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HEPATIC AND EXTRAHEPATIC MICROSOMAL ELECTRON TRANSPORT COMPONENTS AND MIXED-FUNCTION OXYGENASES IN THE MARINE FISH *STENOTOMUS VERSICOLOR*

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Abstract—NADPH-cytochrome *c* reductase, benzo[*a*]pyrene hydroxylase and aminopyrine demethylase activities in hepatic microsomes from the marine fish scup (*Stenotomus versicolor*) were characterized according to dependence of Ph, temperature, ionic strength and Mg^{2+}. The kinetic properties of benzo[*a*]pyrene hydroxylase were variable, depending on protein and substrate concentration, with measured K_m values for benzo[*a*]pyrene between 4×10^{-7} M and 4×10^{-5} M. The K_m for aminopyrine was 7×10^{-4} M, and NADPH-cytochrome *c* reductase had K_m values of 2.1×10^{-5} M and 1.3×10^{-5} M for cytochrome *c* and NADPH, respectively. NADH supported benzo[*a*]pyrene hydroxylation at 10 per cent of the rate seen with NADPH, and no synergism was observed. Aminopyrine demethylation proceeded at least as well with NADH as with NADPH, and there was synergism when combined. NADPH- and NADH-cytochrome *c* reductases were detected in "microsomes" from fourteen extrahepatic tissues, including kidney, testis, foregut, gill, heart, red muscle, hindgut, buccal epidermis, pyloric caecum, spleen, brain, lens, ovary and white muscle. Benzo[*a*]pyrene hydroxylase was detected in all but white muscle, while cytochrome P-450 and aminopyrine demethylase were detectable in fewer tissues. Reduced, CO-ligated absorption maxima in the Soret region were 450 nm for all those but liver (occasionally 449 nm) and heart (about 447 nm). The estimated turnover numbers for benzo[*a*]pyrene hydroxylase and aminopyrine demethylase, and the influence of 7,8-benzoflavone *in vitro* on benzo[*a*]pyrene hydroxylase indicate that the cytochromes P-450 in different fish tissues are not catalytically equivalent.

The principal features of hepatic cytochrome P-450 systems in fish are qualitatively similar to those of better known cytochrome P-450 systems in mammals. Fish hepatic microsomal cytochrome P-450 possesses optical and electron paramagnetic resonance (e.p.r.) characteristics and catalytic functions that are like those of mammalian cytochromes P-450 [1, 2]. Hepatic mixed-function oxygenase (MFO) activity is inducible in fish by treatment with selected compounds [2–4], and there is variation associated with biological factors such as sex [5]. Yet, while basic similarity is apparent, there also are certain intriguing differences between cytochrome P-450 systems in fish and mammals. Among these are findings that untreated members of some fish species have levels of aryl hydrocarbon (benzo[*a*]pyrene; BP) hydroxylase much greater than those observed in untreated mammals [6–8], and the activity is strongly inhibited *in vitro* by 7,8-benzoflavone (7,8-BF) [7, 8]. Likewise, there are differences in the binding of specific substrates to cytochrome P-450 between some of these fish and untreated mammals [9].

NADPH-linked MFO activity in fish liver has received much more attention than that in extrahepatic tissues, presumably because of the dominant role of the liver in foreign compound metabolism. Yet details concerning the features even of hepatic cytochrome P-450 systems in fish have been derived from studies on only a very few species. Furthermore, with some notable exceptions [1, 8, 10] there has been little attention given to components of microsomal electron transport systems other than cytochrome P-450 and its activities in any fish tissue. In light of the large species differences known to exist in cytochrome P-450 MFO systems (e.g. see Ref. 11), the above limitations preclude any extended generalizations concerning these systems in fish. Further studies of hepatic and extrahepatic microsomal electron transport components in diverse fish species are required if a general understanding of these systems in fishes and their role in the disposition and effects of toxic foreign compounds in the water is to be achieved. We herein describe some of the characteristics of these systems in various tissues of the marine teleost *Stenotomus versicolor* (scup or porgy). This species is one of those found normally to posses high levels of hepatic BP hydroxylase activity that is strongly inhibited by 7,8-BF [8].

MATERIALS AND METHODS

Chemicals. Benzo[*a*]pyrene (Gold label) and 7,8-benzoflavone were obtained from the Aldrich Chemical Co., Milwaukee, WI. Glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-PD), nicotinamide adenine dinucleotide phosphate (NADP), reduced NADP (NADPH), reduced nicotinamide adenine dinucleotide (β-NADH), Hepes,* and horse heart cytochrome *c* were obtained from the Sigma Chemical Co., St. Louis, MO.

* Hepes = 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

Sodium succinate was from Fisher Scientific, Medford, MA. Aminopyrine (AP) was obtained from Aldrich and was twice recrystallized from ethanol. Methanol was redistilled; other solvents were used as supplied by Burdick & Jackson, Muskegan, MI.

