</td>
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<td>41.8 ±10.9 b</td>
<td>26.2 ±9.1</td>
<td>1.6</td>
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<tr>
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<td>66.6 ±13.3 c</td>
<td>48.0 ±7.6</td>
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<td>0.89 ±0.05</td>
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<tr>
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<td>43.4 ±11.7</td>
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<td>0.79 ±0.09</td>
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<tr>
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<td>62.7 ±2.9 e</td>
<td>68.2 ±3.3</td>
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<td>0.85 ±0.18</td>
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<tr>
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<td>50.6 ±4.2</td>
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<td>0.67 ±0.06</td>
<td>0.59 ±0.09</td>
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</table>

Note. *Data are given for livers perfused with solutions containing 10 mg/ml BSA then 1 mg/ml BSA. Total PAH concentrations were held constant for the entire experiment. All summary data are given as the mean ± SD.

*Significantly greater than corresponding values at 10 mg/ml BSA.

*Significantly less than the corresponding value at 10 mg/ml BSA.

*Not significantly different from the corresponding value at 10 mg/ml BSA.

in CL$_{H}$ at the 4 tested chemical concentrations (single-factor ANOVA) were observed in several cases. For PYR and BAP, this outcome was generally driven by reduced CL$_{H}$ at the highest tested concentration. There were no significant differences in CL$_{H}$ of PYR or BAP at the 2 intermediate concentrations, which were designed to bracket those used in earlier studies. For 2 of the 3 livers exposed to PHEN, CL$_{H}$ declined starting with the second highest tested concentration. In each case, this CL$_{H}$ was about 10% lower than that measured at the next lower concentration. For the third liver exposed to PHEN, there were no differences in CL$_{H}$ at the 2 intermediate test concentrations. Overall, therefore, although CL$_{H}$ appeared to decline somewhat at very high PAH concentrations, saturation of metabolic activity probably had little or no impact on the results of earlier studies.

Calculation of the Binding Parameter $f_{U}$

Binding constants ($f_{U}$) used in the well-stirred liver model were calculated as the ratio of measured free fractions in liver perfusates and S9 samples (Table 3). An examination of these ratios shows that $f_{U}$ tends to increase with increasing chemical log $K_{OW}$. This finding is consistent with the observed difference in slope terms for the S9 and liver perfusate binding relationships (Figures 3 and 4).

Extrapolation of In Vitro Intrinsic Clearance to Hepatic Clearance by Isolated Perfused Livers

Measured rates of in vitro activity were extrapolated to the intact tissue, resulting in predicted clearance values (CL$_{H}$,in vitro) of 22.5, 58.0, 80.5, 94.0, 1644.7, and 2718.8 ml/min/g liver for NAPH, FLU, ANTH, PHEN, PYR, and BAP, respectively. These values were then used to calculate CL$_{H}$ based on measured $Q_{H}$ values for each liver and binding terms ($f_{U}$) specific to each chemical and BSA concentration. A second set of CL$_{H}$ predictions was obtained by setting $f_{U}$ equal to 1.0. Finally, predicted CL$_{H}$ values obtained under both assumptions were plotted against measured levels of clearance exhibited by perfused livers at each of the 2 tested BSA concentrations (Fig. 8).

Predicted CL$_{H}$ values calculated using chemical-specific binding terms are shown in Figure 8 as open symbols. At 1 mg/ml BSA (Fig. 8A), the correspondence between predicted and observed values was quite good, resulting in points near the 1:1 line of perfect agreement. The greatest difference between predicted and observed values was obtained for NAPH (predicted/observed = 0.49). In contrast, predicted clearance values for livers perfused with 10 g/l BSA (Fig. 8B) tended to underestimate observed values, resulting in points to the left of the line of 1:1 agreement. Importantly, the extent of this disagreement was inversely related to the rate at which chemicals are metabolized. Thus, predicted values for the 2 most rapidly cleared compounds, BAP and PYR, were in good agreement with observed values. Observed clearances for FLU, ANTH, and PHEN were 2.2–3.0 times higher than predicted values. For NAPH, the observed clearance was 6.9 times greater than the predicted value.

