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SUBCELLULAR LOCALIZATION AND PROPERTIES OF CYTOCHROME P-450 AND UDP GLUCURONOSYLTRANSFERASE IN THE RAINBOW TROUT KIDNEY

MAIJA PESONEN and TOMMY ANDERSSON
Department of Zoophysiology, University of Göteborg, Box 25059, S-400 31, Göteborg, Sweden

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Abstract—Rainbow trout kidney was subfractionated by differential centrifugation to obtain preparations suitable for the study of xenobiotic metabolizing enzymes and to ascertain the distribution of these activities in the cell. The cytochrome P-450-dependent monooxygenase, NADPH-cytochrome *c* reductase, and UDP glucuronosyltransferase, which are enzymes important in the biotransformation of xenobiotics, were enriched in the microsomal fraction. Another xenobiotic-metabolizing enzyme, epoxide hydrolase, was enriched in the mitochondrial and microsomal fractions almost to the same extent.

Cytochrome P-450-dependent monooxygenase and UDP glucuronosyltransferase activities were characterized in the trout kidney microsomes. The cytochrome P-450 deethylation of 7-ethoxycoumarin and 7-ethoxyresorufin as well as the glucuronidation of *p*-nitrophenol in the kidney were found to proceed at rates comparable to those occurring in the liver. The difference spectrum of the complex between carbon monooxide and reduced trout kidney microsomes showed a peak at 448.5 nm. Addition of 7-ethoxycoumarin to kidney microsomes produced an absorbance change in difference spectrum similar to the substrate binding spectrum found in rainbow trout liver and rat liver microsomes.

Knowledge about xenobiotic biotransformation processes in aquatic animals have increased considerably during recent years. The biotransformation of many lipophilic compounds to polar products is generally a sequence of reactions. The first step is often catalyzed by cytochrome P-450-dependent monooxygenases. Enzymes involved in subsequent reactions include epoxide hydrolase, glutathione transferase, and UDP glucuronosyltransferase (for refs see 1–3).

As in mammals the major organ involved in xenobiotic metabolism in fish seems to be the liver. Recent studies have, however, indicated that the kidney may also play an important role in xenobiotic biotransformation in fish. Balk *et al.* [4] have shown that the kidney in the Northern pike (*Esox lucius*) accumulated a lipophilic compound, benzo(*a*)pyrene, at comparable levels to those found in the liver. Furthermore, recent studies have shown that the kidney from the scup (*Stenotomus versicolor*) and the rainbow trout (*Salmo gairdneri*) contains relatively high cytochrome P-450-dependent activities, whereas the cytochrome P-450 content is low when compared to the liver [5, 6]. Subcellular localization and characterization of kidney microsomal xenobiotic biotransformation enzymes in fish is, however, poorly investigated.

In our study we have developed a procedure for the subcellular fractionation of rainbow trout kidney. As indicated by enzyme markers, electron microscopy, and spectral measurements, the kidney microsomal fraction obtained with our procedure was suitable for the study on biotransformation enzymes. Thus, we were able to partially characterize the kidney microsomal cytochrome P-450 monooxygenase and UDP glucuronosyltransferase activities in the rainbow trout.

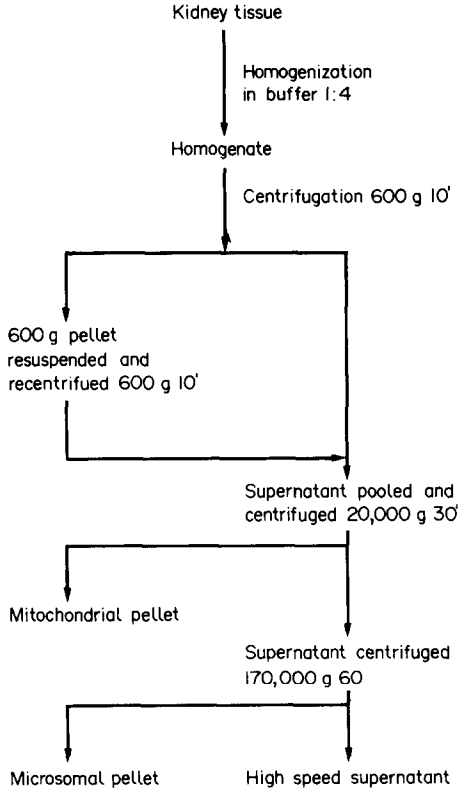

Fig. 1. Scheme for the subcellular fractionation of kidney homogenate from rainbow trout.