Animals. Adult scup or porgy (*S. versicolor*), 80–200 g, were collected by angling in Great Harbor, Woods Hole, MA, or Hadley Harbor, Gosnold, MA between June and October in 1975 to 1977. Fish were generally held in running sea water 19 ± 1° in 800 gallon tanks at the Woods Hole Oceanographic Institution or National Marine Fisheries Service, Woods Hole, for periods of days to months. Fish held for a length of time were fed a diet of chopped smelt and quahogs *ad lib.* every 2 days. The animals were not subjected to any experimental treatment.

Tissue preparation. Animals were decapitated and tissues were excised immediately and placed in ice-cold 0.1 M Na phosphate buffer (PO_4), pH 7.3. With gills only the filaments were taken, and gut samples were opened immediately and the lumen rinsed with buffer. Tissues were minced, and homogenized in 4 vol of 0.1 M PO_4 buffer, pH 7.3, with 1.15% KCl and 3 mM $MgCl_2$, using a Potter–Elvehjem tissue grinder with four passes of the pestle at 1350 rev/min and four at 1900 rev/min. Muscle, gill and gut tissues required at least nine passes at each speed. Portions of any tissue not homogenized were removed and weighed. A microsomal preparation was isolated from a 10,000 *g* supernatant fraction by centrifugation at 40,000 *g* for 90 min. Pellets were resuspended in 0.1 M PO_4, pH 7.3, to concentrations ranging between 1 and 10 mg/ml, depending on the tissue, but liver microsomal fractions were routinely resuspended in 3 vol. buffer/g of liver. Glycogen sediments as a separate layer under the microsomes in this procedure, and it was not included in the resuspensions.

Subcellular fractionation. Scup hepatic tissue was homogenized in an ice bath either in 10 vol. of 0.25 M sucrose, 0.1 M Tris–HCl, pH 7.5, or in 5 vols. of 0.1 M PO_4, pH 7.3, with 1.15% KCl. Two, four, and two strokes of the pestle were used to homogenize the tissue at 800, 1350 and 1350 rev/min respectively. Subcellular fractions were prepared by centrifuging in a SS-34 rotor and a Sorvall RC2-B centrifuge at 600 *g* × 10 min (nuclear), 5,000 *g* × 10 min (heavy mitochondrial), 13,000 *g* × 10 min (mitochondrial), and 40,0000 *g* × 90 min (microsomal). The pellets were washed with the homogenizing buffer, recentrifuged, and the supernatant fractions combined at each step. The washed pellets were resuspended in 0.05 M Tris–HCl, pH 7.8, 1.0 mM EDTA, 10 mM mercaptoethanol, 20% glycerol, or in 0.1 M PO_4, pH 7.3, to a concentration of between 1 and 13 mg protein/ml.

Enzyme assays. Glucose-6-phosphatase (G-6-Ptase; EC 3.1.3.9) was assayed by measuring the production of inorganic phosphate in a reaction mixture containing in a 1.4 ml volume, 50 μmoles cacodylate buffer, pH 6.5, and 30 μmoles G-6-P. The mixture was preincubated for 5 min at 30°, the reaction was initiated by the addition of 0.1 ml enzyme (0.1 to 1.0 mg protein), and the mixture incubated for 10 min at 30°, after which the reaction was terminated by the addition of 1 ml of 10% trichloracetic acid (TCA). A series of "zero time" control reaction mixtures in which enzyme was added after addition of TCA was also run. The denatured protein was sedimented by centrifuging at 1800 rev/min × 9 min or by refrigeration overnight; inorganic phosphate in the supernatant fraction was determined by the method of Flynn *et al.* [12].

NADPH-cytochrome *c* reductase (EC 1.6.2.4) was assayed routinely by a modification of Phillips and Langdon [13], using a reaction mixture containing 0.175 mM NADPH, 80 μM horse heart cytochrome *c*, and 0.2 M phosphate buffer, pH 7.7, ionic strength 0.58 in a total volume of 1.65 ml. The reaction, carried out at 25°, was initiated with NADPH and contained 0.02 to 0.5 mg of microsomal protein depending on the tissue. Reduction of cytochrome *c* was followed at 550 nm using a Cary 118-C recording spectrophotometer. Reduced cytochrome *c* was determined using an extinction coefficient of 21.1 cm^{-1} mM^{-1}. NADH-cytochrome *c* reductase was assayed as above with 0.25 mM NADH replacing NADPH. Reference cuvettes in both cases contained reaction mixtures with no enzyme. One mM KCN was used in certain incubations.

Succinate cytochrome *c* reductase (succ. dehydrogenase; EC 1.3.99.1) was assayed according to Green *et al.* [14]. Reaction mixtures, 1.65 ml volume, contained 10 mg/ml of bovine serum albumin, 80 μM cytochrome *c*, 1 mM KCN, 0.02 to 0.1 mg of microsomal protein/ml, and 0.02 m PO_4 buffer, pH 7.4. Reactions were initiated by addition of sodium succinate to 5 mM, and the reduction of cytochrome *c* was followed at 25° as above.

