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SHORT COMMUNICATIONS

Hepatic cytochrome P-450 isozymes and aryl hydrocarbon hydroxylase in English sole (*Parophrys vetulus*)

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In recent years considerable attention has been focused on the ability of benthic fish to metabolize carcinogens because of the high incidence of liver cancer reported in certain bottom-dwelling fish from industrialized areas [1, 2]. In their extensive research on English sole (*Parophrys vetulus*) from Puget Sound, WA, Malins and coworkers [2, 3] have demonstrated positive correlations among variables, such as the levels of PAH* in sediment, levels of aromatic compounds fluorescing at BaP wavelengths in bile of English sole, and frequency of hepatic lesions, including neoplasms. Accordingly, English sole serves as an excellent model to study carcinogen activation and detoxication in the laboratory. Using BaP as a representative of carcinogenic PAH present in sediment of urban estuaries, we have shown [4, 5] that the values for covalent binding of BaP intermediates to hepatic DNA in BaP-exposed sole are substantially greater than the values reported for the liver of rats and mice. Nishimoto and Varanasi [6] have shown that the liver microsomes of these fish convert BaP, in the presence of DNA, to produce essentially a single adduct, N^2-[10β(7β,8α,9α-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene)-yl]-deoxyguanosine, which is causally linked to skin cancer in rodents [7]. Because the cytochrome P-450 system [8] is involved in both the initial oxidation of carcinogenic PAH (e.g. epoxide formation), and the subsequent formation of ultimate carcinogens (e.g. diol epoxides), studies to evaluate the ability of English sole to metabolize carcinogens should include characterization of the cytochrome P-450 system.

Considerable research on marine and freshwater fish shows that, although piscine cytochrome P-450-dependent monooxygenase systems have many similarities with mammalian systems, important differences, such as lower temperature optima and strong response to "PAH-type" inducers, but refractiveness to "phenobarbital-type" inducers, make the study of piscine systems interesting [9–12]. Purifications of cytochromes P-450 from little skate, rainbow trout and scup have shown the existence of multiple P-450 isozymes [13–15]. Such studies have aided considerably in our understanding of differences in the metabolism of xenobiotics by fish and mammals.

In this paper, we present the results of our initial investigations of cytochrome P-450 isozymes in English sole and their relationship to the metabolism of BaP.

Materials and methods

Juvenile English sole were captured in May, 1985 (water temperature 13°) from Discovery Bay (48°2′N, 122°51′W; twelve fish, 171 ± 15 mm) and President Point (47°46′N, 122°28′W; eleven fish, 172 ± 22 mm), in Washington State. Livers and gall bladders were excised immediately. Livers were frozen in liquid nitrogen and subsequently stored at −70°. Samples of bile were collected from nine of the twelve fish from Discovery Bay. The other three fish, and all of the fish from President Point, did not have enough bile in their gall bladders to sample. Bile samples were analyzed for aromatic compounds fluorescing at BaP wavelengths by HPLC [3]. Liver samples were pooled (1–2 g of tissue/pool) according to sex and, for Discovery Bay fish, by relatively high or low bile fluorescence (see Table 1). Hepatic microsomes were prepared [16], and samples of microsomal protein (50 μg) were analyzed by SDS-PAGE [17]. Immunodetection was performed by electrophoretically transferring the samples to nitrocellulose via Western blotting, and treatment with rabbit anti-trout-LM_2-IgG (LM_2-IgG) and rabbit anti-trout-LM_{4b}-IgG (LM_{4b}-IgG) [14]. Cross-reacting proteins were visualized by staining with ^{125}I-labeled protein A and quantitated by autoradiography [18]. However, because the affinities of trout LM_{4b}-IgG and LM_2-IgG for English sole cytochromes P-450 are not known, quantitations are only relative, and not absolute.

