Biochemical Pharmacology, Vol. 32, No. 7, pp. 1324–1327, 1983.
Printed in Great Britain.

0006-2952/83/071324-04 $03.00/0

Basal levels and induction of hepatic aryl hydrocarbon hydroxylase activity during the embryonic period of development in brook trout

(*Received* 27 *July* 1982; *accepted* 8 *November* 1982)

It is well established that fish have hepatic cytochrome P-450 dependent monooxygenases that are similar in many respects to the more extensively studied mammalian systems [1–6]. These enzymes metabolize a variety of lipophilic xenobiotics and can be induced in fish by experimental exposure to common environmental contaminants, such as commercial polychlorinated biphenyl (PCB) mixtures and petroleum hydrocarbons [3, 7–9]. Much work in mammals has shown that cytochrome P-450 dependent monooxygenases play important roles both in the detoxification of xenobiotics and in pathways leading to the activation of certain compounds to cytotoxic, mutagenic and carcinogenic metabolites [10–12]. In fish, the xenobiotic monooxygenases are likely to be involved in determining the disposition and effects of many lipophilic pollutants [13].

Generally the early developmental stages of fish have been found to be more sensitive to pollutant toxicity than adults [14–16], and the rapidly proliferating tissues of embryos may be particularly sensitive to damage from activated metabolites [17]. However, very little is known about the capacity of these stages to metabolize foreign compounds [18]. We report here on the levels of aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase (AHH) activity in liver homogenates from brook trout embryos and eleutheroembryos*, and the induction of this activity in embryonic liver by the commercial PCB mixture, Aroclor 1254. We believe this to be the first report of the presence and induction of hepatic monooxygenase activity in fish during development prior to hatching.

Protosol, [^{14}C]toluene and a ^{14}C-labeled PCB isomeric mixture (96 μCi/mg and approximately 54% chlorine by weight) were purchased from the New England Nuclear Corp., Boston, MA. All other chemicals were as previously described [18].

Brook trout (*Salvelinus fontinalis*) embryos were obtained by standard techniques at the Sandwich Fish Hatchery, Massachusetts Division of Fisheries and Game, during the natural spawning season in November, 1979. The embryos were from two different pools of eggs. Pool I was a very large pool of eggs stripped from about thirty females and fertilized with milt from about ten males; most of these were used to replenish the hatchery stock. Pool II, obtained 2 weeks later, was stripped from four females and fertilized with milt from four males. Embryos were maintained at the hatchery in flowing spring water at 10° for various periods and then brought back to the laboratory and incubated in the same water in glass dishes at 10°. In the laboratory, water was changed daily, and any dead embryos were removed at frequent intervals. Embryos were not treated with any of the antimicrobial agents normally used at the hatchery. The approximate mean hatching times for pool I and pool II embryos were 44 and 49 days from fertilization respectively.

Yearlings (1-year-old fish), which had been raised at the hatchery from pool I eggs, were obtained in March 1981. Liver whole homogenates from these fish were used for comparison with embryo and eleutheroembryo preparations. Conditions of fish maintenance at the hatchery have been described previously [5].

A portion of the pool II embryos was exposed to Aroclor 1254, which was spiked with the ^{14}C-labeled PCB isomeric mixture to a specific activity of 0.3 μCi/mg. For each treatment sixty-five embryos were incubated in 10 cm glass petri dishes in 30 ml of spring water. PCBs were added in 15 μl of dimethyl sulfoxide (DMSO) to a level of 0.75 or 7.5 ppm (μg/ml), while DMSO alone was added to control embryos. The exposure was initiated 23 days after fertilization and was continued for 21 days. Water was changed and additional inducer added at 48-hr intervals for a total of ten additions. To quantitate PCB levels resulting from the exposure, embryos were washed twice with water, dechorionated, weighed, and then solubilized in 0.3 ml of Protosol in glass scintillation vials. Scintillation mixture (10 ml) and 0.6 N HCl (0.3 ml) were added to each vial, and radioactivity was measured by liquid scintillation counting. Counting efficiency was determined by internal standardization with [^{14}C]toluene.

