INTESTINAL BIOAVAILABILITY AND BIOTRANSFORMATION OF 3-HYDROXYBENZO(*A*)PYRENE IN AN ISOLATED PERFUSED PREPARATION FROM CHANNEL CATFISH, *ICTALURUS PUNCTATUS*

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ABSTRACT:

The intestinal bioavailability and biotransformation of 3-hydroxybenzo(*a*)pyrene, a major metabolite of benzo(*a*)pyrene in many animal species, was investigated in an in situ isolated intestinal preparation from the channel catfish, and in vitro with preparations of catfish intestine and blood. 3-Hydroxybenzo(*a*)pyrene was a good substrate for adenosine 3′-phosphate 5′-phosphosulfate (PAPS)-sulfotransferase and UDP-glucuronosyltransferase in cytosol or microsomes prepared from intestinal mucosa. The benzo-(*a*)pyrene-3-glucuronide and 3-sulfate conjugates were only very slowly hydrolyzed by intestinal β-glucuronidase and sulfatase. The K_m values for PAPS-sulfotransferase and UDP-glucuronosyltransferase were 0.4 and 1 μM, respectively, and V_{max} were 1.61 ± 1.08 nmol benzo(*a*)pyrene-3-sulfate/min/mg of cytosolic protein and 1.08 ± 0.54 nmol benzo(*a*)pyrene-3-glucuronide/min/mg of microsomal protein. Hydrolytic enzyme activities were three orders of magnitude slower. In the in situ intestinal preparation, [3H]3-hy-droxybenzo(*a*)pyrene was readily metabolized to the glucuronide and sulfate conjugates. After 1 h of incubation of 2 or 20 μM [3H]3-hydroxybenzo(*a*)pyrene in the in situ preparation, the luminal contents contained 3-hydroxybenzo(*a*)pyrene, benzo(*a*)pyrene-3,6-dione, benzo(*a*)pyrene-3-sulfate, and benzo(*a*)pyrene-3-glucuronide. Mucosal samples contained these components, as well as some unextractable material. The blood contained mainly benzo-(*a*)pyrene-3-sulfate and an as yet unidentified metabolite of 3-hydroxybenzo(*a*)pyrene bound to hemoglobin. Some, but not all, blood samples contained small amounts of 3-hydroxybenzo-(*a*)pyrene, benzo(*a*)pyrene-3-glucuronide, and benzo(*a*)pyrene-3,6-dione. These studies demonstrate the rapid phase 2 conjugation of a phenolic benzo(*a*)pyrene metabolite in intestinal mucosa, and the transfer of the phase 2 sulfate and glucuronide conjugates to blood.

Omnivorous fish living in polluted environments may be exposed to a variety of organic pollutant chemicals. Exposure may be water-borne, from sediments, or through the diet. Lipophilic compounds, such as polycyclic aromatic hydrocarbons (PAH[2]), are more likely to be taken up through the diet, as they associate with particles in aqueous media (Hattemer-Frey and Travis, 1991; Van Veld et al., 1997). Chemicals present in the diet of omnivorous fish include both parent xenobiotic and metabolites produced by the prey organism.

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[2] Abbreviations used are: PAH, polycyclic aromatic hydrocarbons; BaP, benzo(*a*)pyrene; 3-OH-BaP, 3-hydroxybenzo(*a*)pyrene; BaP-3-glucuronide, benzo-(*a*)pyrene-3-β-D-glucopyranosiduronic acid; BaP-3-sulfate, benzo(*a*)pyrene-3-sulfate; BaP-3,6-dione, benzo(*a*)pyrene-3,6-dione; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; SULT, PAPS-sulfotransferase; UGT, UDP-glucuronosyltransferase; BNF, β-naphthoflavone; HPLC, high-performance liquid chromatography; NCl, National Cancer Institute.

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Fish that inhabit waters polluted by PAH are likely to be exposed in the diet to hydroxylated metabolites and conjugates formed in the food item (e.g., an invertebrate, smaller fish, or plant) as well as the parent PAH. As part of a study designed to investigate the bioavailability and biotransformation of dietary PAH and their metabolites, we have assessed the intestinal bioavailability and biotransformation of 3-hydroxybenzo(*a*)pyrene (3-OH-BaP) in control and β-naphthoflavone (BNF)-induced channel catfish.

3-OH-BaP is a major metabolite of benzo(*a*)pyrene (BaP) in many animal species, including rabbits (Ball et al., 1979), rats (Gozukara et al., 1982), catfish (James et al., 1997), flounder (Little et al., 1984), and lobsters (James, 1989). The major pathways of further metabolism of 3-OH-BaP in the catfish, as in other vertebrates including humans, are expected to be glucuronidation and sulfation, catalyzed by one or more of the UDP-glucuronosyltransferases (UGT) and PAPS-sulfotransferases (SULT), respectively (Fig. 1). The form of UGT catalyzing glucuronidation of 3-OH-BaP in humans is unknown, although other hydroxylated BaP derivatives were metabolized by UGT2B7 and UGT1*6 (Jin et al., 1993). In rats, UGT1A7 had high activity with 3-OH-BaP, and this enzyme was expressed in the gastrointestinal tract as well as other tissues (Grove et al., 1997). The major SULT for sulfation of polycyclic aromatic phenols is likely to be P-PST, also termed SULT-1A1 (Coughtrie et al., 1998). UGT and SULT activities have been demonstrated in intestinal mucosa of
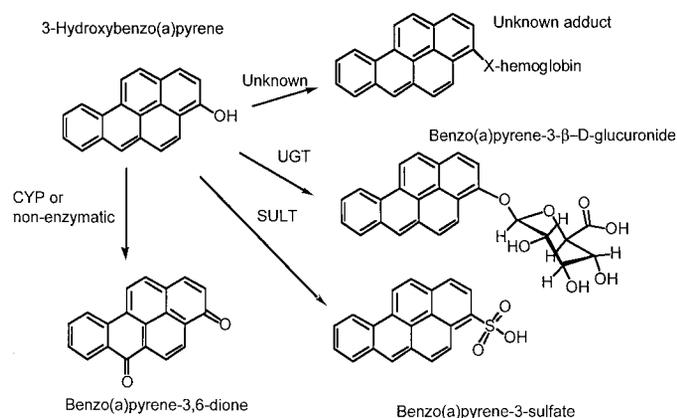
FIG. 1. *Pathways of metabolism of 3-hydroxy-BaP in the channel catfish.*

humans, rats, and catfish (Pacifici et al., 1988; James et al., 1997; Piskula and Terao, 1998). Intestinal bacteria and intestinal mucosa also contain β-glucuronidase and sulfatase activities, which may convert glucuronide and sulfate conjugates formed in the intestine back to parent hydroxylated compound (Massaad et al., 1992). It is also possible that 3-OH-BaP will be converted to BaP-3,6-dione in the intestine. Oxidation of 3-OH-BaP to BaP-3,6-dione may occur non-enzymatically or may be catalyzed by cytochrome P450 or other oxidative enzymes (Glatt and Oesch, 1976; Lind et al., 1978). Another pathway that has been demonstrated for 3-OH-BaP in rodents is conversion to 3-OH-BaP-7,8-dihydrodiol, followed by further metabolism to 3-OH-BaP-7,8-dihydrodiol-9,10-oxide (Owens et al., 1979; Glatt et al., 1987).

