Tissue Distribution and Metabolism of Benzo[a]pyrene in Embryonic and Larval Medaka (Oryzias latipes)

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Received January 19, 2007; accepted August 31, 2007

The need to understand chemical uptake, distribution, and metabolism in embryonic and larval fish derives from the fact that these early life stages often exhibit greater sensitivity to xenobiotic compounds than do adult animals. In this study, a 6-h acute waterborne exposure immediately after fertilization was used to quickly load the egg with benzo[a]pyrene (BaP). This exposure was used to mimic the initial egg concentration of a persistent bioaccumulative toxicant that could result from maternal transfer. We used multiphoton laser scanning microscopy (MPLSM) in combination with conventional analytical chemistry methods to characterize the tissue distribution of BaP and its principal metabolites in medaka embryos and post-hatch larvae. Embryonic metabolism of BaP was evident by MPLSM prior to liver formation or heart development. A major product of this metabolism was identified by liquid chromatography/mass spectrometry as BaP-3-glucuronide. MPLSM showed that metabolites were sequestered within the yolk, biliary system, and gastrointestinal tract. When the gastrointestinal tract became patent a few days after hatch, the metabolites were rapidly eliminated. These findings indicate that some of the earliest embryonic tissues are metabolically competent and that redistribution of BaP and its metabolites occurs throughout development. Rapid metabolism of BaP substantially reduces the body burden of parent chemical in the developing embryo, potentially reducing toxicity. It remains unclear whether metabolism of BaP in medaka embryos leads to the formation of DNA adducts associated with genotoxic effects or yields metabolites that later lead to other toxicity in juveniles or adults.

Key Words: PAH; BaP; fish; metabolism; embryo.

The toxicity of xenobiotics to fish is determined in part by their uptake, tissue distribution, and elimination. Understanding these processes in embryonic and larval fish is particularly important because of their demonstrated sensitivity to many compounds, relative to that of mature animals. Fish eggs are exposed to xenobiotics via maternal transfer and direct uptake from the environment (water, sediments) into which they are deposited. Although the uptake and accumulation of some xenobiotics in fish eggs has been well quantified, little is known about chemical distribution within the egg, including changes during development. Redistribution of a chemical and its metabolites is likely to occur as an embryo utilizes yolk proteins and lipids for energy and growth. This redistribution may be facilitated by development of the cardiovascular system. Lacking direct observations, however, we have thus far only been able to speculate on the extent and timing of these events.

Biotransformation may further impact the toxicity of chemicals in fish early life stages. Morphological and physiological changes associated with normal development are accompanied by the constitutive production of xenobiotic metabolizing enzymes (Binder and Stegeman, 1983, 1984; Guiney et al., 1997; Wang Buhler et al., 1997). Cytochrome P4501A (CYP1A) activity is inducible at the time of liver formation in embryos of killifish (Fundulus heteroclitus) and medaka (Oryzias latipes) (Binder and Stegeman, 1984; Wisk and Cooper, 1992). Expression of the aryl hydrocarbon receptor (AhR) and aryl hydrocarbon receptor nuclear translocator protein (ARNT), two proteins necessary for CYP1A expression, is detectable in killifish embryos at the onset of blood circulation (Powell et al., 2000). zfAhR2, zfARNT2, and zfCYP1A mRNA were detected at 24-h postfertilization (hf) in zebrafish (Danio rerio) embryos, coincident with the onset of blood circulation (Andreassen et al., 2002; Tanguay et al., 1999), and CYP1A protein was detected by immunohistochemistry at 36 hf in zebrafish embryos exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (Andreassen et al., 2002). Thus, the cellular machinery necessary for xenobiotic metabolism in fish is both present and inducible early in development.

Chemical elimination rates can change during early development and may increase dramatically after hatching. For
example, Guiney et al. (1980) reported that in rainbow trout (Oncorhynchus mykiss) the half-life for elimination of $[^{14}C]$-labeled 2,2',6,6'-tetrachlorobiphenyl was 231 days throughout the egg and first two-thirds of the sac fry stage, and then decreased to 15 days in the late sac fry stage. This change in elimination rate just prior to swim-up occurred as the sac fry absorbed their yolk lipids. Similarly, Petersen and Kristensen (1998) showed that elimination of $[^{14}C]$-labeled 2,4',5-trichlorobiphenyl, phenanthrene, and pyrene was minimal in zebra fish throughout the embryonic stage; however, larvae exposed to the same compounds eliminated them rapidly when transferred into clean water. The factors responsible for these observed changes in elimination rate are poorly known. One limitation of measuring radioactivity to determine chemical kinetics is that it does not discriminate between parent chemicals and their metabolites. Measurements of total radioactivity also provide no information regarding chemical distribution to various tissues.

Previously, we used multiphoton laser scanning microscopy (MPLSM) to visualize the tissue distribution of two lipophilic fluorescent chemicals, BODIPY and fluoranthene, in medaka embryos and larvae (Hornung et al., 2004a). BODIPY (Molecular Probes, Eugene, OR) is a nonpolar synthetic dye that is cell permeant and has high affinity for cellular lipids (Gocze and Freeman, 1994), while fluoranthene is a polyaromatic hydrocarbon (PAH) commonly found in sediments and biota from PAH-contaminated sites. The embryos were exposed to BODIPY or fluoranthene for 6 h very early in development (4–10 h) and then imaged through 5 days post-hatch. It was observed that both compounds were largely retained through the embryonic stage of development and did not appear to undergo significant biotransformation (M.W. Hornung, unpublished data).