MATERIALS AND METHODS

Animals. Cultured, immature rainbow trout (*Salmo gairdneri*) ranging in weight between 200 and 400 g were obtained from a hatchery near Göteborg, Sweden. The fish were fed with commercial trout pellets and kept at 10°. A 12:12 light/dark photoperiod daily cycle was used.

Chemicals. 7-Ethoxycoumarin (EC), 7-hydroxycoumarin and styreneoxide were purchased from Aldrich-Europe (Bersee, Belgium); *p*-nitrophenylphosphate, cytochrome *c*, NADH, glucose-6-phosphate and 4-nitrophenol-β-mannopyronidase from Fluka AG (Buchs, Switzerland); diphenylamine from Merck (Darmstadt, F.R.G.); 7-ethoxyresorufin (ER) and resorufin from Pierce Eurochemie B.V. (Rotterdam, Holland); and (^{14}C)styreneoxide from Amersham International PLC (Amersham, U.K.). Other substrates and cofactors needed for enzyme assays were purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals used were of analytical grade.

Preparation of tissue and subcellular fractions. The fish were stunned by a blow to the head and their entire kidney was removed, weighed, and rinsed with ice-cold 0.15 M KCl. Tissue was sliced with a pair of scissors and homogenized in 4 vol. of 0.1 M phosphate buffer (pH 7.4) containing 0.15 M KCl (or in 0.15 M KCl when measuring glucose-6-phosphatase activity) using 10 up-and-down strokes of a Potter-Elvehjem glass–Teflon homogenizer at 800 rpm. Trypsin inhibitor (1.5 mg/ml) was added to the homogenate to prevent protease activity. Subfractionation by differential centrifugation was carried out according to the scheme in Fig. 1. The homogenate was first centrifuged at 600 *g* for 10 min to remove cell debris and neclei. The resultant supernatant was decanted, the pellet resuspended and centrifuged again which was found to increase greatly the recoveries of microsomal enzyme activities (NADPH-cytochrome *c* reductase, EC-*O*-deethylase). The supernatants were combined and further centrifuged at 20,000 *g* for 30 min to remove mitochondria and lysosomes. The microsomes were prepared by centrifuging the postmitochondrial supernatant at 170,000 *g* for 60 min. The resulting microsomal pellet was resuspended in 0.1 M phosphate buffer (pH 7.4) containing 0.15 M KCl and 20% glycerol. Before enzyme activity measurements, the crude homogenate, 600 *g* and 20,000 *g* pellets were rehomogenized in a glass–glass homogenizer by hand using 7 up-and-down strokes. All steps were performed at 0–4°. The liver microsomes were prepared as described by Förlin [7].

Assays. Assays were carried out using freshly prepared subfractions and performed in duplicate. DNA marker for nuclei was extracted according to Schmidt and Tannhauser [8] and determined by diphenylamine reaction [9, 10]. AMP-ase marker for plasma membrane [11], alkaline phosphatase marker for brush border membrane [12], cytochrome oxidase marker for mitochondria [13], *p*-nitrophenyl-α-mannosidase marker for Golgi apparatus [14], β-glycerophosphatase marker for lysosomes [15], catalase marker for peroxisomes [16], glucose-6-phosphatase and NADPH-cytochrome *c* reductase marker for endoplasmic reticulum [17, 18], lactate dehydrogenase (LDH) marker for cytosol [19], and xenobiotic metabolizing enzymes, i.e. EC-*O*-deethylase [20], ER-*O*-deethylase [21], epoxide hydrolase [22], UDP glucuronosyltransferase [23], and glutathione transferase [24], were all determined according to published methods. Protein contents were measured by the method of Lowry *et al.* [25] using bovine albumin as standard. Enzyme incubations were carried out at 20°.

Spectral studies. An Aminco DW 2a UV/VIS spectrophotometer was used for spectral measurements. Difference spectrum of the complex between CO and cytochrome P-450 in microsomal fractions from kidney and liver was assayed by the method of Matsubara *et al.* [26]. Spectral characteristics of kidney microsomes were studied by following the experimental protocol outlined by Estabrook and Werringloer [27].