BP hydroxylase was assayed routinely in a procedure modified from Nebert and Gelboin [15]. Reaction mixtures contained a generating system consisting of 5 mM NADP, 15 mM G-6-P, 3 mM $MgCl_2$ and 2 units of G-6-PD in either 0.1 M phosphate or 0.1 M Tris buffer. After a 15-min preincubation, microsomal protein was added to a final concentration ranging from 0.15 to 1.5 mg/ml depending on the tissue, with liver usually at 0.37 to 0.45 mg/ml. Reactions were initiated by addition of 7.8 μg benzo[*a*]pyrene in 20 μl methanol, and incubations were carried out at 29° for 20 min. The final volume of the reaction mixtures was 0.5 ml and the final pH was 7.0. For determining pH optima, 0.5 mg NADPH replaced the generating system. When studying 7,8-BF inhibition, both 7,8-BF and BP were added in a total of 20 μl methanol. Reactions were stopped with 1 ml acetone and 3 ml hexane. Hydroxylated product was extracted from between 0.1 ml and 2.0 ml hexane, depending on activity, and assayed fluorometrically using a Turner 110 fluorometer, excitation at 396 nm and emission at 520 nm, with authentic 3-OH-BP as a reference standard. All phases of incubation and product extraction were carried out in the dark or under red light.

AP demethylase was determined using NADPH or a generating system as described above, in 0.166 M Hepes, pH 7.6, at a final volume of 1.5 ml. Microsomal protein concentrations ranged between 0.1 and 1.0 mg/ml of reaction mixture, with liver at 0.25 to 0.35 mg/ml. Reactions were initiated by adding 15 μmoles of twice recrystallized AP, incubated for

15 min at 29°, and stopped by adding 0.5 ml of 25% (wt/vol) $ZnSO_4$ and 0.5 ml of saturated BaOH. Formaldehyde was determined as described previously [16, 17]. Formaldehyde standards were incubated and treated as samples.

All enzyme assays were performed using freshly prepared microsomes and were carried out within the range of protein concentrations (estimated according to Lowry *et al.* [18]) and of incubation times which produced a linear response with the liver enzymes. Conditions were standard except where varied as described in the text. Blank values for both MFO activities consisted of reactions carried out without NADPH. Practical limits of detection for these assays were: cytochrome *c* reductases, 0.01 nmole cytochrome *c* reduced/min; BP hydroxylase, fluorescence equivalent to 1.5 pmoles 3-OH-BP; AP demethylase, 0.5 nmole formaldehyde. When some activities were particularly low, e.g. with lens, boiled microsome blanks were included. Extraction efficiency of 3-OH-BP was 50% with liver microsomes, but the data as they appear have not been corrected for this.

Cytochrome P-450 was assayed as before [8], using a Cary 118 recording spectrophotometer. Difference spectra were assayed routinely with CO balanced between reference and sample cuvettes and the sample reduced with $Na_2S_2O_4$, eliminating interference by hemoglobin and affording the observation of contamination by cytochrome oxidase. $Na_2S_2O_4$-reduced spectra were also recorded without CO; 1.5 ml volumes contained 0.25 to 2.3 mg protein/ml. Cytochrome b_5 was assayed as before [8] with liver microsomes. Cytochrome content was determined assuming extinction coefficients of 91 $cm^{-1}\,mM^{-1}$ for cytochrome P-450 and 185 $cm^{-1}\,mM^{-1}$ for cytochrome b_5 [19]. The practical limit of detection of cytochrome P-450 was about 50 pmoles/ml of microsomal resuspension.

RESULTS

Subcellular distribution and requirements for activity. The subcellular distributions of cytochrome P-450, NADPH- and NADH-dependent cytochrome *c* reductases and MFO activities are shown in Fig. 1. By comparison with microsomal and mitochondrial markers it is quite apparent that hepatic MFO systems are principally microsomal in scup. There were MFO and reductase activities present in mitochondrial and "nuclear" fractions also, but of these cytochrome P-450 was detectable only in mitochondrial fractions. The "nuclear" pellet may have contained cytochrome P-450, but there was less than 5 pmoles/mg of protein.

Requirements for microsomal MFO activity in liver and gill are given in Table 1. The data show clearly that native protein, oxygen and NADPH are required for full activity in both tissues. Similarly