In the present study, we extended these observations by imaging the distribution of a second PAH, benzo[a]pyrene (BaP). Preliminary work suggested that MPLSM could be used to detect metabolism of BaP in medaka embryos, and analysis by liquid chromatography (LC) suggested that the primary metabolic product was 3-hydroxybenzo[a]pyrene (BaP-3-OH). We, therefore, developed an MPLSM method to discriminate among BaP-3-OH and conjugates derived from BaP-3-OH based on their excitation spectra (Hornung et al., 2004b). The resulting images in the present study are interpreted in the context of developmental landmarks for medaka as well as existing kinetic data for fish early life stages. Support for conclusions regarding the kinetics of BaP and BaP metabolites is provided by complimentary analytical observations.

MATERIALS AND METHODS

Organisms. Medaka eggs were obtained from breeding stocks maintained on site as previously described (Hornung et al., 2004a). Animals were handled in accordance with guidelines for Animal Care and Use at the U.S. Environmental Protection Agency, Mid-Continent Ecology Division, Duluth, MN.

Reagents. BaP (> 98% pure), methyl-tert-butylether (MTBE), methanol, and acetone were purchased from Aldrich Chemical (Milwaukee, WI). BaP metabolites (BaP-1-OH, BaP-3-OH, BaP-9-OH, BaP-7,8-diol, BaP-9,10-diol, BaP-3,6-dione, BaP-1,6-dione, and BaP-3-sulfate) were purchased from the NCI Chemical Carcinogen Reference Standards Repository (Kansas City, MO). BaP-3-glucuronide was prepared by enzymatic glucuronidation as previously described (Hornung et al., 2004b; see online Supplementary Materials). Ultima Gold and Soluene-350 were purchased from Perkin Elmer (Boston, MA). 7,12-dimethylbenz[a]anthracene (BaP) (14C-BaP 26.0 μCi/mmol in toluene; > 97% pure), tricaine methanesulfonate (MS 222), β-glucuronidase Type B3, and all other chemicals were purchased from Sigma Chemical.

Exposure and sampling. Exposures and incubations were conducted in a 26°C ± 0.5°C water bath under a 16:8-h light:dark cycle using red-filtered fluorescent lighting (filter #19; Rosco, Stamford, CT). Exposure beakers (250 ml) containing 200–300 newly fertilized eggs in 150 ml embryo rearing media (ERM) were placed in 50 ml of ERM in a 100-ml beaker. Animals were transferred to fresh ERM every 48 h for the duration of the incubation period. Unused embryos were killed by anesthetic overdose (buffered MS 222, 400 mg/l).

Multiphoton laser scanning microscopy. The MPLSM system described previously was used to image the tissue distribution of BaP and three BaP metabolites: BaP-3-OH, BaP-3-glucuronide, and BaP-3-sulfate (Hornung et al., 2004b). The method was developed by determining the multiphoton excitation spectra of BaP and its position-3 metabolites in methanol (760–880 nm) and then imaging mixtures spiked into medaka embryo homogenates. Fluorescence detected following excitation at 760 nm, but not at 840 nm or above, indicated that only BaP was present. All three position-3 metabolites produced detectable fluorescence with excitation at 840 nm; however, BaP-3-sulfate exhibited lower fluorescence intensity than either BaP-3-glucuronide or BaP-3-OH. BaP-3-sulfate was not detected with excitation at 860 nm. At 860-nm excitation, fluorescence intensity from BaP-3-glucuronide was detectable but very low compared to that of BaP-3-OH. Only BaP-3-OH produced detectable fluorescence following excitation at 840 nm.

In the present study, further analysis of the relative fluorescence of BaP-3-OH, BaP-3-glucuronide, BaP-7,8-diol, BaP-9,10-diol, BaP-3,6-dione, BaP-1,6-dione, and BaP-3-sulfate was performed at 880 nm. Their relative fluorescence was determined as described previously (Hornung et al., 2004b) with metabolite standards at a concentration of 50 μM in methanol. Additionally, excitation of these metabolites was determined at these four wavelengths in a bovine serum albumin (BSA) solution (2 mg/ml water) as a surrogate medium for egg yolk proteins.

While imaging embryos or hatched larvae, a constant power was maintained at the microscope stage to permit comparisons of relative fluorescence intensity at each excitation wavelength and over time. This was accomplished by normalizing to a fluorescein standard. Fluorescein (100 μM, pH 12.5) was placed in a Cover-well Perfusion Chamber (Grace Biolabs, Bend, OR) mounted on a glass coverslip, with the Nikon 0.5 NA 10× Superfluor lens focused 50 μm into the sample. The fluorescent image of the fluorescein droplet was captured using the internal detectors of the confocal microscope system. Laser intensity at the sample was controlled by adjusting the power through the Pockels cell such that the fluorescent intensity of the images at each of the four excitation wavelengths were in proportional agreement with the published two-photon excitation spectrum of fluorescein (Xu and Webb, 1996).

Embryos sampled from 0 to 3 days postfertilization (dpf) were imaged directly through the chorion, whereas those older than 3 dpf were...
dechorionated for imaging. To orient the 0- to 3-dpf embryos, a Delta-T dish system (Biotechs, Butler, PA) was prepared with 1% agar in which wells of approximately 1 mm diameter were created by removing a plug of agar with a glass Pasteur pipette. The eggs were placed in the bottom of the well, positioned using fine forceps, and a small piece of ice was placed on top of the agar to keep the embryo immobilized during imaging. Embryos sampled after 3 dpf were liberated by enzymatic and mechanical removal of the chorion using a modification of methods described by Hornung et al. (2004a).

In the present study, protease was replaced with hatching liquid collected by hatching batches of control medaka at a density of 10 eggs/ml of ERM (Yasumasu et al., 1989). This shortened the time to release the embryo from the egg for 4 h with protease to 2–3 h in hatching liquid. Dechorionated embryos and newly hatched embryos were immobilized by immersion in N2-saturated water. They were then placed on a glass coverslip or Delta-T dish.

Immobilization on the stage was accomplished by placing several drops of 0.5% agar on the free embryo or larvae just as the agar reached gelling temperature.

A series of images taken from the surface of the organism to a depth of 600 μm were collected using LaserSharp2000 software (Biorad, Hemel Hempstead, UK). Pseudo-colored images were produced using Confocal Assistant software (free-wage version 4.02; 1996) by applying the “Thermol” look-up-table file to the original gray-scale images. Upon completion of imaging at a given wavelength, the embryo-imaging dish was placed in a Petri dish containing sufficient ERM to cover the agar while the laser was being tuned to the next excitation wavelength. After all fluorescent images were collected, embryos were illuminated with the 488 nm visible laser, and a transfer-illuminated image was captured in the same orientation in which the fluorescent images were obtained. Embryos were killed with buffered MS 222 (400 mg/l) at the end of the imaging session.

To determine whether any changes in BaP fluorescence might be due to factors other than metabolism, such as auto-oxidation, immersion oil was spiked with BaP and maintained in an open glass vial in the same exposure chamber used to culture medaka embryos. The fluorescence intensity of the oil droplet was imaged at 760 nm on the day it was prepared and after 4 days. There was no change in fluorescence between the two measurements.

**Exposure and analysis of 14C-BaP and metabolites.** Medaka embryos were exposed to 14C-BaP to determine the extent of metabolism during embryonic development and whether metabolites were retained or eliminated prior to hatching. 14C-BaP was diluted with unlabeled BaP in acetone to obtain a stock solution containing 20 μCi and 1.05 mg BaP/ml. Eight hundred newly fertilized eggs (2–3 hpf) were distributed equally into four beakers, one of which was a vehicle control (0.1% acetone). Eggs were exposed in 50 ml of ERM containing 1 mg/ml of 14C-BaP for 50 h. After exposure, the eggs were rinsed three times with ERM and transferred in groups of 25 to 100-ml glass beakers containing 50 ml of ERM and cultured as described above.

**Sample radioactivity was separated into aqueous- and organic-soluble fractions using a modification of the procedure described by Fay et al. (2000). NaOH in 25% ethanol (0.5 ml, 2 N) was added to 20 eggs or hatched embryos in 2 ml propylene centrifuge vial. Samples were digested in a sonicating water bath for 45 min. Following digestion, the samples were extracted three times with 1 ml hexane, vortexed for 2 min, and centrifuged at 10,000 × g for 5 min. Hexane fractions were combined in a 22-m1 glass scintillation vial, and 18 ml of Ultima Gold scintillation cocktail was added. The remaining NaOH fraction was transferred to a scintillation vial, treated with 0.5 ml of Soluene-350 to digest any remaining tissue fragments, and 20 ml of scintillation cocktail was added. Radioactivity was measured on a Packard 2550 TR/LL scintillation counter using internal standards to correct for sample quenching and counting efficiency. Luminescence correction was enabled in the counting program. Samples were kept protected from light for 2–3 h and then counted for 60 min or until 2-σ of 2% was obtained.**

**Extraction of BaP and hydroxylated BaP metabolites for LC/MS analysis.** Medaka eggs exposed as described above were frozen in groups of 50 at −80°C in 1.5-ml polystyrene vials. Approximately 2 mg of glass homogenization beads (0.1 mm; Scientific Industries, Inc., Bohemia, NY) were added to each vial, and the eggs were manually crushed with a polystyrene pestle in 50 μl of ammonium acetate (2.5mM)/sodium chloride (10%) solution (pH 5.0), followed by homogenization for 2 min with a motorized pestle. Samples were further homogenized with the addition of acetone (2 × 0.5 ml) and transferred to a 15-ml polystyrene vial with three ammonium acetate/sodium chloride solution rinses (1 ml per rinse). Analytes were extracted with MTBE (10 ml), centrifuged (1000 × g, room temperature, 20 min), and 9 ml of the MTBE layer was transferred to a clean 15-ml vial. Samples were evaporated to dryness (N2, 35°C), resuspended with methanol (250 μl), and transferred to a 2-ml polystyrene vial with three rinses of 250 μl of 30% methanol/1% ethanol/0.05% formic acid/2.5mM ammonium acetate. Samples were chilled in an ice/water bath (30 min), and centrifuged (20,000 × g, 5°C, 90 min). The supernatant was transferred to a clean vial, centrifuged a second time, and 250 μl were transferred to a clean autosampler vial for analysis.

**Extraction of BaP-glucuronides and sulfates.** BaP conjugates were extracted from eggs by adding 150 μl water (pH 5.0 with formic acid) and homogenizing for 60 s. Ethanol (175 μl) was added and the samples were homogenized for 120 s. An additional 175 μl ethanol was added and the samples were vortexed for 90 s, chilled for 30 min in an ice/water bath, and centrifuged (20,000 × g, 5°C, 90 min). A 350-μl aliquot of each sample was transferred to a clean 2.0-ml polystyrene vial, centrifuged a second time (20,000 × g, 5°C, 90 min), and transferred to an amber autosampler vial for analysis.

**Enzymatic hydrolysis of glucuronides.** Following an ethanol extraction as described above, samples were reduced to dryness and reconstituted in 500 μl acetate buffer (pH 5.0) with 1000 Units of glucuronidase. Hydrolysis was carried out for 2 h at 37°C and then the reaction was stopped with two additions of 500 μl aceton. Hydroxylated BaP metabolites were extracted with MTBE as described above.

**LC/MS analysis.** Quantification was performed using an Agilent (Palo Alto, CA) model 1100 LC interfaced to an Agilent Mass Selective Detector MS. BaP and hydroxylated BaP metabolites were analyzed using an atmospheric pressure photoionization source operating at 10 V, with the fragmentor set to 65 V. Benzene was used as a dopant to enhance the production of [M+H]+ for BaP. Quadrupole scanning was performed in the positive-selective ion-monitoring mode. The ion used to monitor for BaP was m/z 253, [M+H]+. BaP conjugates were analyzed with electrospray ionization operating at 2500 V on the spray needle and a fragmentor voltage of 65 V. Quadrupole scanning was performed in the positive-selective ion mode to monitor for BaP-glucuronide ions m/z 445 and 462, [M+H]+ and [M−NH4]+, respectively, and in the negative ion mode for BaP-sulfate (ion m/z 347, [M−H]+). Control eggs used to prepare quantification standards were fortified with 0.2–1.4 nmol BaP, BaP-3-OH, or BaP-3-glucuronide per 50 eggs. Detection limits for BaP, BaP-3-OH, and BaP-3-glucuronide based upon recovery from spiked eggs were 5, 2.5, and 10 ng/ml, or 0.4, 0.2, and 0.5 pmol per egg, respectively. Replicate determinations indicated a relative SD for BaP to be 2.2% (n = 7) and for BaP-glucuronide to be 6.9% (n = 5).

**Characterization of BaP metabolites recovered from the glucuronidase reaction was done using a triple quadrupole LC/MS system from Agilent operated in atmospheric pressure chemical ionization mode. The LC was equipped with a C18 reversed phase column (Luna C18(2), Phenomenex, Torrance, CA) with an elution gradient programmed between mobile phase A (1% methanol, 1% ethanol, 2.5mM ammonium acetate, pH 6.2) and B (98% methanol, 1% ethanol, 2.5mM ammonium acetate, pH 6.2) as needed. BaP metabolites were detected at appropriate retention times by monitoring [M+H]+ ions (269 m/z for mono and dihydroxy and 283 m/z for diones). Oxidation of the BaP-hydroxy metabolites at the ionizing electrode of the MS also produced a 283-m/z ion as has been previously reported (Xu et al., 1996).
RESULTS

Relative Fluorescence of BaP Metabolites

The relative fluorescence of several BaP metabolites was compared using multiphoton excitation at 760, 840, 860, or 880 nm. The metabolites substituted at the position-3 carbon exhibited significantly greater fluorescence than the other metabolites (Fig. 1). The intensity of BaP-3-OH was greater than BaP-3-glucuronide in methanol but fluoresced less than BaP-3-glucuronide when in BSA. BaP-9,10-diol fluoresced when excited at 760 nm, with fluorescence greater in BSA than in methanol. BaP-7,8-diol fluoresced weakly at 760 nm in both methanol and BSA. Neither of the two BaP-diols produced detectable fluorescence when excited at wavelengths greater than 760 nm. The two BaP-diones (1,6-dione and 3,6-dione) did not fluoresce at any of the four multiphoton excitation wavelengths in either solution. These results indicate that the fluorescence detected in the eggs and larvae at excitation wavelengths greater than 760 nm may be due to metabolites substituted at the position-3 carbon (or some other untested or undetected metabolites) and that the fluorescence was probably not from BaP-diols and BaP-diones.

Multiphoton Microscopy of BaP and Metabolites in Medaka

MPLSM was used to image the distribution of BaP and metabolites in embryonic and larval medaka. The earliest images were obtained from BaP-exposed embryos that were sampled midway through the 6-h exposure period (3 h) and at the end of exposure (6 h). At 3 h, the medaka were at the 32–64 cell stage. At the end of the 6-h exposure period, the medaka embryos were at the early blastula stage, and the marginal cell nuclei could be observed in the periblast beginning the formation of the yolk (cortical) syncytial layer. When embryos collected at 3 or 6 h of BaP exposure were excited at 760 nm, a low level of fluorescence was observed in the blastula and yolk, while a much stronger signal was seen in the oil globule (Fig. 2). No fluorescence was evident following excitation at 840 nm. These observations suggest that only unmetabolized BaP was present at these early time points.

During the period from the end of exposure at 9 hpf to the next imaging time point at 1 dpf, embryonic development continued through gastrulation and into segmentation. Over this time period the fore-, mid-, and hindbrain became distinct structures, the eye placode formed, and Kupffer’s vesicle formed ventrally at the posterior of the embryonic body (Fig. 3A). BaP-exposed embryos imaged at 1 dpf exhibited strong fluorescence in the oil globules and moderate fluorescence in the yolk when excited at 760 nm (Fig. 3E). Excitation at 840 nm produced detectable fluorescence in the yolk but not in the oil globule (Fig. 3I). This pattern of excitation indicates that the oil globules contained BaP but no BaP-derived metabolites. Fluorescence in the yolk at 840 nm suggested the presence of BaP-sulfate or BaP-glucuronide. The most intense fluorescence signal at 840 nm appeared in the yolk syncytial layer and was most pronounced in the leading edge (Supplementary Fig. S1). This observation suggests that the yolk syncytial layer is capable of metabolizing BaP. Little or no fluorescence was detected at 860 or 880 nm (Figs. 3M and 3Q), indicating that BaP-3-OH was not present (or present at very low levels). No fluorescence was evident in control embryos at any of these excitation wavelengths (Fig. 5).