With the exception of NAPH, CL$_{H}$ values predicted by setting $f_{U}$ equal to 1.0 exhibited excellent agreement with observed clearances. These values, which are shown in Figure 8 as solid symbols, suggest a general improvement in model performance relative to that obtained using chemical-specific binding terms, particularly for compounds exhibiting intermediate rates of clearance (FLU, ANTH, and PHEN). For NAPH, setting $f_{U}$ equal to 1.0 resulted in predicted CL$_{H}$ values that substantially (2.1- to 6.4-fold) overestimated observed clearances.
The goal of this study was to evaluate procedures used to extrapolate measured rates of \textit{in vitro} metabolism to the intact liver. Ultimately, it is hoped that these or similar procedures will be used by environmental risk assessors as part of a larger strategy to predict biotransformation impacts on chemical accumulation by fish (Nichols \textit{et al.}, 2006, 2009a). Presently, however, focused studies are needed to address questions that occur when measured levels of chemical accumulation are used to judge the success or failure of \textit{in vitro-in vivo} extrapolation efforts. In this effort, we used an isolated perfused trout liver preparation to directly evaluate hepatic clearance predictions. An important advantage of this approach is that experiments can be designed to address key questions pertaining to underlying model assumptions.

**FIG. 6.** Dependence of hepatic clearance (CL\textsubscript{H}) on free chemical concentration: Representative data for ANTH. Livers were perfused with a fixed total concentration of ANTH. The concentration of bovine serum albumin in the perfusate was changed from 10 (0–4 h) to 1 (4–8 h) mg/ml. A, Total chemical concentrations: solid dots—afferent values; open circles—efferent values. B, Free chemical concentrations measured by solid-phase microextraction: dots—afferent values; open circles—efferent values. C, Plot of CL\textsubscript{H} versus time. The dashed line denotes the liver perfusion rate. Abbreviation: ANTH, anthracene.

**FIG. 7.** Dependence of hepatic clearance (CL\textsubscript{H}) on total chemical concentration: Representative data for BAP. Livers were perfused with a fixed concentration (1 mg/ml) of bovine serum albumin. The total BAP concentration was increased in a stepwise manner at 3.5, 5.0, and 6.5 h. A, Total chemical concentrations: dots—afferent values; open circles—efferent values. B, Plot of CL\textsubscript{H} versus time. The dashed line denotes the liver perfusion rate. Abbreviation: BAP, benzo[a]pyrene.

**DISCUSSION**

The goal of this study was to evaluate procedures used to extrapolate measured rates of \textit{in vitro} metabolism to the intact liver. Ultimately, it is hoped that these or similar procedures will be used by environmental risk assessors as part of a larger strategy to predict biotransformation impacts on chemical accumulation by fish (Nichols \textit{et al.}, 2006, 2009a). Presently, however, focused studies are needed to address questions that occur when measured levels of chemical accumulation are used to judge the success or failure of \textit{in vitro-in vivo} extrapolation efforts. In this effort, we used an isolated perfused trout liver preparation to directly evaluate hepatic clearance predictions. An important advantage of this approach is that experiments can be designed to address key questions pertaining to underlying model assumptions.
Metabolism extrapolation studies with mammals are generally performed using data obtained from liver microsomes, hepatocytes, or reconstituted enzyme systems (Houston, 1994; Houston and Carlile, 1997; Ito et al., 1998). Here we employed liver S9 fractions because of the regulatory need for an assay system that can be applied to a wide range of fish species. Although procedures for isolation of microsomes and hepatocytes have been developed for several fish species, optimization of these methods requires considerable effort. By comparison, S9 fractions are easy to prepare and can be obtained for any species provided that the liver exists as a discrete tissue (some fish possess a diffuse liver that is difficult to sample). Importantly, all biological material used in this study (S9 fractions and perfused livers) was obtained from the same strain of trout. Potential gender-based differences in metabolic activity were minimized by using sexually immature animals, and all experiments were performed at the temperature (11°C) to which fish had been acclimated.