Absorption changes upon addition of EC to kidney microsomes were obtained as described by Schenkman *et al.* [28]. EC was dissolved in methanol and added in microliter quantities to the sample cuvette. Equivalent quantities of methanol were added to the reference cuvette. The formation of EC-induced spectral change was recorded as the absorbance difference between 350 and 500 nm when 2 μl of 0.1 M EC in methanol was added to the sample cuvette.

Summations of DNA and protein concentrations or enzyme activities in various fractions were 80–115% of the corresponding values in the whole homogenate.

RESULTS

Subcellular distribution of marker enzymes

The distribution of the marker enzymes in the subcellular fractions obtained according to the fractionation scheme in Fig. 1 is shown in Fig. 2. Most of the nuclei were enriched in the 600 *g* pellet, which indicates that the homogenization procedure does not rupture nuclear membranes. Marker enzymes for lysosomes, Golgi apparatus, and peroxisomes were all enriched to the greatest extent in the 20,000 *g* pellet. AMP-ase was enriched (2.5-fold) in the mitochondrial pellet, whereas the marker enzyme for brush-border membranes, alkaline phosphatase, was enriched (4-fold) in the microsomes (Fig. 2). Glucose-6-phosphatase was recovered in all fractions, however, the highest enrichment (2-fold) was found in the microsomal pellet (Fig. 2). Cytochrome oxidase was enriched nearly 5-fold in the 20,000 *g* pellet. NADH-cytochrome *c*-reductase was enriched 4-fold in the 170,000 *g* pellet. However, when the cell fractionation was performed without trypsin inhibitor the activity was mainly found in the cytosol. The high recovery (81%) of total LDH activity in high-speed supernatant indicated low contamination of the membrane fraction with cytosol material and an effective disruption of kidney cells.

In Fig. 2 the distribution of various xenobiotic biotransformation enzymes are also shown. In the microsomal fraction 59% of the total EC-*O*-deethylase activity was recovered and the distribution profile was similar to that of NADPH-cytochrome *c* reductase. The enrichment of the monooxygenase