AHH (EC 1.14.14.1) activity of the microsomal suspensions was assayed radiometrically using BaP as the substrate [16]. The reaction mixture consisted of 450 μl 0.05 M Tris, pH 7.5, 5 mM $MgCl_2$, 1 mM NADPH, and 37.5 μl microsomal suspension (0.2 to 0.8 mg protein). After 15 min preincubation at 25°, the reaction was started by adding 40 nmoles [^{14}C]BaP in 12.5 μl acetone. The reaction was stopped by adding 1.0 ml of 0.15 M KOH in 85/15 (v/v) DMSO/H_2O. Unreacted substrate was extracted with hexane, and the DMSO/aqueous phase was assayed for ^{14}C associated with reaction products. The samples which showed the highest and lowest AHH activities were assayed again for AHH, with the addition of LM_{4b}-IgG or LM_2-IgG, at 0.5, 1.0, 2.0, and 5.0 mg IgG/mg microsomal protein. The microsomes were preincubated at 25° with the LM_{4b}-IgG or LM_2-IgG for 15 min before addition of substrate. Control rabbit IgG had no effect on AHH activity.

Results and discussion

A 16-fold difference was observed in values for pooled hepatic AHH activity of English sole captured from two minimally polluted sites containing low levels of PAH in sediment (Table 1). Fish from Discovery Bay, the less polluted of the two sites with 49 ppb (μg PAH/kg sediment, wet weight) of selected PAH in sediment samples [19], had the two lowest values of AHH activity [36 and 81 pmoles BaP metabolized·min^{-1}·(mg protein)$^{-1}$], whereas all fish from President Point (approximately 1100 ppb PAH in the sediment samples [20]) had high values [330–570 pmol·min^{-1}·(mg protein)$^{-1}$]. Such a wide variation in hepatic AHH activity has been noted in other feral fish species and high values of AHH activity are attributed to exposure of fish to PAH or PAH-type inducers present in their environment [21, 22]. In support of this hypothesis, the data from Discovery Bay show that higher AHH activities were found in fish which showed relatively higher levels of aromatic compounds in bile that fluoresce at BaP wavelengths (Table 1). These data, along with the very low

* Abbreviations: PAH, polycyclic aromatic hydrocarbons; BaP, benzo[*a*]pyrene; HPLC, high pressure liquid chromatography; AHH, aryl hydrocarbon (benzo[*a*] pyrene) hydroxylase; EH, epoxide hydrolase; GST, glutathione-*S*-transferase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; and DMSO, dimethylsulfoxide.

Table 1. AHH activities and concentrations of cytochrome P-450 isozymes in hepatic microsomes of English sole

Pool No.	Sex	Number of fish/pool	Location*	AHH†	LM_{4b}‡,§	LM_2‡
1	M	2	Discovery Bay	36	1.4	12
2	M	3	Discovery Bay	330	13	14
3	F	4	Discovery Bay	81	2.6	9.2
4	F	3	Discovery Bay	200	5.6	12
5	M	3	President Point	350	23	14
6	M	3	President Point	570	30	19
7	F	2	President Point	330	17	23
8	F	3	President Point	440	23	18

* Bile of fish from Discovery Bay was analyzed for aromatic compounds fluorescing at BaP wavelengths (380 nm excitation/430 nm emission), and quantitation was based on fluorescence of a BaP standard. Individual values (ppb) were: Pool 1—36 and 74; Pool 2—120 and 610; Pool 3—90 and 170; Pool 4—250, 1000, and 270. Remaining fish did not have sufficient bile for collection.