The PAHs employed in this effort represent a well-studied class of environmental contaminants. The primary pathway for PAH metabolism by fish involves hydroxylation of one or more aromatic rings followed by sulfation and glucuronidation of hydroxylated products (Varanasi et al., 1989). These hydroxylation reactions are catalyzed predominantly by CYP1A although other CYP enzymes (eg, CYP3A) may contribute substantially (Schlenk et al., 2008).

In vitro rates of clearance for the 6 tested compounds correlated positively with chemical log $K_{OW}$. Several previous investigators have used a single fish species to investigate the metabolism of multiple PAHs. For example, Jonsson et al. (2004) characterized the metabolism of NAPH, PHEN, and PYR in sheepshead minnows by comparing the amount of hydroxylated metabolites (1-OH and/or 2-OH-PAH) in bile to the amount of parent chemical present in whole fish tissue after 36 days of continuous exposure, followed by 9 days of depuration. The results showed that PYR was metabolized to a much greater extent than PHEN or NAPH. Similar observations have been provided by Spacie et al. (1983) and Varanasi and Gmur (1981) based on research with bluegill sunfish (exposed to BAP and ANTH) and English sole (exposed to BAP and NAPH), respectively. Collectively, these studies suggest that metabolism rates for PAHs in fish increase with chemical hydrophobicity.

The importance of hydrophobicity as a determinant of chemical metabolism by CYP enzymes has been studied extensively in mammals, and quantitative structure-activity relationships (QSARs) incorporating measures of this property (generally log $P$, or equivalently log $K_{OW}$) have been developed to describe enzyme binding and intrinsic clearance, as well as inhibition and induction (Hansch et al., 2004; Lewis, 2003; Long and Walker, 2005). In general, nonspecific binding to proteins correlates strongly with chemical log $K_{OW}$. In order to
be metabolized by a CYP enzyme, however, a substrate molecule must bind within the enzyme’s active site. Properties of the active site, therefore, dictate the types of compounds metabolized by a particular enzyme (substrate specificity) and the extent to which this specificity is determined by chemical hydrophobicity. Enzymes of the CYP1A family in mammals have been shown to exhibit a preference for hydrophobic polycyclic aromatic/heterocyclic substrates. However, binding to the active site is not strongly dependent on chemical log $K_{ow}$ ($\text{Long and Walker, 2005}$). Instead, binding appears to be largely controlled by hydrogen bonding interactions. Similarly, substrate binding to the active site of CYP2C9 and CYP2D6 is largely determined by factors other than chemical hydrophobicity. In contrast to these findings, substrate binding to CYP3A4 correlates strongly with log $K_{ow}$ and results in expulsion of water from the active site.

Metabolism rates measured in this study are not sufficient to develop a high-quality QSAR, relating PAH metabolism in fish to chemical hydrophobicity. With additional work, however, it may be possible to develop such a relationship. Moreover, this information suggests that substrate hydrophobicity controls (at least in part) binding within the active site of trout CYP1A (and possibly CYP3A). Although fish possess many different CYPs ($\text{Schlenk et al., 2008}$), CYP1A is thought to be responsible for metabolism of many environmental contaminants.

Initial studies showed that PAH clearance by isolated livers was maintained at a similar level for 8h. This finding confirms earlier work ($\text{Nichols et al., 2009b}$) and demonstrates the physiological and metabolic stability of the preparation. Subsequent studies were conducted to examine the effect of chemical binding in liver perfusates on measured CLH. For NAPBH, FLU, ANTH, and PHEN, CLH increased with a decrease in perfusate BSA content, but these changes were proportionately less than the increase in unbound chemical concentration. For PYR and BAP, changes in binding had little or no impact on CLH. In principle, these results could have been obtained if metabolic activity was near saturation under all tested conditions. Concentration dependence studies with PHEN, PYR, and BAP showed that this was not the case, at least for these compounds. A more likely explanation, suggested by the high $E_{PAH}$ values shown in Table 2, is that the intrinsic capacity of trout livers to clear PAHs is high relative to liver perfusion rates. More specifically, the well-stirred liver model (equation 5) predicts that chemical binding will have little impact on CLH if the product of $f_u$ and $CL_{IN\text{VITRO,INT}}$ >> $Q_H$ (the perfusion-limited case).