With the use of microsurgical instruments, livers were dissected from embryos and eleutheroembryos and carefully separated from gall bladders. Homogenates were prepared from pools of two to twenty livers in 0.05 M Tris–HCl, pH 7.2, 0.25 M sucrose (about 1–3 mg protein/ml) using a 1-ml Potter–Elvehjem tissue grinder. Homogenates were similarly prepared from livers of individual yearlings (4 ml buffer/g liver) and microsomes were sedimented as before [4]. Embryo and eleutheroembryo livers were not weighed since their extremely small size made such measurements impractical; based on protein content, it is estimated that the livers of embryos near hatching weighed less than 50 μg.

Liver homogenates were assayed immediately for AHH activity using the micro-radiometric procedure previously described [18], except that the reaction mixture consisted of 0.05 M Tris–HCl, pH 7.2, 0.40 mM NADPH, 0.060 mM [^{3}H]benzo[*a*]pyrene, 0.8 mg/ml bovine serum albumin and from 2 to 30 μg of liver protein in a final volume of 25 μl, and incubation was for 20 min at 25°. Blanks consisted of the complete reaction mixture without NADPH. The incubation temperature was determined to be optimal using yearling hepatic micosomes, and the reaction with yearling microsomes or liver whole homogenate was linear with time for at least 20 min. AHH activity was linear with liver protein up to 70 μg per reaction mixture. Activities were calculated based on the specific activity of the [^{3}H]benzo[*a*]pyrene, which was determined for each preparation after clean-up by solvent extraction. The limit of detection of this assay procedure with liver homogenates is about 2 pmoles of benzo[*a*]pyrene (BP) metabolites in

* The terminology of fish development of Balon [19] will be used here. The embryonic period is considered to extend from fertilization until the yolk is almost completely absorbed and feeding begins. The embryonic period is divided into three phases: cleavage, embryonic and eleutheroembryonic. The embryonic phase extends from the end of cleavage until hatching, and the eleutheroembryonic phase extends from hatching until the developing fish is no longer dependent on the yolk for nutrition. The term "embryo" will be used here to specifically refer to prehatching developmental stages; the term "eleutheroembryo" will refer to posthatching embryonic stages. Some workers call eleutheroembryos "prolarvae", "yolk sac larvae" or "sac fry".

Table 1. Hepatic aryl hydrocarbon hydroxylase activity in brook trout embryos, eleutheroembryos and yearlings

Stage*		Hepatic AHH† units/mg protein	Hepatic AHH† units/g body weight	Hepatic AHH† units/g total weight
Embryos	(−5)	21 ± 3	26 ± 3	3.3 ± 0.4
Eleutheroembryos	(+9)	63 ± 4	61 ± 10	19 ± 5
Eleutheroembryos	(+33)	58 ± 8	74 ± 1	46 ± 1
Yearlings	(+470)	24 ± 1	39 ± 3	

* The numbers in parentheses indicate the approximate days from hatching. The embryos were in stages 22 and 23 described by Ballard [21].

† AHH units: pmoles benzo[*a*]pyrene metabolites/min. Assays were performed on liver whole homogenates as described in the text. The embryo and eleutheroembryo data are the mean of determinations on two pools of from two to twenty livers ± range. Data for yearlings are the mean ± S.E. of determinations on four individuals. For embryos and eleutheroembryos, body weight refers to the weight of the carcass dissected free of yolk. Over the interval of development examined, this parameter ranged from 0.004 to 0.036 g. Total weight refers to the weight of intact eleutheroembryos and embryos (removed from chorions). This parameter varied relatively little with development: the overall mean value for the two pools of embryos examined was 0.048 ± 0.006 g (± range). The mean weight of yearlings was 100 ± 4 g (± S.E.). The mean ratio of total liver protein to body weight for the embryos and eleutheroembryos was 1.16 ± 0.09 mg protein/g body weight, while the mean value of this parameter for yearlings was 1.67 ± 0.07 (± S.E.).

the final stopped reaction mixture. Protein was measured by the method of Lowry *et al.* [20] using bovine serum albumin as a standard.