By 2 dpf, the oil globules had coalesced into one large oil globule, and the embryonic body nearly encircled the yolk (Fig. 3B). At this time point, the tubular heart had begun to beat and a weak blood circulation could be seen. Pigmentation of the retina was also evident. With excitation at 760 nm (Fig. 3F), the relative intensity of fluorescence in the oil droplet had decreased compared to that observed at 1 dpf and was similar
to that in the yolk. Excitation at 840 nm produced a moderate level of fluorescence in the yolk but no fluorescence in the oil globule (Fig. 3J). Fluorescence was not detected at excitation wavelengths of 860 or 880 nm (Figs. 3M, 3N, 3Q, 3R). As at 1 dpf, these images indicate that conjugated metabolites of BaP were present in the yolk but not in the oil globules.

Between 2 and 3 dpf, the liver rudiment forms and the heartbeat strengthen as the chambers of the heart become fully differentiated (Iwamatsu, 1994). At 3 dpf, the fluorescence in the oil globule at 760 nm was much lower than that of the yolk (image not shown), and by 5 dpf, this signal had diminished to near background (Fig. 3G). Fluorescence intensity in the yolk increased from 3 to 5 dpf at both 760 and 840 nm. Low levels of fluorescence were also evident in yolk at 860 and 880 nm (Figs. 3G, 3K, 3O, 3S), suggesting the presence of some BaP-3-OH or other BaP metabolite that excites at this longer wavelength. The gall bladder and hepatic biliary system were obvious at 3–5 dpf due to strong fluorescence at 760 and 840 nm (Figs. 3G, 3K; Supplementary Fig. S2). The vitelline vasculature during these mid- to late-embryonic stages showed no fluorescence when imaged at the surface of the egg indicating that any circulating BaP or metabolite is below the detection limit of the imaging parameters.

At 7 dpf, just prior to the onset of hatching, the distribution of fluorescence was similar to that in the embryos imaged at 5 dpf, but the fluorescence intensity was greater (Figs. 3H, 3L, 3P, 3T). By the end of the prehatch embryonic stage, the gall bladder had noticeably enlarged and the hepatic biliary system was highly fluorescent at excitation wavelengths greater than 760 nm (Supplementary Fig. S2). In many of these embryos, the common bile duct was enlarged and full of fluorophore. Autofluorescence at an excitation wavelength of 760 nm increased in the gall bladder of control embryos as they neared hatching and in the post-hatch embryos (Fig. 5), but the intensity was far less than in the BaP-treated groups. Intense autofluorescence that saturated the detectors was evident in the pigmented retinal epithelium of control and treated embryos (not shown).

Images of newly hatched BaP-exposed larvae exhibited intense fluorescence at 760, 840, and 860 nm in the yolk, liver, and gall bladder (Figs. 4D, 4G, 4J, 4M). Lower levels of fluorescence were observed at 880 nm in the yolk and gall bladder. Fluorescence also was evident in the anterior portion of the intestine, and over time filled the length of the intestine (Figs. 4D–F; Supplementary Figs. S2–S4). Prior to the gastrointestinal tract becoming patent, the gall bladder continued to enlarge and maintained a fluorescent signal that saturated the detectors at 760 and 840 nm. As post-hatch larvae began to excrete the contents of the gastrointestinal tract, fluorescence in the organism rapidly decreased. By 4 days post-hatch, the embryos had used most, if not all, of their yolk, and the remaining fluorescent signal was in the gall bladder and intestine (Figs. 4F, 4L, 4O). In some embryos, the only remaining strong fluorescence was in the posterior portion of the intestine, indicating that the gall bladder had emptied its contents of BaP metabolites into the intestine. Lower intensity fluorescence was apparent in other tissues including the inner ear, pectoral fin (Fig. 4D), and coincident with intersegmental vessels (Fig. 4D and 4F). However, there is greater autofluorescence in the post-hatch larvae than the embryos (Fig. 5) which could account for some of this low-level fluorescence.

Recovery of $^{14}$C-BaP

A parent or other approach was used to characterize the kinetics of $^{14}$C-BaP and polar metabolites of $^{14}$C-BaP in medaka embryos and larvae. The average amount of parent chemical recovered in hexane immediately following
termination of the exposure was 5.1 pmol per embryo (Fig. 6). At 1 dpf, the amount of parent BaP appeared to decrease slightly, and by 3 dpf, the amount of parent compound had decreased by more than 50% (2.3 pmol per embryo). By the time of hatching, parent BaP was essentially absent from the organisms.

$^{14}$C recovered from the polar NaOH phase exhibited an inverse relationship to parent BaP. Immediately following the exposure, the amount of $^{14}$C recovered in the NaOH phase was very low (0.3 pmol per embryo). By 1 dpf, the amount of $^{14}$C recovered in the polar fraction had increased sevenfold to 2.1 pmol per embryo. On embryonic days 5 and 7, the amount of $^{14}$C in the polar fraction reached a maximum level of 4.4 pmol per embryo. The amount of $^{14}$C in the polar fraction dropped rapidly after hatching, although measurable amounts were still present at the end of the experiment on day 13. Control eggs processed through the same homogenization procedure yielded counts similar to background.

The total amount $^{14}$C recovered on any given day (hexane and NaOH phases) averaged about 6.0 pmol per embryo through all 7 days of embryonic development and then declined rapidly after hatching. Nearly all radioactivity measured after hatching was recovered in the polar fraction.

**LC/MS Analysis**

Embryos and larvae were extracted with MTBE for analysis of BaP and hydroxylated metabolites by LC/MS. BaP levels
declined rapidly during embryonic development and dropped to below the detection limit soon after hatching (Fig. 7). No hydroxylated BaPs (BaP-1-OH, BaP-3-OH, BaP-9-OH, or BaP-7,8-OH) were detected by this method.

Medaka eggs and larvae were also subjected to an ethanolic extraction procedure to recover glucuronide and sulfate conjugates of BaP metabolites. A BaP-glucuronide with an LC retention time consistent with BaP-3-glucuronide was the only conjugated metabolite detected in the embryos. The concentration of this metabolite increased through the embryonic stages, reached a plateau just prior to hatching, and then decreased after hatching (Fig. 7). The sum of recovered BaP and BaP-glucuronide was fairly stable throughout embryonic development, further suggesting that BaP-3-glucuronide was the major metabolite during this time period.

The results of the incubation of egg homogenates with glucuronidase for 2 h confirmed the presence of BaP-3-OH (Fig. 8). This hydrolyzed metabolite was recovered in increasing amounts from eggs at day 3 and day 7 postexposure. No other mono-hydroxy BaP metabolites were recovered from the glucuronidase reaction. Two dihydrodiols and two diones were recovered from the enzymatic hydrolysis reaction. BaP-9,10-diol and BaP-7,8-diol were recovered in high abundance from day 1 postexposure samples. BaP-3,6-dione and BaP-1,6-dione were recovered from the glucuronidase reaction on all three sample days with greater amounts recovered from day 3 and day 7 than from day 1.

**DISCUSSION**

MPLSM was used in combination with conventional analytical methods to investigate the uptake, distribution, and metabolism of BaP in embryonic and larval medaka. The results of this study show that the fish egg is a highly dynamic environment within which developmental, physiological, and biochemical changes dramatically influence the disposition of this PAH. During and shortly after the initial exposure, BaP partitioned primarily into oil globules within the yolk. Redistribution of BaP-derived fluorescence occurred rapidly, however, and changes in signal intensity and distribution were observed throughout development. In a previous study, Hornung et al. (2004b) showed that by using four different excitation wavelengths, it was possible to discriminate fluorescence emitted by BaP and suspected BaP metabolites. In the present effort, conclusions suggested by the interpretation of fluorescent images were confirmed in part by conducting a parent or other analysis of ¹⁴C-BaP–dosed embryos. LC/MS was then used to confirm the predominant metabolic products as BaP-glucuronides.

The partitioning of lipophilic organic chemicals to the oil globule in fish eggs was shown previously in the Atlantic croaker (Micropogonias undulatus). Of the dietary o,p′-DDT transferred to the oocytes from dosed adult females, 95% was extracted from the oil globules and the remainder was in the yolk protein fraction (Ungerer and Thomas, 1996). The rapid loss of BaP-derived fluorescence from the oil globule in medaka embryos in this study was not associated with a dramatic decrease in the apparent size of the globule or a loss of extracted egg lipids (data not shown). In fact, a small oil globule remained in many of the embryos at the termination of the experiment when the yolk reserves had been depleted. Instead, this loss appears to have been linked directly to metabolism of BaP.

Radioactively labeled xenobiotics have been used by several authors to study the uptake and elimination of hydrophobic chemicals in fish early life stages (Guiney et al., 1980; Petersen...
and Kristensen, 1998). As in these earlier studies, total radioactivity in medaka following exposure to ¹⁴C-BaP declined very little during the embryonic stage of development. It is clear, however, that BaP was rapidly metabolized during this time period. This finding demonstrates that although metabolism occurs early in embryonic development, the embryo does not eliminate these metabolites. In addition, it shows that measured levels of total radioactivity can be misleading if the goal is to understand the kinetics of elimination of parent chemical.

The liver is generally considered to be the major organ of xenobiotic metabolism in adult fish. Our observations suggest that medaka embryos possess considerable metabolic activity prior to liver formation. According to the staging of Iwamatsu (1994), the liver anlage does not form until 50 hpf and the liver rudiment appears at nearly 60 hpf in medaka reared at these temperatures. The earliest apparent appearance of BaP metabolites was at 21–24 hpf in the yolk syncytial layer although diffuse metabolic activity may also have been present at this time within the yolk itself.

Xenobiotic metabolizing activity in these early embryonic stages is not entirely unexpected as other enzymes involved in normal growth and development have been detected in fish embryos. Activity and mRNA for enzymes possibly responsible for proteolysis of stored yolk proteins have been measured in fish oocytes and embryos (Kwon et al., 2001). Acetylcholinesterase activity has been detected in early medaka embryos including the yolk syncytial layer (Fluck, 1982). Hsu et al. (2002) reported that female zebrafish deposit mRNA into their oocytes. Using in situ hybridization, they detected mRNA for the cholesterol side-chain cleavage enzyme, CYP11A1, in the embryos during epiboly and later in the yolk syncytial layer. CYP1A protein has also been detected in the trophoblastic syncytium in post-hatch larval sea bream (Sparus auratus; Ortiz-Delgado and Sarasquete, 2004). It is possible that maternally derived P450 mRNA, such as that encoding for CYP1A, is deposited within the medaka oocyte and then

FIG. 5. Trans-illuminated and fluorescence images of control medaka embryos at 1 and 7 dpf, and 1-day post-hatch larvae. Hatching started at 8 dpf. The greatest autofluorescence in control animals was evident in the gall bladder and pigmented cells. Parameters are the same as described in Figure 3. Abbreviations: pigmented cells (pc), gall bladder (gb), liver (lv), oil globule (og), air bladder (ab).

FIG. 6. Kinetics of ¹⁴C-BaP and metabolites in medaka embryos and fry following an acute 6-h exposure to ¹⁴C-BaP beginning within 2 h of fertilization. ¹⁴C in the hexane fraction (BaP, solid circles) and ¹⁴C in the NaOH fraction (BaP metabolites, open squares) from 20 eggs (0–7 dpf) or post-hatch larvae (9–13 dpf) was quantified by liquid scintillation counting. Symbols represent mean and SD of three replicate groups of 20 eggs at each time point. Dashed line represents total recovered ¹⁴C.
translated in the yolk or the yolk syncytial layer. Alternatively, CYP proteins may be deposited within the yolk during oogenesis and become localized or active in the yolk syncytial layer. An interesting possibility suggested by both alternatives is that induction of metabolic activity in female fish due to prior contaminant exposure may lead to higher levels of metabolism in embryos and larvae.

In this study, medaka eggs were exposed to a high concentration of BaP immediately after fertilization and prior to significant embryo development. This was done to rapidly load the egg with chemical and observe the tissue distribution and metabolism throughout development—an exposure scenario similar to that of maternal deposition of a bioaccumulative chemical into the egg with subsequent development in clean water. Data on the transfer of PAHs and their metabolites from maternal compartments to the eggs are generally lacking. Monteverdi and Di Giulio (2000) did demonstrate that exposure of gravid female killifish to BaP resulted in a mix of BaP and hydroxylated metabolites in the oocytes that was similar to the mixture of chemicals associated with vitellogenin in the sera. Whether the presence of metabolites from maternal deposition would alter the tissue distribution and metabolism of parent chemical in the embryo or simply increase the amount of metabolites sequestered in the embryo is unknown.

As post-hatch embryos utilized their yolk reserves and developed a functional gastrointestinal tract, the distribution of the intense fluorescence became limited to the yolk, gastrointestinal tract, gall bladder, and biliary system. Although fluorescence was visible in the gall bladder and could be traced via the common bile duct to the intestine, the metabolism of BaP by the gastrointestinal tract itself cannot be ruled out as a contributor to this fluorescence. AhR and ARNT mRNA were identified in the intestinal mucosal layer in the zebrafish at 48–120 hpf (Andreasen et al., 2002), and CYP1A protein has been detected in the gut epithelium of several fish species following exposure to AhR agonists (Guiney et al., 1997; Ortiz-Delgado et al., 2005). In adult fish, the gastrointestinal tract plays an important role in the metabolism of BaP taken up from dietary sources (Kleinow et al. 1998; Van Veld et al., 1988).

The fish vascular endothelium also is capable of xenobiotic metabolism as evidenced in numerous studies in several species showing inducible CYP1A protein by immunohistochemistry (Andreasen et al., 2002; Cantrell et al., 1998; Guiney et al., 1997; Smolowitz et al., 1991; Toomey et al., 2001). That the vitellin vasculature of the medaka in this study always appeared as a dark non-fluorescent structure in contrast to the intense fluorescence of the yolk, suggests that if metabolism is occurring in these vascular endothelial cells, the metabolites do not remain in those cells but are released rapidly to the circulation. Any circulating BaP or metabolites are also below a detectable concentration as these vessels, including the chambers of the heart, always appeared dark in the images. In the embryo, it may be that only in the most metabolically active tissues do the metabolites appear at sufficient concentrations to be observed by increased fluorescence, such as the yolk syncytium. Alternatively, only after these metabolites reach terminal tissues prior to elimination do they accumulate at sufficient levels to be detected. Similarly, in higher magnification images of the liver of BaP-treated embryos, the fluorescence of the biliary system defines the location of the liver; the hepatocytes have much lower fluorescence (for example see Supplementary Fig. 2). So although the hepatocytes are metabolically competent, they too, do not accumulate sufficient metabolite to reach the fluorescence intensity seen in the biliary system or gastrointestinal tract. In addition, that acute toxicity was not detected in this study further indicates the protective role of metabolism and redistribution of chemicals to organs where they remain sequestered until they are eliminated.

The identification of BaP-3-glucuronide as a product of BaP metabolism in medaka embryos is consistent with previous studies of BaP metabolism in juveniles and adult fish. BaP-1-OH and BaP-3-OH were the major hydroxylated products of BaP following enzymatic hydrolysis of bile from field-collected English sole, common eel, Conger eel, and European flounder (Krah et al., 1987; Ruddock et al. 2002). BaP-1-OH and BaP-3-OH were the major metabolites recovered by hydrolysis of the bile of juvenile turbot injected with BaP (Telli-Karakoc et al., 2002). Assays using fish hepatic microsomes also indicate that BaP-3-OH and BaP-9-OH can be a significant metabolites of BaP (Sikka et al., 1990; Yuan et al., 1997).

BaP-3-OH was the only mono-hydroxy metabolite of BaP detected following glucuronidase treatment in the present...
BaP-3-OH was also the only metabolite tested in this study that exhibited fluorescence with excitation at 880 nm; BaP-3-glucuronide yielded no detectable fluorescence at this wavelength. The fluorescence detected in the medaka embryos and larvae at this longer excitation wavelength may be due to BaP-3-OH that was not conjugated to the glucuronide and was not extractable from the eggs. Alternatively, this fluorescence could be due to other highly fluorescent metabolites of BaP that were not detected and/or extracted by the analytical chemistry methods used here.

The toxicological significance of biotransformation in fish early life stages depends on the activities of the parent compound and its metabolic products. If the parent compound is more toxic than its metabolites, biotransformation would confer protection to the developing embryo. Biotransformation of planar aromatic hydrocarbons occurs via the action of enzymes induced as a result of these compounds binding and activating the AhR. The high sensitivity of fish early life stages to compounds that act via an AhR-mediated mode of action is well established; however, this sensitivity varies widely among species (Elonen et al., 1998;
AhR agonists such as 2,3,7,8-TCDD and several coplanar polychlorinated biphenyls are slowly metabolized in adult fish (Burkhard et al., 2004) and are thus probably not significantly metabolized in fish embryos. In such cases, Hahn (2001) hypothesized that species differences in sensitivity may be related to differences in the affinities of fish AhRs for these chemicals. Evidence of a correlation between low affinity of AhRs for 2,3,7,8-TCDD and low sensitivity to toxicity have recently been reported in birds and frogs (Karchner et al., 2006; Lavine et al., 2005).

BaP, and other PAHs that may be readily metabolized and eliminated, would be less likely to produce AhR-mediated dioxin-like toxicity or would exhibit lower potency than those recalcitrant to metabolism. CYP1A induction and enzyme activity has been shown to decrease over time with PAHs that bind the AhR but are readily metabolized (Billiard et al., 2002; Bols et al., 1999; Riddick et al., 1994). This is likely due to the decrease in the amount of parent chemical available to activate the AhR. Metabolic capacity in the zebrafish was reduced using morpholinos to knock down expression of CYP1A (Billiard et al., 2006). This produced increased toxicity by β-naphthoflavone, indicating that for this PAH-like chemical, metabolism by CYP1A has a protective role. In addition, not all PAHs produce toxicity via an AhR-mediated mechanism. The tricyclic PAHs that are common in weathered crude oil were recently shown to produce cardiac toxicity independent of the AhR (Incardona et al., 2005). Even though these compounds do not act through the AhR, metabolism of the parent chemicals may decrease their acute toxicity. Thus, for compounds that undergo biotransformation, differences in embryonic or larval metabolic activity could contribute to differences in sensitivity during these early life stages.

Alternatively, metabolism may result in increased toxicity by transforming a parent substance into more reactive products. In mammals as in fish, the predominant pathway for BaP metabolism and elimination is ring hydroxylation followed by a Phase II conjugation reaction (glucuronidation or sulfation). The genotoxicity of this compound has been attributed, however, to production of highly reactive BaP-diols and epoxides (recently reviewed by Xue and Warshawsky, 2005). Although produced in small quantities, these reactive compounds can bind and cause DNA damage.

The 7,8-diol is a precursor metabolite leading to DNA adduct formation. This metabolite has been measured in English sole (Parophrys vetulus), starry flounder (Platichthys stellatus) (Varanasi et al., 1986), gulf toadfish (Opsanus beta) (Kennedy et al., 1989), and Ictalurid catfish (Willett et al., 2000). Telli-Karakoç et al. (2002) detected BaP-7,8-diol as the major metabolite in turbot (Scophthalmus maximus) after 1-day exposure to BaP and this corresponded to the presence of a single DNA adduct. By 3-day postexposure, however, BaP-1-OH and BaP-3-OH comprised 60% of the metabolites, and several DNA adducts were detected. In the present study, BaP-7,8-diol and BaP-9,10-diol were recovered following glucuronidase treatment of the ethanol extracts of embryos at 1 post-exposure. Thus, a metabolite known to contribute to DNA damage was present in these animals. The production of genotoxic products in a developing animal could lead to adverse effects in juveniles or adult animals. The use of transgenic medaka (Amanuma et al., 2002; Winn et al., 2000) to detect genetic damage could provide further information on whether metabolism of BaP by embryos and larvae is a protective mechanism. Extending the culture period of BaP-treated embryos to the adult stage may reveal long-term effects of early metabolism of BaP, either as genotoxic or other adverse effects.

Finally, it should be noted that environmental contaminants rarely exist in isolation of one another, but exist instead as component parts of complex mixtures. This is particularly true of PAHs, which include hundreds of known chemical structures. PAHs can influence the uptake and metabolism of one another and produce toxic effects that would not be predicted based upon the addition of their individual toxic potencies (Wassenberg and Di Giulio, 2004; Wassenberg et al., 2005). For example, BaP is a well-known inducer of CYP1A activity. In contrast, fluoranthene can inhibit the induction of CYP1A activity by other chemicals (Willett et al., 2001). MPLSM may provide a means of understanding how multiple PAHs interact to within fish embryos to produce toxicity by comparing the distribution of chemicals administered singly or as part of a defined mixture.

**SUPPLEMENTARY DATA**

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

**FUNDING**

The information in this document has been funded wholly by the U.S. Environmental Protection Agency.

**ACKNOWLEDGMENTS**

The authors thank Rodney Johnson, Kevin Flynn, and Doug Lothenbach for technical advice and assistance, and David Mount and Irv Schultz for critical comments on the manuscript. We also thank Wilson Laboratories, Duluth, MN for assistance with medaka culture, spawning, and egg collection. This project was performed, in part, using compounds provided by the National Cancer Institute’s Chemical Carcinogen Reference Standards Repository operated under contract by Midwest Research Institute, No. N02-CB-07008.

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