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SHORT COMMUNICATIONS

Induction of aryl hydrocarbon hydroxylase activity in embryos of an estuarine fish

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Fish microsomal cytochrome P-450 mono-oxygenases or mixed-function oxidases (MFO), like the well studied mammalian systems, catalyze the biotransformation of a variety of lipophilic foreign compounds [1–3]. Increased levels of hepatic and in some cases extrahepatic MFO activities in fish have been induced by experimental exposure to environmental chemicals including polychorinated biphenyls (PCB's), crude oils and polycyclic aromatic hydrocarbons [4–6]. Such induction also seems to occur in fish inhabiting polluted environments [7–10].

Early developmental stages of fish generally have been found to be more sensitive than adults to pollutant toxicity [11,12]. The capacity of these stages to metabolize foreign compounds may play an important role in determining their tolerance to environmental contaminants. Metabolism of xenobiotics to more polar and readily eliminated products often serves as a detoxification process [13], but with certain compounds such metabolism can result in the formation of cytotoxic, mutagenic and carcinogenic metabolites [14,15] to which embryonic cells can be particularly sensitive [16]. We report here on the capacity of fish embryos to metabolize foreign compounds and to respond to known inducers of fish cytochrome P-450. In particular, we describe the metabolism of benzo[*a*]pyrene (BP), a widespread contaminant of the marine environment [17], by embryos of the estuarine killifish *Fundulus heteroclitus* and the effects of PCBs and petroleum hydrocarbons on this activity.

[^{3}H]BP was purchased from Amersham Searle, Arlington Heights, IL, BP from the Aldrich Chemical Co., Milwaukee, WI, and NADPH from the Sigma Chemical Co., St. Louis, MO. Aroclor 1254 was lot AA-1 from the Monsanto Co., St. Louis, MO, and the No. 2 fuel oil was an American Petroleum Institute reference standard.

Ripe fish were collected at Herring Brook (H.B.) and Wild Harbor (W.H.) marshes (North Falmouth, MA) during the spawning season of *Fundulus* in the spring and summer of 1978. Eggs were stripped and fertilized in the laboratory [18] and treated as described in the tables. The eggs described in Table 1 were from H.B. and those described in Table 2 from W.H.; adults were from H.B. After determining the developmental stage according to Armstrong and Child [19], eggs* were washed twice with filtered seawater and twice with 0.1 M Tris–HCl, pH 7.4, and 0.25 M sucrose, and then homogenized in this buffer (20%, w/v) using a Potter–Elvehjem tissue grinder. The homogenates were fractionated by centrifugation: 5 min at 120 *g*, 10 min at 10,000 *g* (10K) and 90 min at 40,000 *g* (40K). The first fraction containing much large debris was discarded, and the 10K and 40K pellets were resuspended in 0.1 M Tris–HCl, pH 7.0 (0.5–3.0 μl/embryo). This fractionation procedure roughly corresponds to one that yields mitochondrial (10K) and microsomal (40K) fractions from adult fish liver [3], although in the present study the composition of these fractions was not determined. Yolk sac larvae were dissected and the livers, carefully separated from gall bladders, were assayed as dilute whole homogenates in the egg homogenization buffer. The remaining larval carcasses were fractionated and assayed like the eggs.

Aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase (AHH) was assayed by the sensitive radiometric procedure of Van Cantfort *et al.* [20] modified for use on a microscale. The reaction volume was reduced to 25 μl and consisted of 0.1 M Tris–HCl, pH 7.0, 0.400 mM NADPH, 0.060 mM [^{3}H]benzo[*a*]pyrene (about 300 μCi/μmole), 2 mg/ml of bovine serum albumin (BSA), and from 5 to 170 μg of embryonic protein depending on the fraction assayed. Blanks consisted of the complete reaction mixture without NADPH. The reaction was initiated by adding BP in 1 μl of acetone, was incubated at 25° for 30 min, was stopped with 50 μl of 0.15 M KOH in 85% dimethylsulfoxide, and was then extracted three times with 0.375 ml of hexane. Polar metabolites were quantitated by counting 30 μl of the aqueous phase acidified with 10 μl of 0.6 N HCl, in 3 ml of scintillation mixture. The liquid scintillation counting efficiency was determined by internal standardization. The reaction mixture was found to be optimal for adult *Fundulus* hepatic microsomal AHH. The reaction with adult microsomes was linear for 35 min at 25°. Protein was determined by the method of Lowry *et al.* [21] with crystalline BSA as a standard.

AHH activity was considered detectable when the complete reaction mixture differed from the blank at the 0.01 level of significance by Student's *t*-test. Based on the average variance of ten triplicate blanks, it is estimated

Table 1. Aryl hydrocarbon hydroxylase activity in the 10,000 *g* fraction of homogenates of *F. heteroclitus* eggs exposed to Aroclor 1254 and No. 2 fuel oil*

Treatment	Activity (fmoles/min/egg)†
DMSO control	ND‡
20 p.p.b. Aroclor 1254	ND
100 p.p.b. Aroclor 1254	30.3 ± 2.0
200 p.p.b. Aroclor 1254	62.2 ± 3.7
1000 p.p.b. No. 2 fuel oil	38.6 ± 6.1

* Eggs stripped from eleven females were pooled and fertilized with minced testes from four males. For each treatment, approximately ninety eggs were placed in a glass dish with 50 ml of 0.22 μm filtered seawater and incubated at 20°. The exposure was initiated 8.5 hr after fertilization by the addition of the inducing substance dissolved in 50 μl dimethylsulfoxide (DMSO). DMSO alone was added to the control eggs. Water was changed and additional inducer was added six times before assay at day 12 (stages 33–34); p.p.b. = μg/liter.

† fmole = femtomole (10^{-15} mole); mean of three replicates ± S.E.

‡ ND = not detectable. Limit of detection: 17 fmoles/min/egg.

* The term egg is used here to indicate the embryonic stages up to hatching. Yolk sac larvae are embryos that have hatched. The embryonic period extends until complete absorption of the yolk when the true larval period begins.

that, for the power of the t-test to be 0.95 with $P < 0.01$, the sample assayed must have the capacity to produce 3.0 pmoles of polar metabolites during the incubation [22]. The limits of detection indicated in the tables were calculated by dividing this value by the number of eggs per reaction mixture and the incubation time.

AHH activity was not reproducibly detectable in untreated eggs assayed at stages from zygote to shortly before hatching. As shown in Table 1, exposure to the PCB mixture Aroclor 1254 or to No. 2 fuel oil over the course of development resulted in an induction of AHH activity in eggs near hatching (stages 33–34; hatching occurs in stage 34). The induction of AHH by Aroclor 1254 was dose dependent. The induced activity appeared in the 10K fraction, while no activity was detectable in the 40K fraction.

In a separate experiment, *Fundulus* embryos exposed to 200 p.p.b. Aroclor 1254 were assayed at stages 32–33 and shortly after hatching as yolk sac larvae (stages 34–35). As before, there was no detectable AHH activity in the control eggs (Table 2), and substantial activity was present in the 10K fraction of eggs treated with Aroclor 1254. AHH activity was higher in the 10K fraction of these eggs than in those in Table 1 exposed to 200 p.p.b. Aroclor 1254; activity was also detectable in the 40K fraction, perhaps because there were four more water changes with addition of inducer in this experiment.

Unlike control eggs, control yolk sac larvae had detectable AHH activity in all fractions assayed. Comparing the total activity in the two groups of larvae reveals that Aroclor stimulated a greater than 3-fold induction of AHH activity. Induction apparently occured to a greater extent in the extrahepatic tissues of larvae than in larval liver, as liver accounted for more than half of total activity in the controls but less than half in the treated group. In either case the data suggest that extrahepatic tissues of fish embryos may play a significant role in the metabolism of foreign compounds. The reason for a more substantial portion of MFO activity sedimenting with the lighter fraction of yolk sac larvae but with the heavier fraction of eggs is unknown. Chatterjee *et al.* [23], however, reported a similar difference between the sedimental properties of liver microsomal enzymes from fetal and neonatal rats which may have been the result of the formation of vesicles from the fetal hepatic endoplasmic reticulum (ER) that were larger than the microsomes produced from neonatal or adult hepatic ER.

To compare hepatic AHH activities in yolk sac larvae and adults, separate pools of adult liver from three males and three females were assayed as whole homogenates like the larval livers. The mean AHH activity in these two pools (± range) was 71.3 ± 2.6 pmoles/min/mg liver protein, while larval hepatic AHH activities normalized to liver protein were 35.7 ± 5.0 and 87.6 ± 20 pmoles/min/mg protein for control and PCB-treated larvae respectively. Thus, control yolk sac larval liver was about 50 per cent as active as untreated adult liver in metabolizing BP.

The data presented clearly demonstrate the induction of AHH activity in fish embryos exposed to common environmental contaminants. The capacity to metabolize lipophilic foreign compounds and to respond to MFO inducers near the time of hatching, when a large portion of the yolk sac remains, could be adaptive. Fat soluble xenobiotics will tend to concentrate in the yolk and be released into the embryo during yolk absorption; constitutive and induced MFO activities should facilitate the elimination of such compounds.

Fish embryonic MFO activity could also play a role in pollutant-induced lesions, including teratogenic effects, by producing reactive and mutagenic metabolites during organodifferentiation. BP, for example, is a precarcinogen in mammals, that requires metabolic activation by cytochrome P-450 mono-oxygenase activity and the microsomal enzyme, epoxide hydrase [24]. Preparations from adult fish liver have been shown to activate BP to mutagenic and cytotoxic products in a bacterial mutation assay system [25]. The presence of AHH activity, whether constitutive or induced, in *Fundulus* embryos suggests that they, too, have the capacity to activate BP and related compounds. Wales *et al.* [26] have reported that a single 1-hr exposure of rainbow trout embryos to the precarcinogen aflatoxin

Table 2. AHH activity in fractions of homogenates of control and Aroclor-exposed *Fundulus* eggs and yolk sac larvae*

Embryonic material	Fraction	Activity† (fmoles/min/embryo)	
		Control	200 p.p.b. Aroclor 1254
Whole eggs	10K	ND‡	100 ± 5
	40K	ND	11.9 ± 2.3
Dissected larvae	Carcass, 10K	21.5 ± 1.8	133 ± 5
	Carcass, 40K	16.6 ± 2.9	75.9 ± 6.9
	Liver, whole homogenate	51.8 ± 5.8	117 ± 11

* Eggs stripped from six females and fertilized with minced testes from four males were treated under conditions similar to those described in Table 1. At the time of assay, 11 days of development, control eggs were in stage 33 (N = 122), while PCB-treated eggs were in stages 32–33 (N = 121). Control yolk sac larvae (N = 34) were assayed in stages 34–35 after 14 days of development. Aroclor 1254-exposed yolk sac larvae (N = 40) were assayed in the same stages after 16 days of development. Median hatching times for control and Aroclor-treated eggs were 14 and 16 days, respectively. Approximately 90 per cent hatching of the control eggs occured by day 15 after fertilization, whereas about 19 days were required for 90 per cent of the Aroclor-exposed eggs. Hatching success in the two groups was similar: 95 per cent for the controls and 93 per cent for the Aroclor-treated eggs. Water was changed and additional inducer added ten times before eggs were assayed, and fourteen times before treated yolk sac larvae were assayed.

‡ Whole egg data: mean of three replicates ± S.E. Data for larvae represent mean of two replicates ± range.

‡ ND = not detectable. Limits of detection: 10K fraction, 16 fmoles/min/egg; 40K fraction, 9.9 fmoles/min/egg.

B_1 induced a significant number of liver tumours in fish 9–12 months later. Thus, the risk to fish populations, associated with spawning in contaminated environments, may be due in part to bioactivation of foreign compounds during embryonic development.

In summary, exposure of developing *F. heteroclitus* eggs to PCBs or whole No. 2 fuel oil induced aryl hydrocabon hydroxylase activity in eggs near hatching. PCBs induced AHH activity in both the liver and extrahepatic tissues of similarly exposed yolk sac larvae. AHH activity was present both in hepatic and extrahepatic tissues of control larvae but was undetectable in control eggs. The uninduced activity in yolk sac larval liver was about 50 per cent of that in adult liver. Embryonic metabolism of environmental chemicals could be adaptive during yolk absorption but might also contribute to lesions by production of activated metabolites.

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Comparative effects of mixed function oxidase inhibitors on adrenal and hepatic xenobiotic metabolism in the guinea pig

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In recent years, extra-hepatic xenobiotic metabolism has been studied extensively in a variety of tissues including the lung, kidney, intestine, skin and placenta (see Ref. 1). The oxidative metabolism of polycyclic aromatic hydrocarbons (PAH) such as benzo[*a*]pyrene (BP) by cytochrome P-450-containing enzymes has been of particular interest because of the ubiquitous distribution and potential carcinogenicity, mutagenicity and toxicity of PAH. Extrahepatic metabolism of PAH may represent an important detoxifying mechanism, especially in organs that serve as primary portals of entry to the body, but may also result in the production of highly toxic metabolites [1–3]. The results of numerous investigations indicate that the characteristics of PAH-metabolizing enzymes vary considerably from tissue to tissue. For example, responses to inducing agents, well as to enzyme inhibitors, are highly tissue dependent [1–3]. Such differences in enzyme characteristics may play an important part in determining the relative toxicities of PAH in different organs.

Many foreign compounds, including PAH, are rapidly metabolized by adrenocortical tissue, especially that obtained from human fetuses [4–7] or from guinea pigs [8–