Levels of AHH activity in liver homogenates of brook trout embryos, eleutheroembryos and yearlings are shown in Table 1. Hepatic AHH specific activity was about 3-fold higher in eleutheroembyros at both 9 and 33 days from hatching than in embryos 5 days prior to hatching. Thus, there was a substantial increase in AHH activity in brook trout liver around the time of hatching, which was apparently followed by little change in activity during later eleutheroembryonic development. However, hepatic AHH activity had declined in year-old fish. It is noteworthy that specific activity was essentially the same in liver homogenates from brook trout embryos and yearlings.

A similar pattern emerged when hepatic AHH activity was normalized to body weight (Table 1), reflecting the fact that the ratio of total liver protein to body weight changed relatively little with development. However, near the time of hatching the embryonic body accounted for only about 10–15% of the total embryo weight, the majority of the weight being due to the yolk. If the weight of the yolk is included, then embryos near hatching have substantially less hepatic capacity to metabolize BP per unit total weight than yearlings have per unit body weight, in spite of the fact that activities per g body weight were similar in the two groups. As a result of both growth of embryo body at the expense of yolk and the increase in specific activity after hatching, hepatic AHH activity normalized to total weight continually increased over the interval of development examined.

Levels of AHH activity in homogenates of liver from control embryos and those exposed to Aroclor 1254 are shown in Table 2. The embryos were assayed approximately 5 days before hatching (data from the control group were also included in Table 1). Animals treated with Arclor 1254 at both doses had levels of hepatic AHH activity that were induced 4-fold. The lack of an observed dose dependency suggests that the induced levels of activity represent the maximal extent of the response in these trout embryos.

The data presented here demonstrate that liver homogenates from brook trout embryos and eleutheroembryos are active in metabolizing BP and establish that embryonic liver of trout is competent to respond to Aroclor 1254 as an inducer of monooxygenase activity. Since hepatic AHH specific activity in trout eleutheroembryos was more than twice the level in sexually immature yearlings (Table 1), and since we have found that levels of activity in yearlings are at least as high as those in nonspawning adults*, we may conclude that the livers of brook trout eleutheroembryos are capable of metabolizing BP at a considerably greater rate than those in nonspawning adults. This is in contrast to the results of a previous study [18], in which we found that eleutheroembryos of the estuarine killifish, *Fundulus heteroclitus*, had hepatic AHH with specific activity of about half that in adults. Thus, the ontogenic patterns of hepatic AHH activity in brook trout and *Fundulus* appear distinct. The two species differ further in that extrahepatic tissues of *Fundulus* eleutheroembryos carried out a substantial portion of the total BP metabolism measured *in vitro*, while we could not detect extrahepatic activity in brook trout eleutheroembryos.

The developmental pattern of hepatic monooxygenase activity in brook trout reported here resembles that in chickens. Levels of hepatic microsomal cytochrome P-450 and various monooxygenase activities in chicken embryos near hatching are similar to those in adult chickens [22–24]. Certain activities such as aniline hydroxylase rise to more than twice the adult level within 1 day of hatching and then gradually fall off to the adult level during the following week [22]. The ontogenic patterns of monooxygenase activities in both brook trout and chickens contrast with those seen in the common laboratory mammals at comparable stages of development. Typically during late

* Hepatic AHH activities in nonspawning adult males (>4-year-old) and male yearlings obtained in the Spring of 1977, were 14 ± 2 (N = 3) and 15 ± 1 (N = 7) pmoles/per min/per mg microsomal protein (± S.E.), respectively, determined by analysis of fluorescent metabolites as previously described [5]. These results are not directly comparable to those in Tables 1 and 2, since hepatic microsomes rather than homogenates were assayed, and the fluorimetric rather than the radiometric AHH assay was used.

Table 2. AHH activity in liver whole homogenates from control and Aroclor 1254 exposed brook trout embryos

Exposure* group	(ppm PCBs† in whole embryos at time of assay)	AHH activity‡ (pmoles benzo[*a*]pyrene metabolites/min/mg protein)
Control	—	18 ± 5
0.75 ppm	64 ± 4	72 ± 7
7.5 ppm	229 ± 6	72 ± 13

* Embryos were exposed to Aroclor 1254 as described in the text and were assayed 44 days after fertilization, approximately 5 days before hatching, in stage 22B described by Ballard [21].

† PCBs were measured as described in the text. Data are the means of three replicates ± S.D. and are expressed on a wet weight basis for embryos removed from chorions.

‡ For each group assays were performed on a homogenate of a single pool of sixteen to twenty livers. The data are the means of five replicates ± S.D.

fetal development in nonprimate mammals, hepatic monooxygenase activities are extremely low compared with those in adults, and increase after birth in a pattern dependent on both the substrate and species [25, 26].

Fish embryos can accumulate lipophilic pollutants as a result of direct exposure [27–29] and are also likely to be loaded with a fraction of the body burden of such compounds present in their parents [30, 31]. Guiney *et al.* [31] have shown that residues of 2,5,2′,5′-tetrachlorobiphenyl in experimentally exposed rainbow trout redistribute during gametogenesis and are transferred to ova and sperm. In fish embryos the yolk serves as a sink for fat soluble xenobiotics, and these compounds will be released into the tissues during yolk absorption [28, 30]. Constitutive and induced embryonic monooxygenase activities may serve the adaptive function of facilitating the elimination of such compounds. However, the presence of monooxygenase activity in fish embryos and eleutheroembryos indicates that these stages are likely to be susceptible to the deleterious effects of a variety of toxins requiring metabolic activation. Induction of higher levels of monooxygenase activity could potentiate such effects. In an *in vivo* carcinogenesis study, Wales *et al.* [32] have shown that rainbow trout embryonic liver can activate the precarcinogen, aflatoxin b_1. However, these investigators did not examine the metabolism of aflatoxin by the embryonic tissue. Further work is necessary to elucidate the role that monooxygenase activities play in determining both the fate and effect of organic pollutants in the early developmental stages of fish.

In summary, hepatic AHH specific activity in brook trout embryos near hatching was at about the same level as in sexually immature yearlings or nonspawning adults, and the embryonic liver was competent to respond to Aroclor 1254 as an inducer of AHH activity. Around the time of hatching in brook trout, there was about a 3-fold increase in hepatic AHH specific activity, so the livers of the eleutheroembryos were considerably more active in metabolizing BP than those of yearlings. The presence of substantial monooxygenase activity during late embryonic development in fish may facilitate the elimination of lipophilic xenobiotics present in the yolk, but may also contribute to the formation of activated metabolites and toxic lesions.

Acknowledgements—This research was supported by NSF Grant OCE77-24517 and OCE80-18569. We thank Lloyd Raymond, Massachusetts Division of Fisheries and Game, for providing the embryos used here. This work was submitted by R. L. B. in partial fulfillment of the requirements for the degree of Doctor of Philosophy, granted by the Massachusetts Institute of Technology–Woods Hole Oceanographic Institution Joint Program in Biological Oceanography. Contribution No. 5212 from the Woods Hole Oceanographic Institution.

Department of Biology
Woods Hole Oceanographic Institution
Woods Hole, MA 02543, U.S.A.

ROBERT L. BINDER*
JOHN J. STEGEMAN

* To whom correspondence should be addressed. Current address: Department of Pharmacology and Toxicology, Medical College of Wisconsin, P.O. Box 26509, Milwaukee, WI 53226, U.S.A.

REFERENCES

1. J. T. Ahokas, O. Pelkonen and N. T. Karki, *Cancer Res.* **37**, 3737 (1977).
2. J. R. Bend and M. O. James, in *Biochemical and Biophysical Perspectives in Marine Biology* (Eds. D. C. Malins and J. R. Sargent), Vol. 4, p. 125. Academic Press, New York (1978).
3. C. R. Elcombe and J. J. Lech, *Toxic. appl. Pharmac.* **49**, 437 (1979).
4. J. J. Stegeman, R. L. Binder and A. M. Orren, *Biochem. Pharmac.* **28**, 3431 (1979).
5. J. J. Stegeman and M. Chevion, *Biochem. Pharmac.* **29**, 553 (1980).
6. J. J. Stegeman, in *Polycyclic Hydrocarbons and Cancer* (Eds. H. V. Gelboin and P. O. P. Ts'o), Vol. 3, p. 1. Academic Press, New York (1981).
7. L. Forlin, *Toxic. appl. Pharmac.* **54**, 420 (1980).
8. M. O. James and P. J. Little, *Chem. Biol. Interact.* **36**, 229 (1981).
9. J. F. Payne and W. R. Penrose, *Bull. environ. Contam. Toxic.* **14**, 112 (1975).
10. A. H. Conney, *Pharmac. Rev.* **19**, 317 (1967).
11. J. R. Gillette, J. R. Mitchell and B. B. Brodie, *A. Rev. Pharmac.* **14**, 217 (1974).
12. S. S. Thorgeirsson and D. W. Nebert, *Adv. Cancer Res.* **24**, 149 (1977).
13. J. J. Lech and J. R. Bend, *Environ. Hlth Perspect.* **34**, 115 (1980).
14. W. W. Kuhnhold, D. Everich, J. J. Stegeman, J. Lake and R. E. Wolke, in *Proceedings: Conference on Assessment of the Ecological Impact of Oil Spills*, p. 667. American Institute of Biological Sciences, Washington (1978).
15. J. M. McKim, *J. Fish. Res. Bd Can.* **34**, 1148 (1977).
16. H. Rosenthal and D. F. Alderdice, *J. Fish. Res. Bd Can.* **33**, 2047 (1976).
17. R. D. Harbison, *Environ. Hlth Perspect.* **24**, 87 (1978).
18. R. L. Binder and J. J. Stegeman, *Biochem. Pharmac.* **29**, 949 (1980).
19. E. K. Balon, *J. Fish. Res. Bd Can.* **32**, 1663 (1975).

20. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
21. W. W. Ballard, *J. exp. Zool.* **184**, 7 (1973).
22. G. Powis, A. H. Drummond, D. E. MacIntyre and W. R. Jondorf, *Xenobiotica* **6**, 69 (1976).
23. C. F. Strittmatter and F. T. Umberger, *Biochim. biophys. Acta* **180**, 18 (1969).
24. J. M. Machinist, E. W. Dehner and D. M. Ziegler, *Archs Biochem. Biophys.* **125**, 858 (1968).
25. G. F. Kahl, D. E. Friederici, S. W. Bigelow, A. B. Okey and D. W. Nebert, *Devl Pharmac. Ther.* **1**, 137 (1980).
26. C. R. Short, D. A. Kinden and R. Stith, *Drug Metab. Rev.* **5**, 1 (1976).
27. M. B. Eldridge, T. Echeverria and S. Korn, *J. Fish. Res. Bd Can.* **35**, 861 (1978).
28. W. W. Kuhnhold and F. Busch, *Meeresforsch. Rep. Mar. Res.* **26**, 50 (1977/1978).
29. P. D. Guiney, M. J. Melancon, Jr., J. J. Lech and R. E. Peterson, *Toxic. appl. Pharmac.* **47**, 261 (1979).
30. G. J. Atchison, *J. Great Lakes Res.* **2**, 13 (1976).
31. P. D. Guiney, J. J. Lech and R. E. Peterson, *Toxic. appl. Pharmac.* **53**, 521 (1980).
32. J. H. Wales, R. O. Sinnhuber, J. D. Hendricks, J. E. Nixon and T. A. Eisele, *J. natn. Cancer Inst.* **60**, 1133 (1978).

Biochemical Pharmacology, Vol. 32, No. 7, pp. 1327–1328, 1983.
Printed in Great Britain.

0006-2952/83/071327-02 $03.00/0

Kinetics of deamination of 5-aza-2′-deoxycytidine and cytosine arabinoside by human liver cytidine deaminase and its inhibition by 3-deazauridine, thymidine or uracil arabinoside*

(*Received* 13 *July* 1982; *accepted* 10 *November* 1982)

5-Aza-2′-deoxycytidine (5-aza-dCyd) was shown to be a potent antineoplastic agent against tumor cells *in vitro* [1] and against leukemic cells in mice [2, 3]. This agent is now undergoing clinical trial at this institution in leukemic patients resistant to conventional chemotherapy [4]. Because of the rapid plasma elimination half-life seen in man [4], we were interested to study the interaction of 5-aza-dCyd with human liver cytidine deaminase and to compare it with 5-aza-cytidine (5-aza-Cyd) and cytosine arabinoside (ara-C), nucleoside analogs which are used currently for the clinical treatment of leukemias. Also, we tested the potential interference of various substances with the deamination of 5-aza-dCyd, 5-aza-Cyd or ara-C. The potential inhibitors chosen were 3-deazauridine (3-DU), thymidine (Thd), and uracil arabinoside (ara-U). 3-DU and Thd have been shown to enhance the antileukemic action of 5-aza-dCyd [5, 6] and ara-C [7, 8]. Ara-U is the deaminated catabolite of ara-C and may attain higher plasma concentrations than the parent compound, especially when high dose ara-C therapy is administered [9].

5-Aza-dCyd was obtained from Chemapol (Prague, Czechoslovakia); 5-aza-Cyd and ara-C were obtained from the Drug Development Branch, National Cancer Institute (Bethesda, MD); cytidine (Cyd), deoxyuridine and uridine were purchased from P-L Biochemicals (Milwaukee, WI); and deoxycytidine (dCyd) and Thd were purchased from Boehringer Mannheim (West Germany). 3-DU was obtained from Ash Stevens Inc. (Detroit, MI), and uracil arabinoside was purchased from Calbiochem (San Diego, CA).

Human cytidine deaminase was prepared from freshly frozen liver which was obtained at autopsy from a normal adult. The enzyme purification was done according to Wentworth and Wolfenden [10]. The final purification step yielded a specific activity of 8.2×10^{-3} units/mg protein. Protein concentrations were determined by the method of Lowry *et al.* [11]. One unit of activity was defined as that amount of enzyme required to deaminate 1 μmole cytidine/min under the following conditions. The deamination of the substrates was followed by measuring the decrease in absorbance at 291 nm for Cyd, dCyd and ara-C, whereas the deamination of 5-aza-Cyd and 5-aza-dCyd was followed at 272 nm. Kinetic measurements were also done at 247 nm for 5-aza-dCyd and gave similar results as at 272 nm. A recording Gilford DU-2 spectrophotometer was used with a thermally regulated block. Assays were conducted at 25° in 20 mM potassium phosphate buffer, pH 7.4. Changes in extinction coefficients corresponding to complete deamination of substrates were determined enzymatically from the change in absorbance after prolonged incubation with the enzyme for all substrates. In the case of Cyd, dCyd, and ara-C, a similar molar extinction coefficient was found enzymatically and with pure substrates and products. For the two 5-aza-analogues the deaminated products did not have any detectable absorbance at 272 nm. The changes in molar extinction coefficients using a 10 mm light path were: Cyd, −1297; dCyd, −1169; ara-C, −1350; 5-aza-Cyd, −904; and 5-aza-dCyd, −750.

The reaction products of the 5-aza-dCyd deamination were analyzed by TLC and high pressure liquid chromatography (HPLC). Excess enzyme and substrate were incubated at 8° during 8 hr at pH 7.4. TLC separation was achieved on Avicel cellulose plates (1000 μm thick) using the solvent system *n*-butanol–acetic acid–water (100:10:30) [12] at 4° and gave an R_f value of 0.24 for 5-aza-dCyd. HPLC separations were made after a trichloroacetic acid (TCA) precipitation of a similar reaction mixture followed by a neutralization with a concentrated phosphate buffer. An aliquot (20 μl) was injected on a C_{18} μBondapak (10 μm) reverse-phase column using a 10 mM potassium phosphate buffer (pH 6.8) as the eluent at a flow rate of 1.5 ml/min. A variable wavelength u.v. detector was used. The retention time for 5-aza-dCyd was 5 min.

The different K_m and V_{max} values for the substrates studied are shown in Table 1. It can be seen that the two natural substrates, Cyd and dCyd, showed the lowest K_m for the enzyme. Ara-C showed an intermediate K_m as compared to the natural substrates and the two 5-aza-analogues, which had the highest K_m for the human liver cytidine deaminase. On the other hand, the enzyme had a similar V_{max} for all the substrates studied, and this would indicate that, once

* This work was supported by Grant MA-6356 from the Medical Research Council of Canada, by the Conseil de la Recherche en Santé du Québec, and by LEUCAN.