In the present study, experiments were conducted in situ with an isolated perfused segment of the intestine to investigate the bioavailability and biotransformation of two low, environmentally relevant concentrations of 3-OH-BaP in living catfish. Other studies were conducted in vitro to determine the properties of microsomal UGT and cytosolic SULT activities with 3-OH-BaP, the properties of BaP-3-sulfatase and BaP-3-glucuronidase in subcellular fractions of intestinal mucosa, and the hemoglobin binding of 3-OH-BaP.

Experimental Procedures

Chemicals. Ring [G^{3}H]-3-OH-BaP, 417 mCi/mmol, 98% radiochemically pure, was purchased from Chemsyn Science Laboratories through the NCI chemical carcinogen repository. On HPLC analysis, this material showed a minor ^{3}H-labeled peak that comigrated with BaP-3,6-dione, but a single fluorescent peak at the retention time of unlabeled 3-OH-BaP. Unlabeled 3-OH-BaP, BaP-3,6-dione, BaP-3-glucuronide, and BaP-3-sulfate were obtained from the Midwest Research Institute through the NCI chemical carcinogen repository. The unlabeled compounds showed single UV (280 nm) and fluorescence (excitation 375 nm, emission 435 nm) peaks by HPLC analysis. Other chemicals and cosubstrates for assays were the purest grade available and were obtained from Fisher Scientific (Fairlawn, NJ) or Sigma (St. Louis, MO).

Fish Source, Maintenance, and Induction. Channel catfish used in these studies were male and female, body weight 600 to 1800 g. Groups of four to six individual catfish were used for each experimental set. They were raised at the Louisiana State University Ben Hur aquaculture farm and transferred as juveniles to tanks in the laboratory equipped with flow-through well water for 3 to 12 months before use. The water temperature was $20.3 \pm 0.6°C$, total hardness was 26.4 ± 5.3 mg of $CaCO_3$/liter and pH was 7.96 ± 0.31 over the maintenance and experimental period. The fish were grown and maintained on a commercial chow diet (Silvercup trout chow, Sterling Farms, UT) fed at 2 to 3% body weight per day. Induced fish were fed chow containing 10 mg of BNF/kg of chow at the 2% body weight per day for 2 weeks before use. The induced fish were not fed (in situ studies) or were fed one meal of control chow (in vitro studies) the last day before use in experiments, to permit removal from

intestine of BNF residues that might inhibit enzyme activity (James et al., 1997).

Preparation of Intestinal Subcellular Fractions, and Blood Thrombocytes. Cytosol and washed microsomes were prepared from intestinal mucosa scraped from the proximal two-thirds or distal one-third of the intestine, as described previously (James et al., 1997). Thrombocytes were prepared from EDTA-treated whole catfish blood by a literature method (Young et al., 1985). Catfish blood, 5 ml, was drawn into a glass tube containing 0.1 ml of 15% disodium EDTA. After mixing, the whole blood was centrifuged at 200g for 10 min at 4°C. The plasma was transferred to a clean tube and centrifuged at 16,300g for 10 min at 4°C. The pellet was resuspended in 2 ml of 0.9% NaCl and re-centrifuged at 16,300g for 10 min. The washed pellet was resuspended in 2.5 ml of buffer containing 0.25 M sucrose, 5 mM EDTA, 0.05 M Tris-Cl, pH 7.4, and 0.2 mM phenylmethylsulfonyl fluoride, homogenized, and used in assays.

Enzyme Assays. All enzyme assays with 3-OH-BaP were conducted under subdued light to retard nonenzymatic breakdown. Microsomal 3-OH-BaP UGT activity was measured by monitoring the fluorescence of BaP-3-glucuronide (Singh and Wiebel, 1979), as described previously (James et al., 1997). Assays were conducted in 0.1 M Tris, pH 7.5, with 5 mM MgCl$_2$, 200 μM UDPGA, 0.1 to 5 μM 3-OH-BaP, and 20 to 50 μg of microsomal protein per tube, final volume 0.5 ml. It was found that addition of Lubrol to microsomes increased activity, with maximal activation at a concentration of 1 to 1.5 mg of Lubrol/mg of microsomal protein. Thus, microsomes were treated with 1 mg of Lubrol/mg of protein before addition to assay tubes. The tubes were incubated for 5 to 10 min at 35°C and then the reaction was stopped by adding 2 ml of ice-cold methanol. The tubes were centrifuged to precipitate protein and 2 ml of the supernatant was added to 0.5 ml of 1 N NaOH. The fluorescence of BaP-3-glucuronide was measured at excitation 300 nm, emission 421 nm, and quantitated against a standard curve with authentic BaP-3-glucuronide.

SULT activity with 3-OH-BaP was measured as follows. Intestinal cytosol, 10 to 20 μg, was incubated with 0.1 to 5 μM 3-OH-BaP, 1 mg of bovine serum albumin, and 20 μM PAPS in 0.1 M Tris, pH 7, final volume 0.5 ml for 5 min at 35°C. In studies of the apparent K_m for PAPS, assays were carried out with 0.1 to 20 μM PAPS and 2 μM 3-OH-BaP. Studies with blood thrombocyte homogenates were as above, but used 100 μg of protein, 1 μM 3-OH-BaP, and 20 μM PAPS. The reaction was stopped by adding 2 ml of ice-cold methanol. The tubes were centrifuged to precipitate protein and 2 ml of the supernatant was added to 0.5 ml of 1 N NaOH. The fluorescence of BaP-3-sulfate was measured at excitation 294 nm, emission 415 nm, and product formation was quantitated against a standard curve with authentic BaP-3-sulfate.

β-Glucuronidase and sulfatase activities were measured in intestinal microsomal and cytosolic fractions with BaP-3- β-D-glucopyranosiduronic acid, or BaP-3-sulfate as respective substrates. Both microsomal and cytosolic fractions were used for both assays. Assay tubes contained 0.5 M Tris-Cl buffer, pH 8.5, 0.5 mg of microsomal or cytosolic protein, 10 μM BaP-3-β-D-glucopyranosiduronic acid, or 10 μM BaP-3-sulfate, and water to 0.5 ml. Tubes were incubated in the dark at 25°C. Some incubations were conducted with nitrogen-flushed solutions in capped tubes, to ensure that the 3-OH-BaP released by hydrolysis was not air-oxidized before measurement. After 60 min (β-glucuronidase) or 120 min (sulfatase), 2 ml of ice-cold methanol was added and tubes were vortex-mixed to stop the reaction. The tubes were placed on ice for 10 min to allow the protein to flocculate, then centrifuged at 2000 rpm for 10 min. The supernatant, 2 ml, was mixed with 0.5 ml of 1 M NaOH and the fluorescence measured at excitation 440 nm, emission 515 nm. The amount of 3-OH-BaP formed was quantitated using a standard curve prepared with authentic 3-OH-BaP.