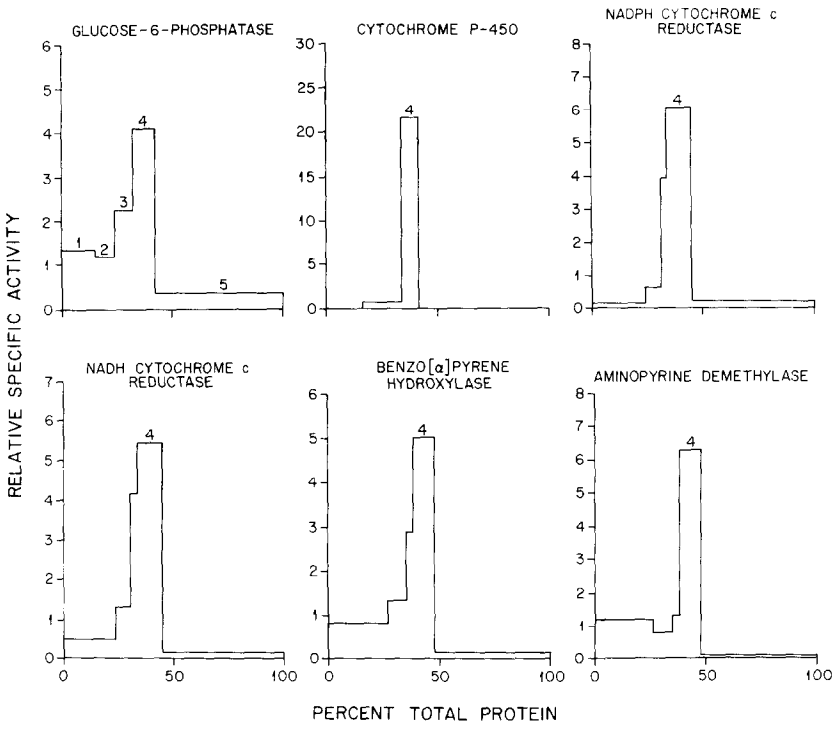

Fig. 1. Subcellular distribution of hepatic cytochrome P-450, cytochrome reductases and mixed-function oxygenase activities in *S. versicolor*. Relative specific activities were calculated according to DeDuve *et al.* [20]. Key: (1) nuclear, (2) heavy mitochondrial, (3) light mitochondrial, (4) microsomal and (5) supernatant fractions. The relative specific activities of succinate-cytochrome *c* reductase in mitochondrial, microsomal and supernatant fractions were 4.1, 0.10 and 0.06 respectively. Data represent means of at least three determinations

Table 1. Requirements for MFO activity with scup (*S. versicolor*) liver and gill microsomes

Incubation conditions	Liver				Gill	
	BP hydroxylase (units/mg)*	(%)	AP demethylase (units/mg)*	(%)	BP hydroxylase (units/mg)*	(%)
Complete†	685 ± 40‡	100	4.0 ± 0.5	100	12.2 ± 2.4	100
Minus NADPH		1		4		1
Minus Mg^{2+}§		130		87		
CO		14		21		17
N_2		95		100		99
N_2 (sealed)		55				53
Boiled microsomes		< 1		< 7		< 1
NADPH§	714 ± 47	100	3.8 ± 0.4	100		
NADH§	72 ± 11	10	4.8 ± 0.7	128		
NADPH + NADH§	791 ± 52	111	9.6 ± 0.8	254		

* Units are pmoles 3-OH-BP equivalents · min^{-1} (BP hydroxylase) and nmoles HCHO · min^{-1} (AP demethylase).

† Activity in complete reaction mixtures with an NADPH generating system.

‡ All data are averages of three determinations on separate pools of tissue ± S.E.M.

§ The NADPH generating system was replaced by 1 mg/ml NADPH and/or 1 mg/ml NADH in these reactions. In the case of Mg^{2+} dependence, NADPH replaced the generating system in reactions both with (100 per cent) and without Mg^{2+}.

there is strong inhibition of both BP hydroxylase and AP demethylase activities by CO. We observed an inhbition of BP hydroxylase in liver microsomes by 3 mM Mg^{2+}, a concentration lower than that found inhibitory in mammals [15, 21], yet 3 mM Mg^{2+} appeared to promote activity of AP demethylase in scup; however, the difference was not significant. The activities of both BP hydroxylase and AP demethylase were supported by NADH as well as NADPH (Table 1). BP metabolism supported by NADH was about 10 per cent that with NADPH, like the activity in mammalian and rainbow trout liver [6], and the activity with both coenzymes present was additive. AP demethylase, on the other hand, proceeded at least as well with NADH as with NADPH, and the activity when both were present was synergistic, but only moderately so.

pH, ionic strength and temperature dependence. BP hydroxylase had a fairly broad peak of activity between pH 6.8 and 8.0, but maximal activity occurred between pH 7.0 and 7.2. AP demethylase exhibited a similar broad range, with a peak between pH 7.4 and 7.6, and NADPH-cytochrome *c* reductase had a sharp peak of activity at pH 7.74. In scup, as in mammals, the activity of NADPH-cytochrome *c* reductase was maximal at fairly high total ionic strength. The activity between 0.4 and 0.6 was 25 per cent greater than that at lower or higher ionic strengths. Both AP demethylase and BP hydroxylase increased in activity up to 0.1 ionic strength and then remained constant up to 0.2 (AP) or 0.3 (BP).

The temperature optima for BP hydroxylase and NADPH-cytochrome *c* reductase were quite similar—near 30° (Fig. 2). AP demethylase had quite

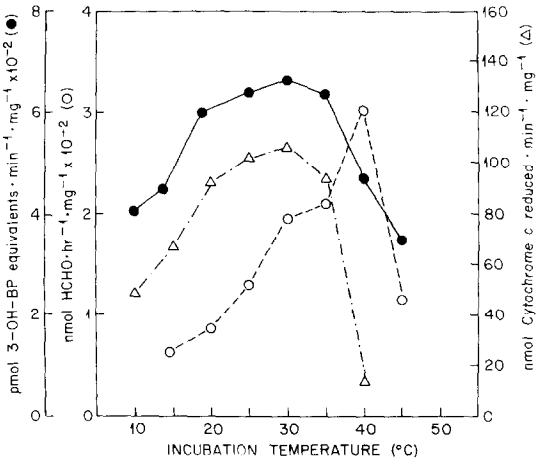

Fig. 2. Temperature dependence of NADPH-cytochrome *c* reductase, benzo[*a*]pyrene hydroxylase and aminopyrine demethylase in *S. versicolor* liver microsomes. Incubations were for 10 min at the temperatures indicated. Each point represents the mean of three determinations on one pool (reductase) or replicate determinations on two to three pools (MFO) of tissue.