Measured rates of in vitro activity were extrapolated to the perfused liver using information (average tissue weight and measured $Q_H$) specific to each preparation. Additional experiments were performed to calculate a set of chemical-specific binding terms ($f_u$) based on measured free fractions in liver perfusates ($f_{U,\text{PERF}}$) and S9 fractions ($f_{U,S9}$). Based on their measured log $K_{ow}$ values (ranging from 3.30 to 6.13), we expected to see large differences in binding of the 6 test compounds in both liver perfusates and S9 fractions. Measured free concentrations, expressed as the log of the bound/free ratio and regressed against chemical log $K_{ow}$ (Figures 3 and 4), confirmed this outcome. However, correlation coefficients ($r^2$) for these fitted relationships were substantially different (0.99 for S9 fractions and approximately 0.5 for both perfusate solutions). Previously, $\text{Endo and Goss (2011)}$ analyzed albumin binding data for 83 neutral organic compounds. Although binding correlated positively with log $K_{ow}$, the fitted model that described this relationship had a relatively high SD, and it was suggested that molecular size considerations dictate, in part, these interactions. Taken together, the present findings suggest that PAH binding in liver S9 fractions is largely controlled by nonspecific partitioning to lipid and proteins, whereas binding in liver perfusates is influenced by more complex interactions with BSA.

Trout liver S9 fractions rapidly metabolized both BAP and PYR, resulting in predicted CLH values that were essentially equal to the tissue perfusion rate. Observed clearance rates also approached the rate of tissue perfusion with the result that observed and predicted values for both tested conditions (1 and 10mg/ml BSA) were in good agreement. This outcome provides some support for procedures used to extrapolate in vitro metabolism to the intact tissue. It provides little information, however, regarding the accuracy of measured in vitro metabolism rates and $f_u$ values except to confirm that the product of these terms is high relative to $Q_H$.

Compounds that are metabolized at low-to-moderate rates provide a stronger test of metabolism extrapolation procedures, including potential binding effects. For FLU, ANTH, and PHEN, CLH rates predicted at 1 mg/ml BSA were within a factor of 2 of measured values under both modeled assumptions regarding $f_u$ ($f_{U,\text{PERF}}/f_{U,S9}$ or $f_u = 1.0$). For each of these compounds, however, measured levels of binding at 1 mg/ml BSA are similar to values obtained from the S9 system, resulting in $f_u$ values close to 1.0 (0.68–1.92). Measured and predicted CLH rates for FLU, ANTH, and PHEN at 10 mg/ml BSA may provide a better test of chemical binding effects. In this instance, predicted values obtained assuming that $f_u = f_{U,\text{PERF}}/f_{U,S9}$ underestimated observed levels of clearance by a factor of 2–3. Improved agreement between measured and predicted values was obtained when $f_u$ was set equal to 1.0.

NAPBH was the least well metabolized of the 6 compounds examined. Figure 8 shows that clearance values predicted using calculated binding terms substantially underestimated observed clearances. Unlike the other compounds, however, setting $f_u$ equal to 1.0 did not result in improved agreement between predicted and observed values. Instead, adoption of this binding assumption resulted in predicted clearances considerably higher than measured values.

The high levels of $E_{PAH}$ noted for several chemicals suggest that a well-stirred liver model may be inadequate when extrapolating in vitro data to the intact tissue. Of concern is the question whether high rates of metabolism create concentration gradients within the tissue, impacting CLH. This
possibility is accounted for by other models of liver function including the parallel tube (Pang and Rowland, 1977) and dispersion models (Roberts and Rowland, 1985). When intrinsic clearance rates are high, $CL_{int}$ predictions generated by these more complex models diverge from those of the well-stirred model, tending generally to be lower (Houston and Carlile, 1997; Ito and Houston, 2004). Under these conditions, the maximum attainable value of $E_{pph}$ may be substantially less than 1.0. For BAP in particular, the fact that measured $CL_{int}$ rates were consistently lower than predicted values suggests that a more complex liver model may be needed.