In Situ Isolated Perfused Intestinal Segment. The perfusion technique was as described by Kleinow et al. (1998). Briefly, fish were anesthetized with tricaine methane sulfonate (MS-222) at induction and maintenance doses of 106 and 86 mg/liter, respectively. Afferent and efferent vessels supplying the prospective intestinal segment were cannulated. Following cannulation and ligation of collateral vessels, the efficiency of perfusion and the length of perfused intestine were determined by blanching. Upon subsequent establishment of steady-state blood flow with donor blood, inflow and outflow were measured to determine the perfusion integrity of the isolated preparation. Once the borders of the perfused intestinal segment were determined and ligatures

placed around the intestine, the preparation was ready for infusion of the 3-OH-BaP.

In Situ Studies. A solution of 2 or 20 μM 3-OH-BaP, 4 ml, was administered into the lumen of the isolated perfused intestinal segment as described previously (Kleinow et al., 1998). The 3-OH-BaP was partitioned into a semipurified micellar solution formulated from 2.5 mM monooleoyl rac glycerol, 10 mM sodium taurocholate, 2.5 mM myristic acid (14:0), 2.5 mM palmitic acid (16:0), 2.5 mM stearic acid (118:0), 2.5 mM linoleic acid (18:2), and 0.9% saline. Blood flow was checked every 10 min, and the isolated segment massaged every 5 min over the 60-min perfusion. Whole blood was collected throughout the perfusion, whereas postinfusate, washed intestinal epithelial scrapings, and perfusion integrity checks (liver, kidney, and anesthetic water) were harvested at the end of the perfusion. Samples for metabolite determination (preinfusate, postinfusate, intestinal epithelial cells, and blood) were placed under nitrogen and frozen ($-20°C$) until analysis. Separate samples of blood, tissue, and postinfusate were digested in 1 N NaOH prior to neutralization and measurement of total radioactivity by liquid scintillation counting.

Extraction of Tissue Samples from in Situ Experiments. The chemical composition of radioactivity in samples from the in situ experiments was determined by solvent extraction and HPLC analysis. Because of the tendency of the [3H]3-OH-BaP to oxidize rapidly on exposure to air, all manipulations were done in a large glove box filled with nitrogen. Samples of the catfish blood or postinfusate were extracted with $5\times$ volume of acetonitrile:methanol (9:10) three times. The intestinal epithelial cell (mucosa) samples were homogenized in 0.2 to 0.5 ml of 0.05 M phosphate buffer, pH 7.4, before extraction as above. Aliquots of the pooled organic extracts and of the remaining aqueous phase were counted for radioactivity. The organic phase extracts were evaporated to dryness under N_2 and stored at $-80°C$ until HPLC analysis. For blood samples that had substantial percentages (25–65%) of unextractable radioactivity in the aqueous phase, further studies were conducted to investigate the nature of the bound radioactivity. Some aqueous phase samples were acidified with HCl to a final concentration of 0.2 to 1 M HCl. The HCl suspension was heated at 80°C for 1 to 3 h, then re-extracted with acetonitrile:methanol as above. Samples of the extract and the neutralized (NaOH) aqueous phase were counted for radioactivity. The organic phase was analyzed by HPLC.

The recovery of 3-OH-BaP from tissue samples was determined by adding 1.2 nmol [3H]3-OH-BaP to 1 ml control catfish blood or 0.5 g of control intestinal mucosa homogenized with 0.5 ml of 0.05 M phosphate buffer, pH 7.4. The spiked samples were extracted under nitrogen as described above. The recovery of BaP-3-glucuronide or BaP-3-sulfate from mucosa was determined by adding unlabeled conjugate, 5 nmol, to 0.5 g of control intestinal mucosa homogenized with 0.5 ml of 0.05 M phosphate buffer, pH 7.4. The spiked samples were extracted as above, and the conjugates followed by fluorescence spectrophotometry.

To verify the identity of the conjugate metabolites of [3H]3-OH-BaP, samples of the dried extracts from mucosa were incubated with 1 unit of β-glucuronidase in 0.5 ml of 0.1 M acetate buffer, pH 5, or with 0.14 unit of sulfatase in 0.5 ml of 0.01 M Tris buffer, pH 7.5, under nitrogen at 35°C overnight. Standards containing BaP-3-glucuronide or BaP-3-sulfate, and control incubations without enzyme were also done under each set of conditions. After incubation, the samples were evaporated to dryness under nitrogen and reconstituted with mobile phase for HPLC analysis.

HPLC Analysis. Samples were analyzed by gradient or isocratic reverse-phase HPLC. The gradient system was as described previously for analysis of benzo(a)pyrene metabolites (James and Little, 1983). The isocratic mobile phase was 70% methanol in 0.005 M aqueous tetrabutylammonium phosphate. The residue of the organic extracts was dissolved in 0.15 ml mobile phase. The solution was filtered through a 0.45-μm nylon centrifuge filter and 50 μl of the filtrate was injected onto the pre-equilibrated 25- $\times$ 4.6-cm C_{18} HPLC column. The column was eluted at 1 ml/min, and peaks were detected by on-line radiochemical (Flo-one beta; Packard, Meriden, CT) and fluorescence (Shimadzu model RF 525, set at excitation 375 nm, emission 435 nm) detection. The retention times of standard compounds are shown in Table 1.

Binding to Hemoglobin. After it was shown that a substantial part of the radioactivity present in blood was not extractable from whole blood, the binding of 3-OH-BaP and BaP-3-glucuronide to hemoglobin was investigated.

TABLE 1

HPLC separation of 3-hydroxy-benzo(a)pyrene and metabolites

Both separations used a Beckman/Altex ultrasphere C_{18} column, 250 $\times$ 4.5 mm with a 50 $\times$ 4.5 mm guard column. Mobile phase A was isocratic, 70% methanol with 0.005 M tetrabutylammonium phosphate at a flow rate of 1 ml/min. Mobile phase B used a flow rate of 0.7 ml/min. The elution started with 55% methanol for 1 min, followed by a linear gradient to 86.5% methanol over 30 min, then a gradient to 100% methanol from 45 to 47 min. The column was eluted with 100% methanol for 23 min, then returned over a linear gradient to the starting condition of 55% methanol. For both systems, standard compounds were identified by in-line UV (280 nm) and fluorescence (excitation 375 nm, emission 435 nm) detection.

Compound	Retention Time	
	Isocratic System A	Gradient System B
	min	
BaP-3-glucuronide	9.0	5.4
BaP-3-sulfate	15.0	8.0
BaP-3,6-dione	22.5	35
3-Hydroxy-BaP	53.0	43
BaP	>85	57

[3H]3-OH-BaP in methanol solution, 2 nmol, was added to incubation tubes, and the methanol removed under nitrogen. A suspension of 100 mg of bovine ferric hemoglobin or human ferrous hemoglobin in 1 ml of 0.05 M potassium phosphate buffer, pH 7.4, was added to the incubation tubes, so that the final concentration of [3H]3-OH-BaP was 2 μM, and the hemoglobin concentration (10 g/dl) was similar to normal catfish blood. Tubes were incubated at 35°C in air or under nitrogen for 30 min then extracted with methanol:acetonitrile (10:9) as described above for blood to remove unbound 3-OH-BaP. A sample of the aqueous phase was taken for quantitation of the bound radioactivity. In some studies, the remaining aqueous phase was evaporated to dryness under nitrogen then suspended in 4 ml of 0.3 M HCl and heated under N_2 at 80°C for 2 h.

Statistical Analysis. Excel software was used to analyze data for statistical significance by Student's t test.

Results

Enzyme Assays. High UGT and SULT activities with 3-OH-BaP were present in intestinal fractions (Table 2). UGT activity was found only in microsomes, and SULT activity only in cytosolic fractions (data not shown). The maximal rates were similar for glucuronidation and sulfation, but apparent K_m values for 3-OH-BaP were lower for sulfation (0.4 μM) than glucuronidation (1 μM). As was found in previous studies (James et al., 1997), neither UGT nor SULT activities in intestinal microsomes or cytosol, respectively, were affected by pretreatment with dietary BNF (data not shown). Catfish blood thrombocyte homogenates were shown to be capable of a very low rate of sulfation of 3-OH-BaP, 4.6 $\pm$ 0.1 pmol of BaP-3-sulfate formed/h/mg of protein (mean $\pm$ S.D., $n = 4$).

Hydrolysis of BaP-3-glucuronide or BaP-3-sulfate conjugates by β-glucuronidase and sulfatase proceeded very slowly in intestinal samples (Table 3). Conducting the incubations under nitrogen did not affect the amount of 3-OH-BaP measured. No clear subcellular fractionation of the hydrolytic activities was found, although proximal intestinal microsomes had more β-glucuronidase activity than did cytosol. The activities reported in Table 3 were measured at pH 8.5, the pH of the in situ intestinal preparation. In studies conducted with 9-OH-BaP conjugates at the pH optima for β-glucuronidase (pH 5) and sulfatase (pH 6.7), hydrolytic activities were 20-fold (glucuronidase) to 1000-fold (sulfatase) slower than the forward conjugation reactions (data not shown).

In Situ Experiments and Analysis of Samples. After the 1-h incubation period, 3-OH-BaP-derived radioactivity was present in blood and washed intestinal mucosa as well as in the solution remaining in the intestinal lumen (postinfusate). The total amount of radioactivity transferred into mucosa and blood related to the concentration

TABLE 2

Properties of intestinal microsomal UGT and cytosolic SULT with 3-OH-BaP

	3-OH-BaP			Cofactor, K_m Apparent
	K_m Apparent	V_{max}	K_m/V_{max}	
	μM	*nmol/min/mg*		μM
UDP-Glucuronosyltransferase	1.02 ± 0.39	1.08 ± 0.54	1.40 ± 1.29	5 ± 1 UDPGA
	(n = 5)			(n = 3)
PAPS-Sulfotransferase	0.40 ± 0.33	1.61 ± 1.08	0.25 ± 0.19	1.1 ± 0.3 PAPS
	(n = 3)			(n = 3)

Values shown are mean ± S.D. for the indicated number of control catfish, *n*.
Assays were conducted at pH 7.5 (UGT) or 7.0 (SULT) under conditions described under *Experimental Procedures*.

TABLE 3

Glucuronidase and sulfatase activities in catfish intestinal microsomes and cytosol

	Rate at pH 8.5, pmol 3-OH-BaP formed/min/mg protein			
Substrate	Microsomes		Cytosol	
	Proximal	Distal	Proximal	Distal
BaP-3-glucuronide	2.0 ± 0.3	0.42 ± 0.21	0.07 ± 0.03	0.20 ± 0.09
BaP-3-sulfate	0.44 ± 0.16	0.17 ± 0.08	0.87 ± 0.05	0.37 ± 0.26

Values shown are mean ± S.D., n = 4. The rates were obtained at pH 8.5, the pH of the buffer used in the in situ studies, in the presence of 10 μM substrate.

infused and was approximately 10 times higher in fish incubated with 20 μM 3-OH-BaP than 2 μM 3-OH-BaP (Figs. 2 and 4, top left panels). BNF pretreatment did not significantly affect the total concentration of radioactivity found in blood, mucosa, or postinfusate at the two concentrations of 3-OH-BaP infused (Figs. 2 through 4, top left panels). Minimal amounts of radioactivity were found in the liver or kidney of the catfish, or in the anesthetic water, indicating that the isolated intestinal preparation maintained its integrity throughout the perfusion (data not shown).

Studies of the extraction efficiency, and stability of the 3-OH-BaP under the conditions of analysis were conducted with the infusate solution (i.e., the micellar solution in which the 3-OH-BaP was solubilized), mucosa and blood samples spiked with [3H]3-OH-BaP. The solvent extraction conditions used were highly efficient for the infusate solution (99.8 ± 0.1% extracted) and the mucosa (99.2 ± 0.2% extracted). With spiked blood, however, the extraction efficiency fell to 81.9 ± 0.3%. HPLC analysis of extracts of the infusate solution and spiked mucosa showed that 98.1% of the extracted radioactivity was 3-OH-BaP. Extracts of spiked blood contained <2% 3-OH-BaP and most of the [3H] was in the form of BaP-3,6-dione. BaP-3-glucuronide and BaP-3-sulfate were efficiently extracted from mucosa by acetonitrile:methanol, 9:10, with 99.8 ± 0.3% of each conjugate recovered in the solvent. The efficiency of extraction from blood was not determined, but previous studies with [3H]BaP-9-glucuronide and [3H]BaP-9-sulfate showed that >98% of the conjugates were extracted from spiked blood samples (James et al., 1996).

Although the amount of radioactivity taken up into mucosa was dose-dependent, there was no induction effect on the uptake of 3-OH-BaP into mucosa (Fig. 2). The radioactivity in the mucosa of the experimental fish was readily extracted (98 ± 1%) into the acetonitrile:methanol mixture, and analysis of the samples showed the 3-OH-BaP that was taken up by the mucosa was extensively conjugated with sulfate and glucuronide. The identity of each of the conjugate peaks was confirmed by incubations with hydrolytic enzymes, which converted the appropriate conjugate to 3-OH-BaP (data not shown). In all but three of the 21 individual fish studied in all treatment groups, more BaP-3-sulfate than BaP-3-glucuronide was present in mucosa after the 1-h incubation period. Although sulfation predominated in most fish, there was considerable interanimal variability in the extent of conju-
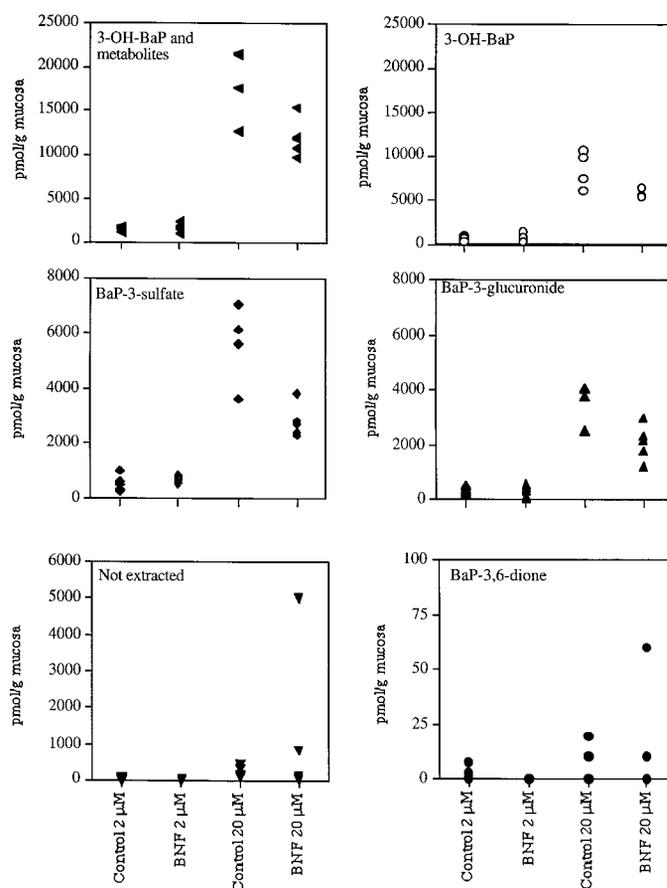

FIG. 2. *Concentrations of 3-OH-BaP and metabolites in mucosa from isolated intestinal segments of individual catfish in each of four groups 1 h after introduction of a micellar solution of [3H]3-OH-BaP into the lumen.*

The top left panel shows total molar equivalents calculated from the radioactivity present in mucosa. Other panels show the concentrations of each individual metabolite. The groups were control or BNF-induced channel catfish infused with 2 μM [3H]3-OH-BaP and control or BNF-induced catfish infused with 20 μM 3-OH-BaP.
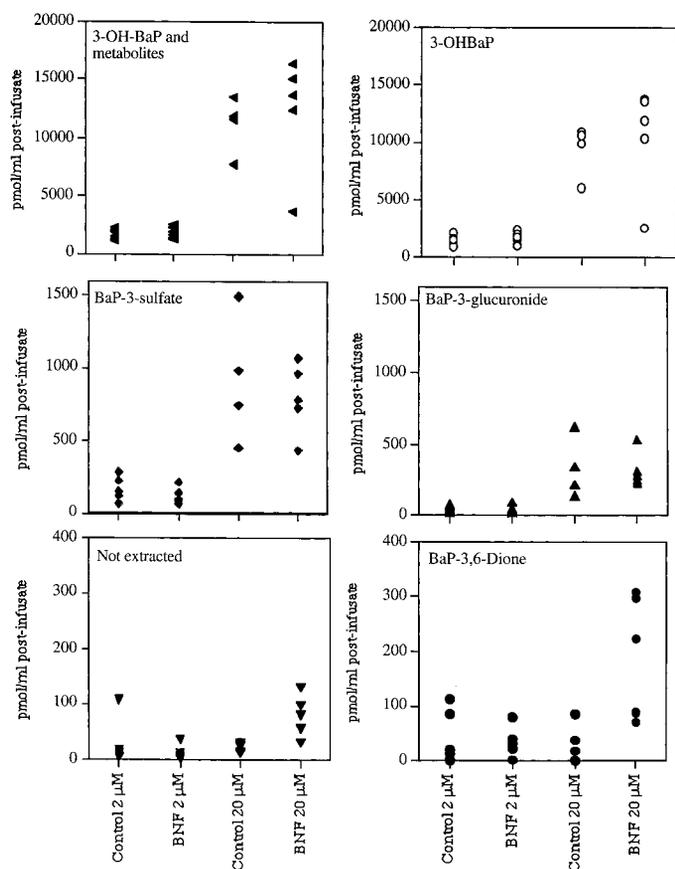
FIG. 3. *Concentrations of [³H]3-OH-BaP and metabolites in the fluid "postinfusate" present in the isolated intestinal segments of individual catfish at the end of the 1-h in situ incubation of [³H]3-OH-BaP.*

The top left panel shows total molar equivalents calculated from the radioactivity present in the postinfusate. Groups are as described for Fig. 2.
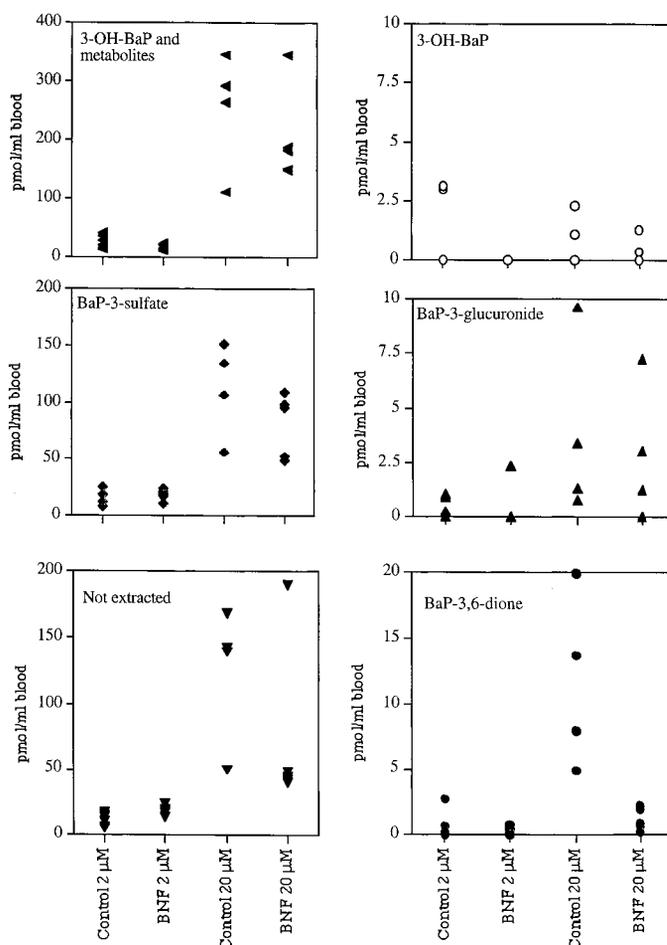

FIG. 4. *Concentrations of [³H]3-OH-BaP and metabolites in blood of individual catfish collected over a 1-h in situ incubation.*

The top left panel shows total molar equivalents calculated from the radioactivity present in blood. Groups are as described for Fig. 2.

gation by either pathway, so that in any one treatment group, the mean concentration of BaP-3-sulfate in mucosa was not significantly higher than the mean concentration of BaP-3-glucuronide (Fig. 2, middle panels). BaP-3,6-dione was found in only eight of the 20 mucosa samples analyzed (see Fig. 2). Mucosa from fish incubated with 20 μM 3-OH-BaP, and two of the fish incubated with 2 μM 3-OH-BaP, contained small amounts (<1–240 pmol/g) of an unidentified metabolite. This metabolite eluted from HPLC at 65 min under the conditions shown in Table 1, i.e., between 3-OH-BaP and BaP. The radioactivity that was not extracted into acetonitrile:methanol was not further characterized.

Metabolites of 3-OH-BaP were present in the postinfusate. Greater than 99% of the radioactivity in postinfusate was extracted into acetonitrile:methanol, and HPLC analysis showed there was almost three times as much sulfate as glucuronide in postinfusate in all treatment groups (Fig. 3, middle panels). Since the amount extracted was similar to that extracted from 3-OH-BaP-spiked samples, the nature of the nonextracted radioactivity was not investigated. In each individual fish, the extracts of postinfusate samples contained more sulfate than glucuronide, in one case over 20-fold higher. Postinfusate extracts from some fish contained small amounts of BaP-3,6-dione. The unidentified metabolite that eluted at 65 min was found in all postinfusate samples from fish incubated with 20 μM 3-OH-BaP, and from four of the fish incubated with 2 μM 3-OH-BaP. In fish incubated with 20 μM 3-OH-BaP, this unknown accounted for 319 ± 214 and 441 ± 243 pmol of 3-OH-BaP/ml of postinfusate (mean ± S.D., *n* = 4) in controls and BNF-treated fish, respectively.

For blood samples, only 57.8 ± 11.7% of the radioactivity was extracted into the acetonitrile:methanol. All of the extracts of catfish blood contained BaP-3-sulfate, which was the major metabolite in blood (Fig. 4). Significantly smaller (*p* < 0.001), and variable amounts of BaP-3-glucuronide, BaP-3,6-dione, and 3-OH-BaP were found in blood extracts (Fig. 4). Nine of the 21 blood sample extracts contained no detectable BaP-3-glucuronide, <0.02 pmol/ml. Eight of these blood samples were from fish infused with 2 μM 3-OH-BaP. Unchanged 3-OH-BaP was detected in 6 of the 21 blood samples. BaP-3,6-dione was detected in 14 of the 21 blood samples, including all of the high dose (20 μM) catfish. Ten of the 21 blood extracts analyzed contained the unidentified metabolite that eluted from HPLC at 65 min, and found also in mucosa and postinfusate.

In analyzing the data to examine movement of the major metabolites, BaP-3-sulfate and BaP-3-glucuronide, from the site of formation in mucosa, conjugate gradients from mucosa to blood, and mucosa to postinfusate were calculated for each individual fish. In blood samples with no detectable BaP-3-glucuronide conjugate, values for the BaP-3-glucuronide concentration were arbitrarily set at half the detection limit, i.e., 0.01 pmol/ml, rather than zero, so that these samples could be included in the gradient calculations. The calculated conjugate gradients, grouped by treatment, are shown in Table 4. BaP-3-sulfate and BaP-3-glucuronide concentration gradients were established in a bipolar fashion from the mucosa to the postinfusate and from the mucosa to the blood at both the 2 and 20 μM 3-OH-BaP concentra-

TABLE 4

Gradients of BaP-3-sulfate, BaP-3-glucuronide, and total 3-OH-BaP equivalents from mucosa to blood and mucosa to postinfusate following perfusion/incubation of catfish in situ intestinal preparations with micelles containing either 2 or 20 μM [^{3}H]3-OH-BaP for 60 min

Catfish were either controls or treated with β-naphthoflavone (10 mg/kg diet) for 14 days.

Treatment, Concentration of 3-OH-BaP	Mucosa to Blood Gradients[a]			Mucosa to Post-infusate Gradients		
	Total 3-OH-BaP Equivalents[b]	BaP-3-Sulfate	BaP-3-Glucuronide	Total 3-OH-BaP Equivalents	BaP-3-Sulfate	BaP-3-Glucuronide
Control, 2 μM	61.7 ± 11.5	36.4 ± 4.5	17,300 ± 7,900	0.95 ± 0.13	3.6 ± 0.3	8.8 ± 3.0
BNF, 2 μM	44.3 ± 7.3	37.9 ± 5.9	24,200 ± 8,600	0.93 ± 0.10	7.0 ± 1.3	8.8 ± 2.3
Control, 20 μM	78.5 ± 12.1	53.0 ± 7.0	1,944 ± 679	1.76 ± 0.39	6.9 ± 1.2	14.7 ± 5.1
BNF, 20 μM	64.3 ± 9.3	37.6 ± 5.6	18,600 ± 10,900	1.32 ± 0.50	3.8 ± 0.6	7.5 ± 1.6

[a] The gradients shown are the ratio of metabolite concentration in mucosa to that in blood or postinfusate. (For example, a mucosa to blood gradient of 10 represents a mucosa metabolite concentration 10 times that in blood). Gradient concentrations were calculated for each individual fish and presented as mean ± S.E. ($n = 6$ for 2 μM control and BNF; $n = 4$ for 20 μM control, and $n = 5$ for 20 μM BNF). Note that in the groups infused with 2 μM 3-OH-BaP, three control and five BNF-induced fish had no detectable BaP-3-glucuronide (concentration set at half the limit of detection for purposes of calculations). In the groups infused with 20 μM 3-OH-BaP, one BNF-treated fish had undetectable BaP-3-glucuronide. Mucosa to blood gradients were significantly steeper ($p < 0.01$) than mucosa to postinfusate gradients for the glucuronide and sulfate conjugates and total 3-OH-BaP molar equivalents.
[b] The total 3-OH-BaP equivalents were calculated from the total [^{3}H]3-OH-BaP molar equivalents per gram of mucosa or per milliliter of blood or postinfusate.

tions. Mean BaP-3-sulfate concentrations in postinfusate were 3.6- to 7-fold lower than mucosa and BaP-3-glucuronide was 7.5- to 14.7-fold lower. Mean BaP-3-sulfate in blood was 36.3- to 53-fold lower than mucosa, whereas BaP-3-glucuronide concentrations were 1,944- to 24,200-fold lower (Table 4). More BaP-3-sulfate was produced and/or retained in the mucosa than the corresponding glucuronide for all treatments. The relative concentrations of the two major metabolites differed in each of the three compartments. BaP-3-sulfate concentrations were 0.7- to 3.3-fold greater than the glucuronide in the mucosa, 1.4- to 5.4-fold greater in the postinfusate, and a minimum of 10-fold greater in the blood.

The nature of the radioactivity that was not extracted from blood by acetonitrile:methanol was further investigated. Some of this radioactivity (about 25%) could be released from the blood residue by incubation in 0.2 N HCl at 80°C for 3 h, followed by extraction with acetonitrile:methanol, 9:10. HPLC of the released radioactivity showed that the major component (84 ± 9.8%) eluted with the same retention time as BaP and was fluorescent. In some samples, this was the only radioactive peak released from the bound material, and in others peaks that comigrated with BaP-3,6-dione or more polar products were observed. Although the major metabolite hydrolyzed from blood comigrated with BaP, its identity was not verified by other means, as there was insufficient material in the extracts for determination of its molecular structure. Incubation of 10 g/dl bovine ferric hemoglobin or human ferrous hemoglobin with [^{3}H]3-OH-BaP also resulted in bound radioactivity, which could be partially hydrolyzed by HCl to products similar to those released from catfish blood. The rate of binding of 2 μM 3-OH-BaP to bovine ferric hemoglobin was linear for 30 min, and reached a plateau thereafter (Fig. 5). Varying the concentration of 3-OH-BaP between 0.03 and 0.4 μM showed that formation of the bound product to ferric or ferrous hemoglobin was dose-dependent (Fig. 6). Formation of the hemoglobin adduct did not require an oxygen atmosphere, as similar rates of formation were observed when the incubation was conducted under nitrogen (data not shown). Although the bound products were not fully characterized in incubations with catfish blood, ferric, or ferrous hemoglobin, the unbound radioactivity was shown to differ between these samples. Incubation of 3-OH-BaP with catfish blood or ferrous hemoglobin gave only BaP-3,6-dione in the acetonitrile:methanol extract, whereas incubation with ferric hemoglobin gave over 85% unchanged 3-OH-BaP and less than 15% BaP-3,6-dione.

Discussion

The in vitro studies showed that catfish intestinal mucosa contained SULT and UGT activities, with apparent K_m values for 3-OH-BaP in
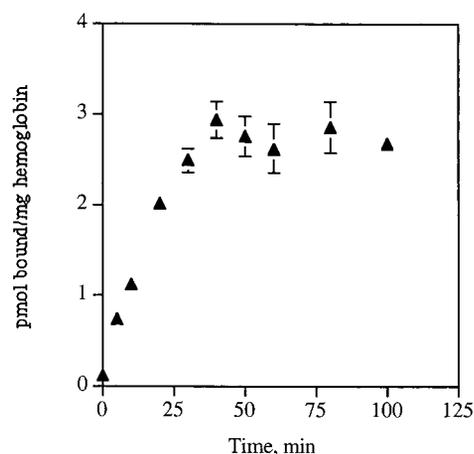

FIG. 5. *Time course of binding of [^{3}H]3-OH-BaP to hemoglobin.*

A 10-g/dl solution of bovine ferric hemoglobin in 0.05 M potassium phosphate buffer, pH 7.4, was incubated with 2 μM [^{3}H]3-OH-BaP, and samples were removed at the times indicated. Unbound material was extracted into acetonitrile:methanol and the amount bound calculated by scintillation counting of bleached samples of the residue.

the micromolar range. The apparent K_m values for the cosubstrates, PAPS and UDP-glucuronic acid, were low and within the concentrations likely to be found in cells (Mulder, 1990). This indicated the potential for efficient conjugation of low dietary concentrations of 3-OH-BaP. Sulfatase and β-glucuronidase activities were much lower than rates of the forward reactions at the pH of the in situ preparation and at the optimal pH for each enzyme, suggesting that once formed, the conjugates would be stable in the intestinal cell. Although catfish treated with BNF, 10 mg/kg diet for 2 weeks before use had higher intestinal CYP1A-dependent activities, UGT activity with 3-OH-BaP was not induced under this treatment regimen (James et al., 1997). SULT activity was unaffected by BNF treatment. It was shown in previous studies that BNF was inhibitory to both UGT and SULT, with IC$_{50}$ values for both pathways of about 50 μM (James et al., 1997). The induction protocol followed in this study included a 1-day period of no BNF treatment prior to use in the in situ study, to avoid the potential for inhibitory BNF residues in mucosa, and indeed none was observed.

In an in situ preparation of perfused catfish intestine, 3-OH-BaP was extensively conjugated to BaP-3-sulfate and BaP-3-glucuronide over the course of 1 h. There was some oxidation to BaP-3,6-dione. There was apparently no conversion of 3-OH-BaP to 3-OH-BaP-7,8-dihydrodiol even in induced fish, although others have reported fur-
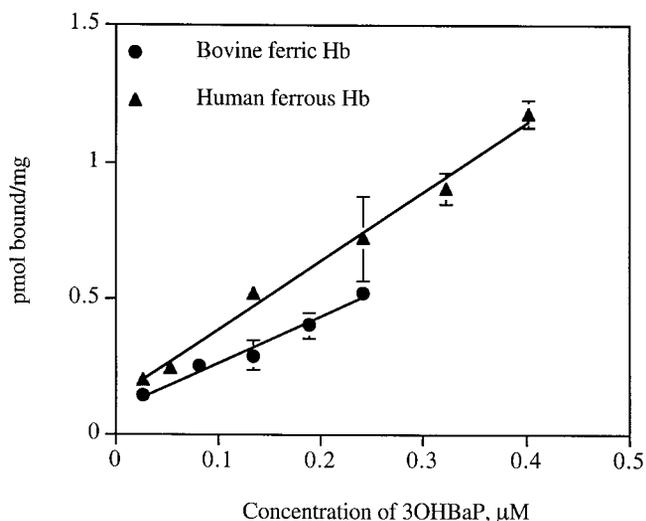
FIG. 6. *Concentration dependence of binding of [³H]3-OH-BaP to bovine ferric hemoglobin and human ferrous hemoglobin.*

Incubations with 10 g/dl hemoglobin in 0.05 M potassium phosphate buffer, pH 7.4, were conducted for 30 min with varying concentrations of [³H]3-OH-BaP as shown. Following extraction of unbound material with acetonitrile:methanol the amount bound was determined by scintillation counting.

ther oxidation of 3-OH-BaP by CYP1A as an activation pathway (Ribeiro et al., 1985; Glatt et al., 1987). This may be because conjugation was more favorable than oxidation by CYP1A in the catfish intestine. Under the conditions used (initial concentration of 2 or 20 μM [³H]3-OH-BaP in the lumen), more BaP-3-sulfate was formed in mucosa than BaP-3-glucuronide in most of the fish studied. A possible explanation was that 3-OH-BaP, on entering the enterocyte, encountered the cytoplasmic SULT enzyme before reaching the inner membrane bound UGT enzyme. SULT had a somewhat lower K_m than UGT for 3-OH-BaP, and although the difference was not marked, the K_m difference may have influenced the major conjugation pathway taken. Examination of K_m/V_{max} for control fish (Table 2) showed that sulfation was generally the favored pathway, but there were large interindividual variations. Once formed, BaP-3-sulfate appeared to be transported out of the cell more efficiently than BaP-3-glucuronide. At the 1-h termination of the perfusion, considerably more BaP-3-sulfate than glucuronide was found in the lumen (postinfusate fluid) and in blood. Gradient differences were consistently larger between the mucosa and the blood rather than at the postinfusate/mucosa interface suggesting that an inherent dichotomy existed between the two pathways of metabolite movement. Likewise, the relative gradients established for the two metabolites changed between the two pathways. The mucosa/blood gradient difference was 36.6- to 638-fold larger for BaP-3-glucuronide than that for BaP-3-sulfate suggesting that BaP-3-sulfate transport into the blood was either selectively less restricted than BaP-3-glucuronide or some feature influencing the disposition of BaP-3-glucuronide was operative. Differences in metabolite concentration in the blood were clearly disproportionate to the concentration differences evident in the mucosa. These trends were much less evident on the luminal side.

Previous studies in our laboratories have examined the movement of presynthesized BaP conjugates (BaP-9-sulfate; BaP-9-glucuronide) from the intestinal lumen through the intestinal mucosa to the "systemic" circulation in the identical catfish in situ preparation (James et al., 1996). Both BaP-9-sulfate and BaP-9-glucuronide were able to transverse from the lumen to the systemic circulation such that the highest concentrations were observed in the postinfusate, then the mucosa followed by the blood. In large measure, the material reaching

the systemic circulation following dosing with BaP-9-sulfate or BaP-9-glucuronide was the administered conjugate. Germane here is the fact that mucosal and blood BaP-9-sulfate concentrations were nearly equivalent to those of BaP-9-glucuronide when both compounds were independently administered into the lumen at equimolar concentrations. Dramatic differences, as noted in the current study, between BaP-3-sulfate and BaP-3-glucuronide were not evident with BaP-9-sulfate or BaP-9-glucuronide at the blood mucosa interface. The low blood concentrations of BaP-3-glucuronide could not be explained by differences in the physical-chemical properties of the positional isomers of the hydroxylated BaP conjugates. The estimated log P of 3-OH-BaP was 5.3, of the 3- and 9-sulfate conjugates approximately 3.9 and of the glucuronide conjugates 3.0 (Ghose and Crippen, 1986, 1987). If transport from the mucosa to the blood had been influenced solely by lipophilicity, we would have expected similar concentrations of BaP-3-sulfate and BaP-3-glucuronide in blood, based on the findings with preformed BaP-9-sulfate and BaP-9-glucuronide.

Several significant differences between these studies may have contributed to these findings and may lend insight into the operative processes. Foremost, the feature that stood out is the amount of compound-derived radioactivity that was unextractable from the blood in each of the three studies. Following luminal administration of BaP-9-sulfate, 5.7 to 13.2% of total radioactivity was unextractable from the blood, 5.1 to 10.2% was unextractable for BaP-9-glucuronide, and between 22 and 61% for 3-OH-BaP in the current study. Although the composition of the bound product was not determined, evidence here and elsewhere suggested that both 3-OH-BaP and BaP-3-glucuronide were reactive with components of the blood and formed bound unextractable products (N. Sugihara and M. O. James, manuscript in preparation). The reaction of BaP-3-glucuronide with blood to form these bound products potentially lowered the detectable BaP-3-glucuronide levels in the blood and subsequently changed the applicable and observed gradients. If the relative proportions of BaP-3-sulfate/BaP-3-glucuronide in the mucosa or postinfusate were extrapolated to the blood using BaP-3-sulfate blood concentrations as a point of reference, it could be inferred that 34 to 75% of the total bound radioactivity was related to the BaP-3-glucuronide conjugate depending on the treatment. It was shown that during the enzymatic hydrolysis of BaP-3-glucuronide by glucuronidase, a BaP derivative was formed that bound to DNA to a far greater extent than either the 3-OH-BaP or its glucuronide (Kinoshita and Gelboin, 1978). A similar process may have operated in forming hemoglobin adducts. Although such a comparison is interesting, the applicability is speculative at this time given the unknown composition of the bound product.

Other plausible mechanisms may have contributed to the differing bipolar gradients established at the apical (lumen- postinfusate) and basolateral (blood) surfaces of the intestinal mucosa. The first of these may be related to the relative ease of access on the apical side and the relative distance and complexity on the basolateral side. The brush-border membrane and unstirred water layer were the primary barriers to diffusion on the apical side, whereas elements of the basolateral membrane, lamina propria, and vascular endothelium were operative on the basolateral side. In this case, these differences would be further exaggerated by the greater functional surface area on the intestine luminal surface (apical mucosa surface), which would permit better bidirectional diffusion back into the intestinal lumen. Similarities in the relative movement of BaP-3-sulfate and BaP-3-glucuronide across the apical surface and contrasting differences across the basolateral surface also suggest that regional differences in these barriers exist. In support of this concept are studies with isolated intestinal brush-border and basal membranes of fish and mammals that have demonstrated regionality in composition as related to membrane lipids,

membrane proteins, cholesterol, and the presence and density of transporters (Pind and Kuksis 1986; Simons and van Meer, 1988; Crockett and Hazel, 1995; Schulthess and Hauser, 1995; Kleinow et al., 2000). Although physiochemical investigations have indicated that differences in lipid and cholesterol composition may influence membrane fluidity in the fish intestine (Crockett and Hazel, 1995), no information is currently available for fish relating these features to xenobiotic movement.

The unchanged 3-OH-BaP found in postinfusate probably represented a fraction that was not taken up into mucosa, rather than material produced by hydrolysis of BaP-3-sulfate or BaP-3-glucuronide. Very little hydrolytic activity was found in mucosal microsomes and cytosol for either conjugate. Both conjugates were stable in postinfusate fluid. In other organ systems, such as aortic smooth muscle, BaP-3-sulfate was readily formed but rapidly hydrolyzed by sulfatase (Yang et al., 1986a,b); however, the in vitro finding of low hydrolytic activity in the catfish intestine suggests the conjugates are relatively stable in this system. BaP-3,6-dione concentrations in postinfusate were lower than the conjugates in all samples except the group treated with BNF and infused with 20 μM 3-OH-BaP. Although low, BaP-3,6-dione concentrations in postinfusate were higher than in mucosa, suggesting that the major source of the BaP-3,6-dione was nonenzymatic breakdown of 3-OH-BaP in the postinfusate, rather than transfer from mucosa.

In blood, a substantial fraction (22–61%) of the radioactivity present could not be extracted by acetonitrile:methanol and appeared to be covalently bound to a blood protein. Acid hydrolysis liberated about 25% of the nonextractable radioactivity into an extractable form. Incubation of hemoglobin or methemoglobin with 3-OH-BaP resulted in formation of a bound form suggesting the unextractable material was bound to the hemoglobin molecule. There was little difference in the covalent binding of 3-OH-BaP to the reduced or oxidized forms of hemoglobin (Fig. 6); however, the unbound material differed, with substantially more conversion of 3-OH-BaP to BaP-3,6-dione in the presence of whole blood or ferrous hemoglobin. This was consistent with the better oxygen-carrying capacity of the reduced form of hemoglobin, and iron-catalyzed oxidation of the 3-OH-BaP. The nature of the material bound to hemoglobin is under investigation.

In summary, 3-OH-BaP was readily conjugated with sulfate and glucuronide in catfish intestine. There was no evidence for further oxidative metabolism to 3-OH-BaP-7,8-dihydrodiol. Both of the conjugates, but preferentially BaP-3-sulfate, were secreted into the intestinal contents. BaP-3-sulfate was the predominant metabolite in blood and very little unchanged 3-OH-BaP or BaP-3-glucuronide was found in blood. It was not clear if this was due to preferential transport of the sulfate conjugate from mucosa into blood, or to a combination of relatively reduced transport of 3-OH-BaP and BaP-3-glucuronide and the propensity of 3-OH-BaP and BaP-3-glucuronide to react with hemoglobin and perhaps other blood proteins. Although conjugation is often thought to prevent systemic uptake, this was not the case for the relatively lipophilic BaP-3-sulfate.

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