Table 2. Apparent K_m and V_{max} of scup (*S. versicolor*) hepatic microsomal BP hydroxylase, AP demethylase and NADPH-cytochrome *c* reductase

Activity	(M) K_m*	V_{max}†
BP hydroxylase‡		
40 μg microsomal protein/ml		
[BP] 0.08–4.0 μM (2)§	4.0×10^{-7} (± 1.5)	290 (± 90)
400 μg microsomal protein/ml		
[BP] 0.2–0.8 μM (3)	4.5×10^{-7} (± 3.8)	23 (± 12)
[BP] 2.0–20 μM (4)	3.8×10^{-5} (± 2.8)	991 (± 187)
AP demethylase‡		
Aminopyrine (2)	7.0×10^{-4} (± 1.1)	4.8 (± 0.6)
NADPH-cytochrome *c* reductase		
Cytochrome *c* (1)	2.1×10^{-5}	182
NADPH (2)	1.3×10^{-5} (± 0.2)	133 (± 2)

* K_m values were estimated using least squares regression analysis of Lineweaver–Burke plots. Numbers in parentheses are ± range.

† Units are pmoles 3-OH-BP equivalents produced, nmoles HCHO produced or nmoles cytochrome *c* reduced, per min per mg of microsomal protein.

‡ Ten-min reaction times were used for both BP hydroxylase and AP demethylase.

§ Number of determinations.

a different pattern of activity with a sharp optimum at 40°, but there was a plateau at 30°, and the 40° optimum might be considered anomalous. Scup do prefer warm water (between 20 and 25°), but do not encounter temperatures above 30° in the waters from which these animals were taken. Extending the incubation time for BP hydroxylase to 2 hr confirmed a temperature optimum near 30° for this activity. The thermal lability of scup hepatic P-450 systems was assessed with BP hydroxylase. There was marked inactivation by incubation for 15 min at temperatures above 40°, and at 43° only 30 per cent of the original activity remained, indicating clearly that the system in scup hepatic microsomes was quite susceptible to thermal inactivation, more so than in mammals [15].

Kinetic parameters. The kinetics of BP hydroxylation with microsomal preparations were difficult to determine accurately because of variation with the concentration of protein and the concentration range of BP. At higher microsomal protein concentrations the substrate saturation curve was sigmoidal and the corresponding double reciprocal plot allowed the calculation of two distinct K_m values (Table 2), 4.5×10^{-7} M between 0.2 and 0.8 μM BP, and 3.8×10^{-5} M between 2 and 50 μM BP. Values between 0.8 and 2.0 μM BP could not be included in the calculation of either K_m because of the sigmoidal nature of the substrate saturation curve. At lower protein concentrations, no sigmoidal pattern in substrate saturation was observed. Here, the double-reciprocal plot allowed only a single K_m, about 4.0×10^{-7} M.

Aminopyrine demethylase, measured by formaldehyde generation, did not exhibit kinetic complexities like those seen with BP hydroxylase. Substrate saturation provided a peak of activity at 10 mM AP, with a K_m of 7.0×10^{-4} M (Table 2). There was strong apparent substrate inhibition above this concentration, and only 40 per cent of the peak activity was seen at 100 mM AP. The apparent K_m of NADPH-cytochrome *c* reductase for NADPH was 1.3×10^{-5} M. However, the substrate saturation curve for NADPH deviated from the classical hyperbolic form; the reductase activity plateaued between about 100 and 150 μM NADPH, then showed a further increase beyond 200 μM. Cytochrome *c* showed simple substrate saturation behavior with an apparent K_m of 2.1×10^{-5} M (Table 2).

Tissue distribution. Microsomal protein, cytochrome P-450 content and reduced, CO-ligated absorption maxima, and NADPH- and NADH-dependent cytochrome *c* reductase activity of various tissues are given in Table 3. Cytochrome P-450 was present in the highest amounts in liver, followed by heart, pyloric caecum, kidney, hindgut, testis and gill. Cytochrome P-450 may have been present in red muscle preparations but contamination by cytochrome oxidase, indicated by a CO-bound $Na_2S_2O_4$-reduced peak at 430 nm, cast doubt on the identity of the shoulder appearing at about 450 nm. Our methods did not reveal cytochrome P-450 in the remaining tissues examined. In liver samples the absorption maximum of CO-bound, reduced cytochrome P-450 was usually at 450 nm, although occasionally the peak was at 449 or 449.5 nm. In heart, the peak was shifted to the blue, appearing at about 447 nm, yet the appearance of this peak only under conditions appropriate for cytochrome P-450, and with no detectable cytochrome oxidase contamination, permits consignment to this family of hemoproteins. In some extrahepatic tissues, most noticeably gill, there was a peak at about 420–421 nm, of unknown identity (not cytochrome P-420), but this peak did not interfere with P-450 determination. Cytochrome b_5, analyzed only in liver samples, had an NADH-reduced Soret absorption maximum at 424 nm and a minimum at 411 nm [8], and a specific content of 0.066 ± 0.016 (S.D., N = 11) nmoles/mg of microsomal protein.