With the possible exception of PYR and BAP, however, the use of a more complex liver model would not result in improved agreement between predicted and observed levels of $CL_{int}$ because predicted rates were generally lower than measured values. Instead, current findings for NAPH, FLU, ANTH, and PHEN suggest either that trout S9 fractions exhibit lower intrinsic activity than the intact tissue or that the binding term $f_U$ calculated as $f_{UPH}^{PERF}/f_{U,S9}$, underestimates the availability of hydrophobic substrates to metabolizing enzymes of the liver. For these 4 compounds, the results are consistent with previous reports showing that measured $CL_{int}$ (in vivo, in situ, or by perfused livers) generally exceeds that predicted by in vitro-in vivo extrapolation procedures, which incorporate chemical binding terms (plasma and incubation medium; Grime and Riley, 2006). Utilizing data for over 100 drugs, Halifax et al. (2010) assessed the ability of microsomes and hepatocytes derived from human livers to predict measured levels of in vivo intrinsic clearance. Predictions based on in vitro data were consistently lower than measured in vivo clearance values; for microsomes, the average prediction bias was about 5-fold, whereas that determined for hepatocytes was about 4-fold.

One potential cause of discrepancies between measured and predicted levels of in vivo hepatic clearance is that cells within the intact tissue possess active transporters that move chemicals into cells against their concentration gradient (Lu et al., 2006; Webborn et al., 2007). Procedures used to isolate hepatocytes or prepare subcellular fractions may alter or destroy this activity. This possibility does not, however, explain greater in vivo clearance of compounds that readily diffuse across cell membranes, including the PAHs used in this effort, or chemicals for which facilitated transport is the principal mechanism of cellular uptake. Alternatively, improvements to the S9 assay itself may result in improved agreement between observed and predicted clearance values.

Unfortunately, the results of this study provide little insight into the role of chemical binding as a determinant of hepatic clearance. Although setting $f_U=1.0$ resulted in some improvement in predicted levels of hepatic clearance for several compounds, the extent of underprediction observed under the alternative assumption ($f_U = f_{UPH}/f_{U,S9}$) was comparable to the normal levels of prediction bias noted above. In order to explore binding effects more fully, it would be necessary to study hydrophobic compounds with log $K_{ow}$ values comparable to PYR and BAP but which possess relatively low rates of in vitro clearance (eg, similar to that of NAPH).

The results of this study provide qualified support for procedures currently being used to extrapolate in vitro hepatic metabolism data for fish to the whole animal. Although predicted clearance rates tended to underestimate observed values, these differences were relatively small (less than 8-fold, and generally much closer). From the perspective of supporting bioaccumulation assessments in a regulatory setting, this study suggests that accurate predictions of in vivo hepatic clearance can be obtained for compounds that are rapidly metabolized in vitro, even if they are very hydrophobic (and therefore highly bound), provided that the liver blood flow rate is well known. Assuming that the liver is the primary site of metabolism, this information can be used with confidence to predict metabolism impacts on chemical accumulation. Confidence in predicted levels of in vivo hepatic clearance declines when measured rates of in vitro activity are low due to a consistent bias toward underprediction of in vivo clearance, uncertain binding effects, and the difficulty of making these measurements.

Complicating matters further, in vitro studies cannot begin to address the complexity inherent to any real world setting. As an example, the PAHs evaluated in this effort are generally present in the environment as a complex mixture. Some of these compounds are known inducers of biotransformation enzymes, whereas others may act as inhibitors. Likely difference among fish species and life stages provides additional challenges. Nevertheless, it may be possible to use in vitro methods to bin compounds (ie, “high,” “medium,” and “low”) according to their metabolic stability. This information could be then used during early stages of a chemical assessment to screen for likely impacts on chemical accumulation and as a means of prioritizing limited in vivo testing resources. With experience, it also may be possible to develop empirical corrections to deal with the issue of prediction bias (Hallifax et al., 2010). Experimental procedures developed in this study, including direct measurement of hepatic clearance by perfused livers, provide a strong basis for such efforts.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES