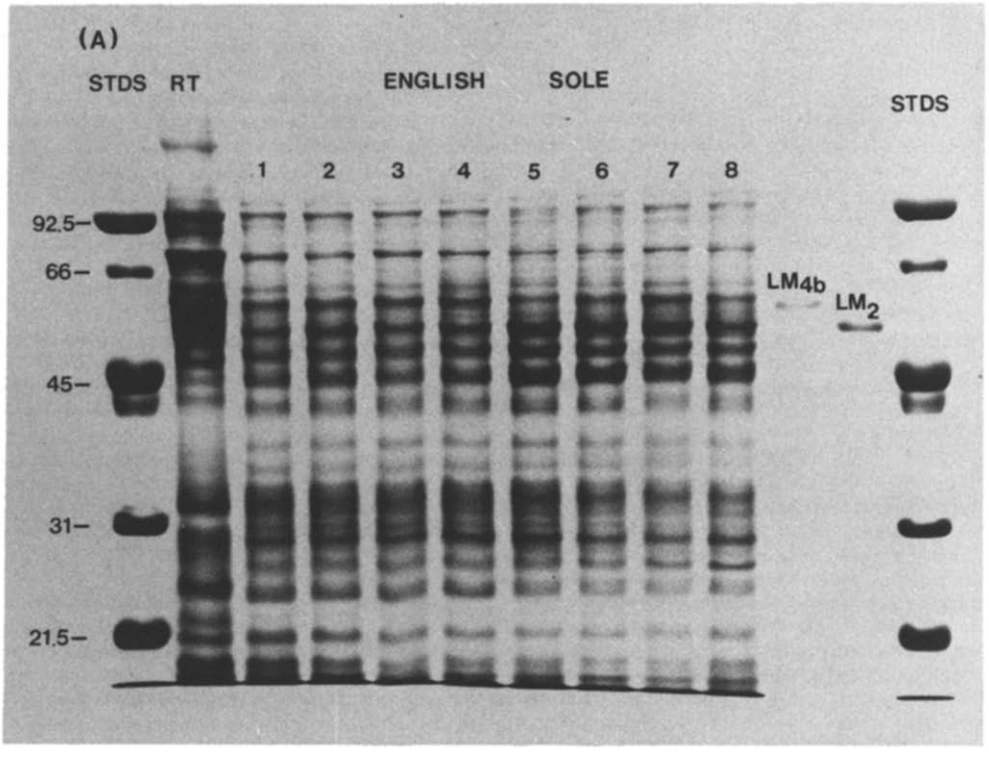
† Expressed in pmoles product formed·(mg protein)$^{-1}$·min^{-1}.

‡ Expressed in pmoles trout LM_{4b} (or LM_2) equivalents·(mg protein)$^{-1}$.

§ Because $RTLM_{4a}$ cross-reacts with $RTLM_{4b}$-IgG, quantitation may include any LM_{4a}-type isozymes, if present in English sole.

sediment concentrations of PAH found in Discovery Bay, also suggest that the fish with high AHH activities had migrated recently from a contaminated area. However, this is only a partial explanation because marine sediment within a relatively small area can often be heterogeneous with respect to concentrations of xenobiotics [20]. Thus, the relationship observed between hepatic AHH activity and BaP-fluorescence in bile of English sole (Table 1) shows how such biochemical variables can provide useful information on the xenobiotic exposure of feral fish in their environment, and emphasizes that the measurement of xenobiotics in sediment or water alone may not give accurate information on xenobiotic exposure of fish.

The electrophoretogram of sole liver microsomes (Fig. 1A) shows a number of bands in the range of molecular weight (50,000–58,000) of cytochromes P-450 purified from fish species such as rainbow trout, little skate and scup [13–15]. The autoradiograms of the Western blot of liver microsomes (Figs. 1B and C) show that two separate cytochromes P-450 in sole cross-reacted with antibodies to purified trout liver cytochromes P-450, LM_2 (mol. wt 54,000) and LM_{4b} (mol. wt 58,000) respectively. Williams and Buhler [14] have purified and characterized five hepatic cytochromes P-450 (LM_1, LM_2, LM_3, LM_{4a}, LM_{4b}) from rainbow trout; of these P-450s, LM_{4b} is highly responsive to PAH-like inducers and was demonstrated to be closely