NADPH-dependent and NADH-dependent cytochrome *c* reductase activities were detected in "microsomes" of all tissues examined, and the highest activities of both were found in liver, pyloric caecum and kidney. In general, the activity of NADH-cytochrome *c* reductase was about two to five times greater than that of NADPH-cytochrome *c* reductase except in hindgut, spleen and lens,

Table 3. Microsomal protein, cytochrome P-450, NADPH-cytochrome *c* reductase and NADH-cytochrome *c* reductase in scup (*S. versicolor*) tissues*

Tissue	mg microsomal protein / g tissue	Cytochrome P-450 Absorption maximum†	Cytochrome P-450 pmoles / mg mic. protein	NADPH-cytochrome *c* reductase (units/mg)‡	NADH-cytochrome *c* reductase (units/mg)‡
Liver	12.4 ± 0.5§ (50,12)‖	449–450	613 ± 71 (38,9)	106 ± 5 (30,5)	179 ± 5 (15,3)
Kidney	4.4 ± 0.8 (31,6)	450	100 ± 36 (17,2)	47 ± 2 (15,3)	91 ± 5 (9,2)
Testis	4.2 ± 1.1 (8,2)	450	23 ± 6 (8,2)	13 (6)	62 (6)
Foregut	4.5 ± 1.3 (10,2)		ND (10,2)	14 (4)	61 (4)
Gill	3.4 ± 0.4 (33,7)	450	~ 20 ± 5 (17,2)	13 (6)	26 (6)
Heart¶	4.2 ± 0.8 (25,5)	~ 447	260 ± 57 (15,3)	11 (6)	53 (6)
Red muscle¶	5.8 ± 1.3 (21,4)	450	? (17,2)	3.8 (6)	52 (6)
Hindgut	4.9 ± 1.0 (17,4)	450	81 ± 40 (11,2)	20 (6)	5 (6)
Buccal epidermis	1.1 ± 0.2 (27,4)		ND (17,3)	9 (5)	44 (5)
Pyloric caecum	8.3 ± 1.8 (17,4)	450	153 ± 48 (11,2)	58 (4)	121 (4)
Spleen	3.6 ± 0.4 (23,4)		ND (11,2)	60 (6)	4 (6)
Brain	2.2 ± 0.4 (29,5)		ND (17,3)	11 (6)	15 (6)
Lens	3.5 ± 0.4 (21,4)		ND (9,2)	2.0 (5)	1.5 (9,2)
Ovary	5.8 ± 0.3 (9,2)		ND (9,2)	7 (5)	16 (5)
White muscle	5.1 ± 1.1 (17,3)		ND (9,2)	0.7 (6)	4.4 (9,2)

* Tissues are ranked according to BP hydroxylase activity, Table 4.

† CO-bound, reduced vs CO Soret absorption maximum.

‡ Units are nmoles cytochrome *c* reduced/min/mg of microsomal protein.

§ Means ± S.E.M. Values without S.E.M. are the means of replicates on a single pooled sample. ND = not detected.

‖ Numbers in parentheses are total N of fish followed by N pooled groups in the determination. Single numbers in parentheses represent a single pool of N fish.

¶ The reductase activities for these tissues were determined with 1 mM KCN. Without KCN, heart and red muscle had 3.4 and 0.1 units NADPH activity/mg, and 45 and 42 units NADH activity/mg respectively. Liver reductase activities were unchanged by KCN.

Table 4. Benzo[*a*]pyrene hydroxylase and aminopyrine demthylase in scup (*S. versicolor*) tissues

Tissue*	BP hydroxylase (units/mg)†	AP demethylase (units/mg)‡
Liver	685 ± 40§ (40,9)‖	4.0 ± 0.5 (40,9)
Kidney	223 ± 59 (27,5)	2.4 ± 1.7 (18,4)
Testis	59 ± 2 (8,2)	3.3 (3)
Foregut	13 (6)	0.2 (6)
Gill	12 ± 2.4 (26,5)	0.4 ± 0.2 (9,2)
Heart	9.9 ± 3.5 (11,2)	0.2 (3)
Red muscle	6.1 ± 2.8 (17,3)	1.4 ± 0.8 (14,3)
Hindgut	6.0 ± 4.0 (13,3)	ND (3)
Buccal epidermis	2.4 ± 0.9 (17,3)	ND (17,3)
Pyloric caecum	1.0 ± 0.5 (14,3)	ND (3)
Spleen	0.8 ± 0.3 (11,2)	0.1 (3)
Brain	0.7 ± 0.2 (17,3)	ND (9,2)
Lens	0.7 ± 0.5 (21,4)	ND (9,2)
Ovary	< 0.5 (5)	ND (5)
White muscle	< 0.5 (9,2)	ND (9,2)

* Tissues are ranked according to BP hydroxylase activity.

† Units are pmoles 3-OH-BP equivalents produced/min/mg of microsomal protein.

‡ Units are nmoles HCHO produced/min/mg of microsomal protein.

§ Means ± S.E.M. Values without S.E.M. are the means of replicates on a single pooled sample. ND = not detected.

‖ Numbers in parentheses are total N of fish followed by N pooled groups in the determination. Single numbers in parentheses represent a single pool of N fish.

where NADPH-dependent activity exceeded NADH-dependent activity. Red muscle, on the other hand, had about fourteen times more NADH- than NADPH-dependent activity when assayed with KCN, and almost no NADPH activity without KCN. NADH/NADPH-dependent activity ratios were also higher without KCN in heart microsomes.

Heart, red muscle, and gill are very rich in mitochondria, and the higher NADH/NADPH-dependent activity ratios in red muscle and apparent lack of NADPH-dependent activity without KCN may have been due to the influence of both cytochrome *c* reductase and cytochrome oxidase in contaminating mitochondrial fragments. This possibility was assessed further by determining the amount of succinate cytochrome *c* reductase in these preparations. In red muscle preparations (obtained from pooled tissue of seven fish) the activity of succinate cytochrome *c* reductase was 64.2 ± 13.0 units (nmoles cytochrome *c* reduced · min^{-1})/mg of protein, while in heart it was 18.1 ± 3.0 units/mg and in gill it was 6.0 ± 2.0 units/mg. Consistent with the appearance of cytochrome oxidase contamination, these data suggest that the contamination by mitochondria was substantial in red muscle "microsomes", but in heart and also gill it was much less so. By comparison, in liver and kidney preparations there was negligible succinate cytochrome *c* reductase activity (2.1 ± 1.2 and 1.8 ± 1.1 units/mg respectively).

BP hydroxylase activity was observed in all but one of the tissues examined (Table 4). In white skeletal muscle the activity was at the limits of

Table 5. Influence of 7,8-benzoflavone on microsomal BP hydroxylase in scup (*S. versicolor*) tissues

Tissue	0	% Activity remaining* at 7,8-benzoflavone concentration: 1.25×10^{-7} M	5×10^{-6} M	1×10^{-4} M
Liver	100 ± 9†	96.6 ± 7	63.0 ± 15	16.0 ± 3
Kidney	100 ± 3	82.2 ± 5	72.1 ± 6	43.1 ± 3
Gill	100 ± 14	‡	‡	79.0 ± 6
Testis	100 ± 5	‡	‡	100.0 ± 2
Red muscle	100 ± 2	‡	‡	125.0 ± 15

* Activity in reactions containing 7,8-BF as compared to identical reations without 7,8-BF equalling 100 per cent. One hundred per cent activities are like those in Table 4.

† Data are means of replicate determinations with pooled tissues from eight fish, ± range.

‡ Not determined due to insufficient material.

detection, and is thus suspect. As reported previously [8], the activity in scup liver was quite high, and substantial activity was also found in kidney, testis, foregut, gill and heart. The estimated turnover number for this activity, expressed in pmoles 3-OH-BP/min/nmole of P-450, in liver, kidney, testis and gill, ranged from 600 to 4000, much higher than that estimated in this laboratory for hepatic microsomal BP hydroxylase from untreated mice (about 60 pmoles/min/nmole of P-450) [8]. Heart, hindgut and pyloric caecum had lower estimated turnover numbers. Detectable levels of AP demethylase were found only in eight of the fifteen tissues examined. The estimated turnover numbers for AP demethylase in kidney, testis and gill were all much higher than that in liver, and in fact were higher than than seen in untreated mice (about 700 nmoles HCHO/hr/nmole of P-450) [8].

Influence of 7,8-benzoflavone on BP hydroxylase. Highly active BP hydroxylase in untreated scup liver has been reported to be strongly inhibited by 10^{-4} M 7,8-BF [8], this is similar to the situation observed with 3-MC-induced cytochrome P-448 in some mammals [22]. The influence of 7,8-BF on BP hydroxylase in several scup tissues (Table 5) differed quite markedly in the pattern of inhibition/stimulation. At 10^{-4} M 7,8-BF this activity in liver was inhibited by more than 83 per cent, while in kidney there was 57 per cent inhibition by the same concentration of 7,8-BF. There was little or no inhibition seen in gill or testis and, if anything, there was a slight stimulation of activity in red muscle.

DISCUSSION

The temperature optima for scup MFO and NADPH-cytochrome *c* reductase were like those of rainbow and brown trout [6, 23, 24] and dogfish [1] MFO, but were lower than those seen with mammalian enzymes, consistent with the poikilothermal nature and the habitat temperature of these fish. The pH dependence of scup MFO was, however, rather unlike that seen with freshwater rainbow trout. Pedersen and Hershberger [6] noticed a sharp optimum at 7.4 with BP hydroxylase, and Dewaide and Henderson [23] found AP demethylase to have an optimum at pH 8.0.

Complications in the determination of BP hydroxylase kinetic properties in mammalian microsomes have been ascribed to assay conditions that effect either substrate or product. These include sample, amount of protein, concentration range of BP, and duration of incubation [25–27]. With scup liver microsomes we too observed variable kinetics with differences in range of either BP or protein concentration, establishing that this problem exists in fish as well. This confounds comparisons with the results of others, yet the K_m values we obtained with low protein, generally considered to be more accurate, were quite low. The K_m for formaldehyde production from AP with scup microsomes was 7×10^{-4} M, like that seen with certain untreated rats—8×10^{-4} M [28, 29], but lower than the 2–3×10^{-3} M for rainbow and brown trout [23, 24]. The V_{max} for AP demethylase in scup (4.8 nmoles HCHO/min/mg of protein; 54 nmoles/g of liver/min) was slightly greater than that reported for rainbow trout (1.0 μmoles/g of liver/hr) [23] or brown trout (25–33 nmoles/g of liver/min) [24]. The dependence of scup liver NADPH-cytochrome *c* reductase on pH and high ionic strength was very similar to that seen with purified or lipase-solubilized reductases from rats [13, 30], but the K_m values for cytochrome *c* and NADPH seen here were somewhat higher than found with rats.

It is assumed that the scup hepatic microsomal cytochrome b_5 system functions in reactions similar to those in mammals, principally fatty acid desaturation, although this was not measured here. However, NADH-cytochrome *c* reductase and cytochrome b_5 may also be involved in the function of cytochrome P-450 in xenobiotic metabolism by scup liver microsomes. The transfer of electrons from NADH to cytochrome P-450 has been shown to proceed by two pathways, one via microsomal NADH-cytochrome *c* reductase and cytochrome b_5, and the other via NADPH-cytochrome *c* reductase [31]. The rate of NADH-supported activity varies with the substrate [32], and synergism has generally been identified only with type I substrates [33]. With scup microsomes we also found that NADH synergism of NADPH-supported activity occurred with the type I substrate AP, but not with BP. Unlike other systems studied, the activity of scup AP demethylase supported by NADH alone equalled or exceeded that supported by NADPH; the reason for this is unclear. Such relatively high rates of NADH-supported metabolism have been observed for chlorobenzene with microsomes from phenobarbital-treated rats [34] and with metabolism of BP by outer mitochondrial membranes of 3-MC-induced rats [35].

To our knowledge this is the first report of cytochrome P-450 and MFO levels in several of these extrahepatic tissues in fish. Pohl *et al.* [1] reported several MFO activities, but not cytochrome P-450, in seven extrahepatic tissues of little skate, including kidney, gill, spleen, spiral valve, stomach, pancreas and heart. Pedersen and Hershberger [6] detected BP hydroxylase in heart and posterior kidney but not in gill, muscle or blood of trout. The levels of extrahepatic and extrarenal MFO in scup were higher in those tissues proximate to the environment, hence to pollutant exposure (i.e. gill and some alimentary tract tissues), or in tissues with metabolic functions believed to involve cytochrome P-450, such as testis. Ovary, which could also have steroidogenic functions, had very low activity, which we believe to be due to inclusion of gametic as well as gonadal "microsomes" in this gravid sample. The pyloric caecum, part of the alimentary tract, also had low MFO activity, which is somewhat surprising considering the levels of cytochrome P-450 and reductase activity in this tissue. However, it is extremely difficult to obtain this tissue without causing the introduction of bile into the lumen. The tissue was washed but the bile may yet contribute to a decline in catalytic function *in vitro*.

Brain and lens were among tissues having the lowest certain MFO activity. The BP hydroxylase present in lens tissue could be implicated in the formation of chemically induced cataracts. Fish, as

do mammals, develop cataracts in response to some hydrocarbons*, and while the mechanism by which cataracts are initiated is not clear, the formation of electrophilic metabolites may stimulate this disease. We are unaware of other reports of hydroxylase activity in lens, but there is a report linking susceptibility to chemically induced cataracts with the inducibility of hepatic "AHH" in genetically responsive mice [36], though this may involve transport of metabolites from the liver.

Some investigators have found the activity of hepatic BP hydroxylase, but not of AP demethylase, in untreated fish to be much greater than in mammalian counterparts [7, 8, 23, 24]. In some, this hydroxylase activity can be strongly inhibited by 7,8-BF [7, 8]. The strong inhbition by 7,8-BF in scup liver may be a feature of environmentally induced cytochromes P-450 [8], but in any case this characteristic does not extend to BP hydroxylase activity in other scup tissues. The different effects of 7,8-BF *in vitro* on BP hydroxylase from various tissues implies that the cytochromes P-450 are different in these tissues. Disparity in estimated turnover numbers for both BP hydroxylase and AP demethylase in various tissues supports this contention by suggesting that cytochromes P-450 in these tissues are not catalytically equivalent. The functional importance of these systems to processes in some tissues, such as heart, red muscle or lens, is uncertain. Nevertheless, the presence of BP hydroxylase, whether it is an incidental catalytic function of constitutive cytochromes P-450 or not, suggests that many tissues in fish can metabolize and possibly activate polynuclear aromatic hydrocarbons to mutagenic derivatives, as can the liver [37].

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