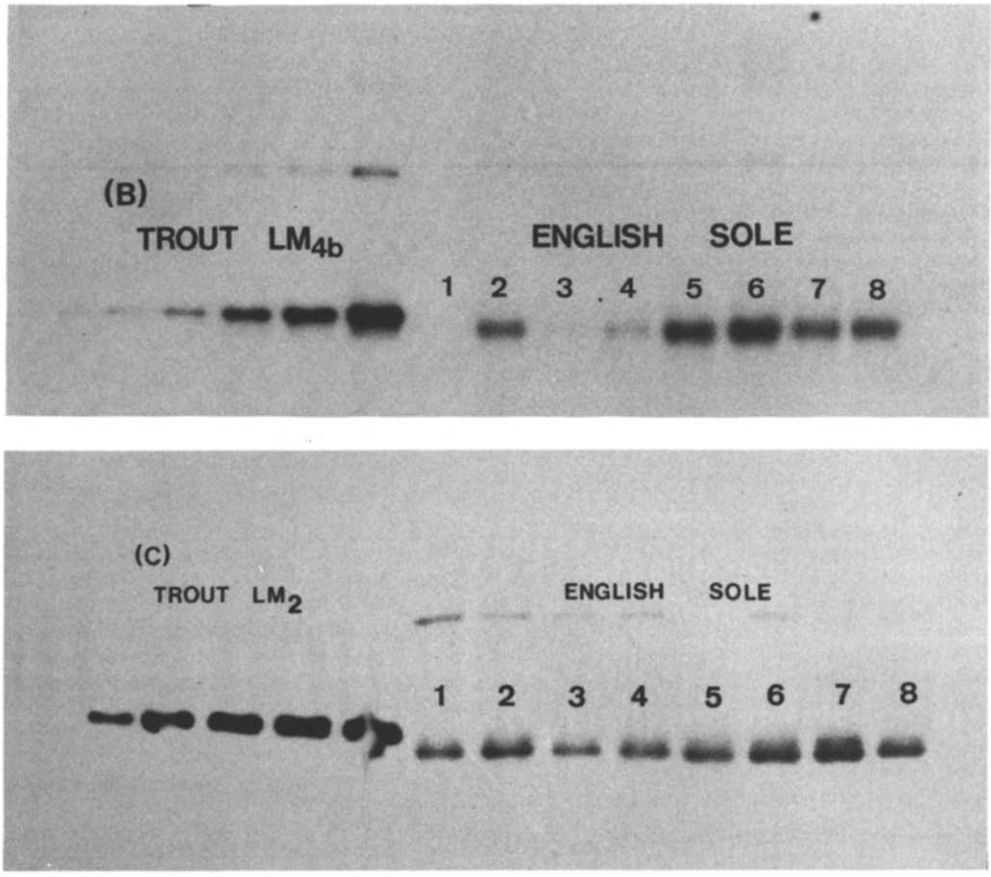


Fig. 1. (A) Electrophoretic separation by SDS-PAGE. Standards (STDS) are phosphorylase B (92,500), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500). RT, rainbow trout hepatic microsomal protein. (B) Autoradiogram of Western blot of hepatic microsomal proteins cross-reacting with rabbit IgG to purified trout LM_{4b}. (C) Autoradiogram of Western blot of hepatic microsomal proteins cross-reacting with rabbit IgG to purified trout LM_2 (see Table 1 for details).

related to a P-448 type cytochrome. The form of cytochrome P-450 in English sole that cross-reacted with LM_{4b}-IgG had a slightly lower molecular weight of about 54,000. The results in Fig. 2 show that the concentration of LM_{4b} in sole liver microsomes was highly correlated ($r = 0.96$) with AHH activity. Studies with purified trout liver cytochrome LM_{4b} have shown that in reconstituted systems this isozyme efficiently metabolizes BaP and produces a preponderance of bay-region diols [14]. The present results showing that sole liver microsomes contain a form of P-450 that cross-reacted with trout LM_{4b}-IgG is in agreement with the findings of BaP metabolism by sole liver microsomes [6, 23] or the intact fish [24, 25], showing a tendency to produce BaP 7,8-dihydrodiol, which can be metabolized to the ultimate carcinogen, BaP 7,8-dihydrodiol-9,10-epoxide [6].

The second form of cytochrome P-450 detected in sole liver microsomes had an estimated molecular weight of about 52,000 and cross-reacted with LM_2-IgG (Fig. 1C). LM_2 is believed to be a constitutive isozyme in trout and is correlated with aflatoxin metabolism, but not with BaP metabolism [26]. The present results with sole liver microsomes show that AHH activity did not correlate as well ($r = 0.71$) with the relative concentration of LM_2 as it did with the relative concentration of LM_{4b} (Table 1). The ratio of sole liver cytochrome cross-reacting with LM_2-IgG to the cytochrome cross-reacting with LM_{4b}-IgG was substantially greater than one for those microsomal preparations having the lowest AHH activities; however, as AHH activity increased, the ratio diminished and was less than one for microsomal preparations having the highest AHH activities (Table 1). Moreover, the addition of LM_2-IgG to sole hepatic microsomes did not inhibit AHH activity, whereas the addition of LM_{4b}-IgG efficiently reduced AHH activity to 10% of the original value in two samples of microsomes showing the highest and lowest AHH activities (Fig. 3). Thus, it appears that of these two hepatic cytochromes P-450 in sole, only the isozyme with cross-reactivity to trout LM_{4b}-IgG was appreciably active towards BaP. The results showing the presence of detectable levels of this isozyme, believed to result from PAH exposure, in fish from relatively uncontaminated areas are consonant with findings for other feral fish showing a certain degree of induction of cytochrome P-450-dependent monooxygenases even in pristine areas [21, 22, 27]. However, the possibility that the low levels of the isozyme cross-reacting with LM_{4b}-IgG may represent the basal levels in liver of English sole cannot be ruled out. It is obvious that detailed studies on characterization of cytochrome P-450-dependent monooxygenases along with other xenobiotic metabolizing enzymes, such as EH, GST, and UDP-glucuronyl transferase, are needed to provide a better understanding of the effect of environmental pollution on the ability of English sole to metabolize xenobiotics, as well as certain important endobiotic compounds (e.g. steroids). In that context, preliminary results [28] show that, while hepatic GST activity

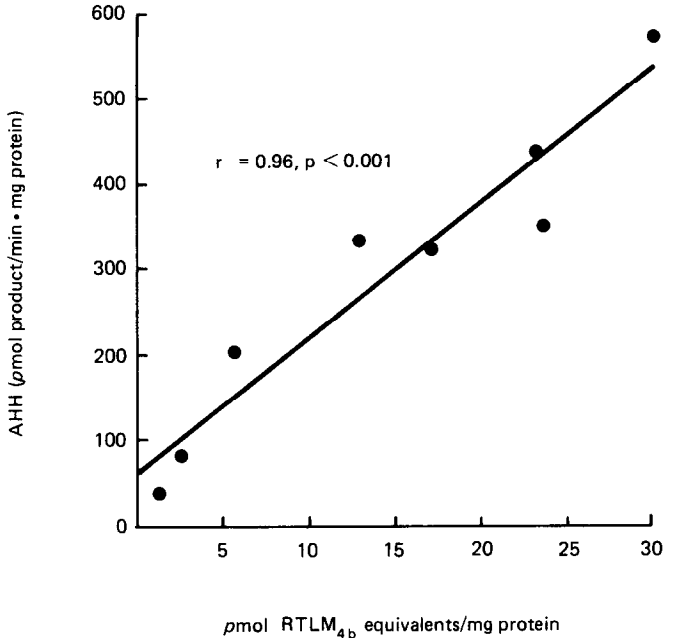


Fig. 2. Correlation between levels of AHH activity and rainbow trout LM_{4b} ($RTLM_{4b}$) equivalents in hepatic microsomes from English sole.

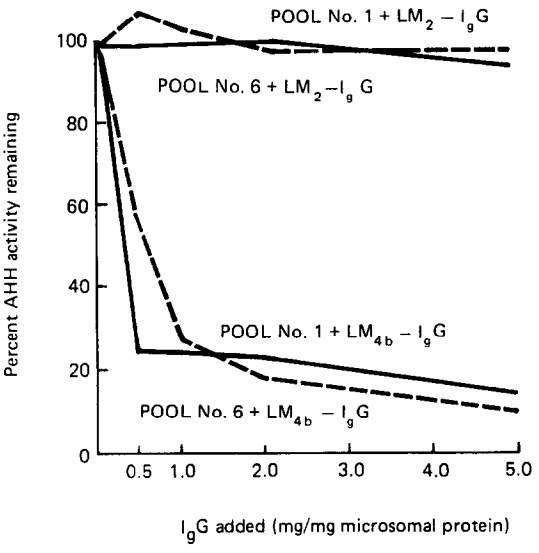


Fig. 3. Effect of addition of rabbit-anti-trout-LM_{4b} (LM_{4b}-IgG) and rabbit-anti-trout-LM_2 (LM_2-IgG) on AHH activity of hepatic microsomes of English sole [pools 1 (——) and 6 (– – –); see Table 1 for details].

is substantially higher in sole sampled from industrialized areas as compared to relatively uncontaminated areas, no such difference is observed in the activity of EH.

No sex-related differences in levels of (a) hepatic AHH activities, (b) aromatic compounds fluorescing at BaP wavelengths in bile, or (c) the two cytochrome P-450 isozymes were apparent in these juvenile fish. In studies of BaP metabolism *in vivo*, no sex-related differences are noted in juvenile English sole [4, 5, 24]. However, detailed studies with adult fish at different times of the year and at different stages of gonadal maturity are needed to assess whether sex-related differences exist in the ability of adult English sole to metabolize xenobiotics.

In summary, immunochemical techniques utilizing rabbit antibodies raised to two trout liver cytochrome P-450 isozymes have shown the presence of at least two cytochromes P-450 in liver of English sole sampled from relatively uncontaminated areas. The level of isozyme that cross-reacted with the rabbit antibody to trout LM_{4b}, a cytochrome P-448 type isozyme, was strongly correlated with hepatic AHH activity; the isozyme that cross-reacted with LM_2-IgG did not correlate as well with hepatic AHH. Moreover, LM_{4b}-IgG strongly inhibited AHH activity in sole liver microsomes, whereas LM_2-IgG did not have any discernible effect on AHH activity.

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Oxidation products are responsible for the resistance to the action of collagenase conferred on collagen by (+)-catechin

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Among many other pharmacological properties [1, 2], (+)-catechin [or (+)-cyanidanol-3], a flavonoid, has been attributed [3, 4] a stabilizing effect on collagen which renders collagen more resistant to the action of mammalian collagenase (EC 3.4.24.3). However, in aqueous solutions (pH 6 to 8), (+)-catechin spontaneously becomes oxidized and polymerizes [5–7]. We report here that the resistance to the action of collagenase conferred on collagen by its preincubation with (+)-catechin is likely to be due to the action on collagen of oxidation products derived from (+)-catechin and not to the direct action of intact (+)-catechin.

Materials and methods

[^{14}C]collagen-coated Multiwell plates, prepared from acid-soluble guinea-pig skin (type I) collagen, were used to monitor collagen degradation following our described technique [8], using as a source of collagenase trypsin-activated mouse bone culture fluids [9] containing approximately 10 units of collagenase/ml (1 ml/well) or occasionally, bacterial collagenase (EC 3.4.4.19), either crude (Type I, from Sigma, St Louis, MO; 2 mg/well) or highly purified (Type VII, from Sigma; 20 μg/well). For some experiments, the plates were coated with pepsin-extracted bovine articular cartilage (type II) collagen [10] or with pepsin-treated acid-soluble type I collagen (2 mg/ml in 0.5 M-acetic acid, incubated for 48 hr at 4° with pepsin, 0.2 mg/ml, then dialyzed against pH 7.5 buffer). All collagens were ^{14}C-labelled by acetylation [11]. Whenever required, the collagen films covering the plates were pretreated with (+)-catechin by incubating the collagen-coated plates for up to 2 days at 35° with, into each well, 1 ml of TNCN buffer (50 mM-Tris/HCl, pH 7.5, with 0.14 M-NaCl, 5 mM-$CaCl_2$ and NaN_3, 0.2 mg/ml) containing the drug [(+)-catechin chlorhydrolysinate, kindly provided by Dr P. Niebes, Continental Pharma, Mont-Saint-Guibert, Belgium]; the plates were then extensively rinsed with TNCN buffer to remove unbound catechin. Control plates were treated with TNCN buffer alone. Sodium dodecyl sulfate polyacrylamide slab gel electrophoresis of collagen degradation products was done as previously reported [12], after incubation of collagen with mammalian collagenase for 18 hr at 25° in the presence or not of 0.5 mM (+)-catechin. The oxidation of catechin was followed either directly by taking the complete absorbance spectra of the preparations in a Perkin-Elmer model 555 spectrophotometer, or indirectly, after a colorimetric reaction with *p*-dimethylaminocinnamic aldehyde [13].

Results and discussion

Preincubation of collagen (type I)-coated plates for 2