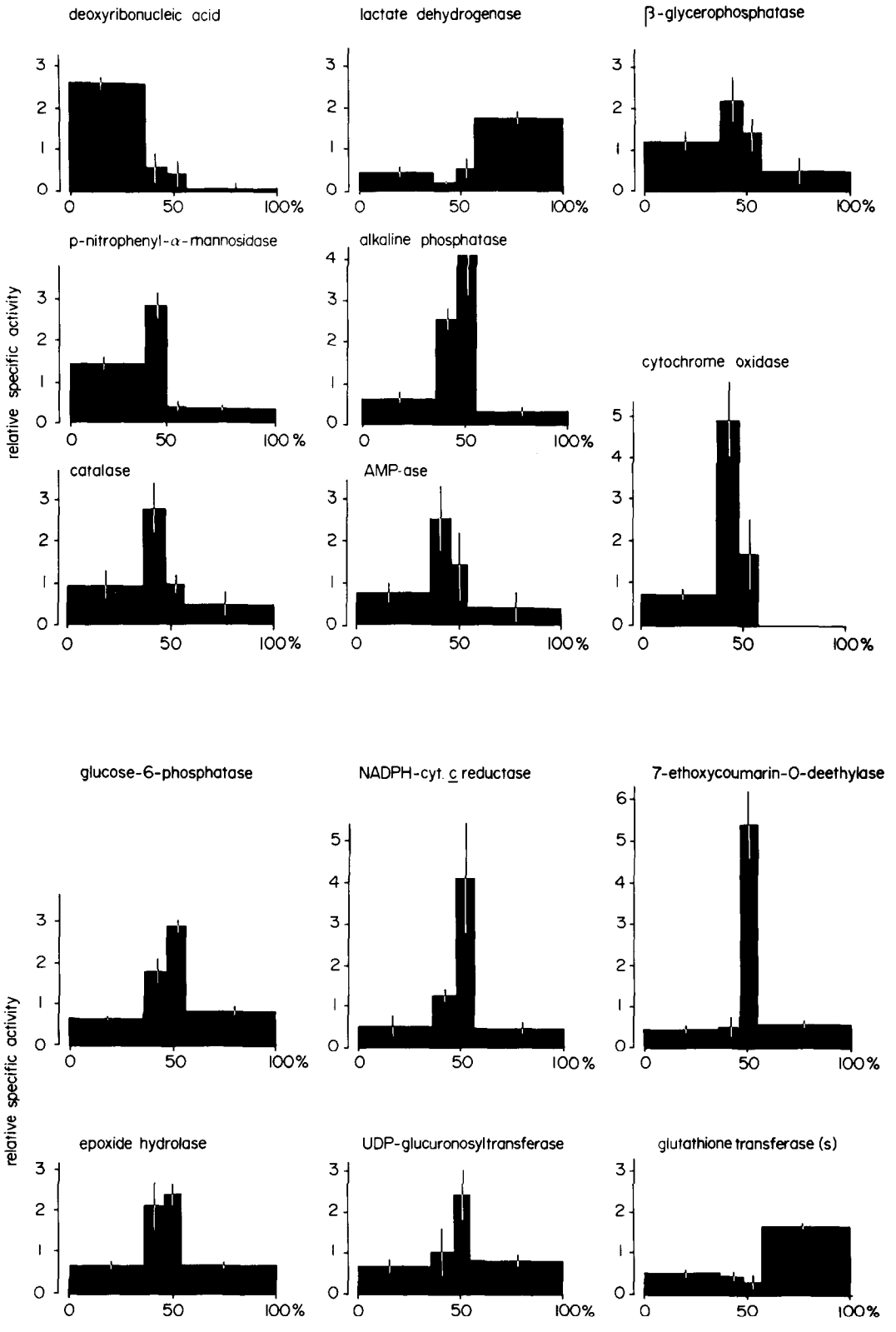

Fig. 2. The distribution of DNA, marker enzymes, and various xenobiotic metabolizing enzymes after subcellular fractionation of rainbow trout kidney by differential centrifugation. Fractions (from left to right) are 600 *g*, 20,000 *g* and 170,000 *g* pellets and cytosol. The values are presented as DeDuve plots [39] and are means of relative specific activities (RSA) ± SE. RSA = the per cent of total activity/per cent of total protein.

activities in the microsomal pellet was greater than that of the conjugating enzyme, UDP glucuronosyltransferase, which has a distribution pattern similar to that of glucose-6-phosphatase. Epoxide hydrolase activity with styrene oxide as substrate was enriched in the microsomal and mitochondrial fractions almost to the same extent. In the soluble fractions 77% of the total glutathione transferase activity towards 1-chloro-2,4-dinitrobenzene was recovered.

Electron microscopy of the microsomal pellet (data not shown) revealed that the pellet contained predominantly microsomal vesicles and some melanin granules. No identifiable nuclei, mitochondria, lysosomes, or peroxisomes were found.

Characterization of xenobiotic metabolizing enzymes

The difference spectra of the complex between carbon monoxide and cytochrome P-450 obtained from kidney and liver microsomes are shown in Figs. 3 A and B, respectively. The microsomes in both sample and reference cuvettes were first bubbled with carbon monoxide. Dithionite was then added to the sample cuvette only and the resulting spectra showed absorption maxima at 427 and 448.5 nm in the kidney microsomes and at 449 nm in the liver microsomes. According to this procedure, the cytochrome P-450 content (expressed as mean ± SE of 5 animals) in kidney and liver microsomes was 0.029 ± 0.002 and 0.125 ± 0.004 nmol/mg protein, respectively.

To ensure that cytochrome b_5 did not interfere with the measurement of cytochrome P-450 in kidney microsomes the difference spectra caused by carbon monoxide was recorded (Fig. 4). The subsequent recording permitted evaluation of oxyhemoglobin contamination (a peak at about 419 nm) in the sample. The addition of a few grains of the chemical reductant, sodium dithionite, to the sample cuvette resulted in the reduction of cytochrome b_5 and cytochrome P-450 and the appearance of an absorbance band at about 449 nm. The addition of a few grains of sodium dithionite to the contents of the reference cuvette permitted the recording of the difference spectrum of the CO complex of reduced cytochrome P-450 (cytochrome b_5 is reduced in both cuvettes and cancelled from the difference spectrum). This caused a shift of the peak from 449 nm to 452 nm (Fig. 4).

The difference spectrum of cytochrome b_5 was determined in a separate experiment using the same dilution conditions as above. NADH was used to reduce cytochrome b_5 which was characterized by an absorption maximum at about 424 nm and a trough at about 410 nm (Fig. 4).

The addition of EC to the sample cuvette containing microsomes produced a spectral shift with a peak at 415 nm and a trough at 430 nm (Fig. 5). The magnitude of spectral change observed was

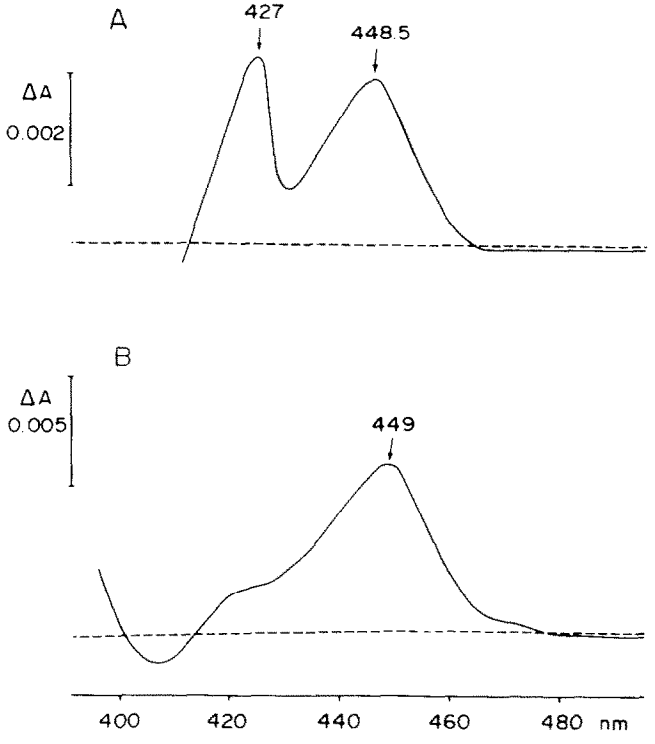

Fig. 3. The difference spectra of the complex between carbon monoxide and the reduced cytochrome P-450 from rainbow trout kidney (3A) and liver microsomes (3B) determined by the method of Matsubara *et al.* [26]. Microsomal suspension was divided between two cuvettes and a base line of equal light absorbance was recorded. Both cuvettes were bubbled with carbon monoxide and a few grains of sodium dithionite were added to the sample cuvette. Kidney and liver microsomal fractions were diluted in 0.1 M potassium buffer, pH 7.4, to give a protein concentration of 0.72 and 0.82 mg per ml, respectively.

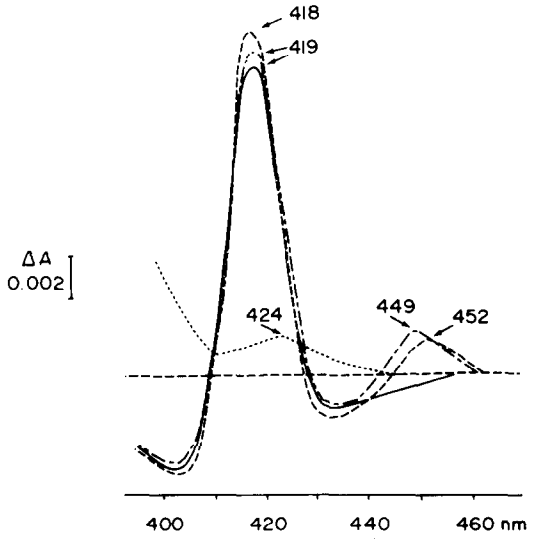

Fig. 4. Difference spectra obtained with rainbow trout kidney microsomes. The diluted microsomal suspension (0.72 mg microsomal protein per ml) was divided into two cuvettes and a base line of equal light absorbance was determined. The contents of a sample cuvette were then gassed gently with carbon monoxide and spectrum was recorded (———). A few grains of sodium dithionite were further added to the contents of sample cuvette and the difference spectrum (—·—·—·) was recorded. A similar amount of sodium dithionite was then added to the reference cuvette and the spectrum (— — — —) was recorded. The difference spectrum of b_5 was determined in a separate experiment using the same dilution of microsomes as described above. After establishing a base line, NADH (0.24 mM final concentration) was added to the contents of the sample cuvette and the difference spectrum (. . . .) was recorded.

dependent on the concentration of EC in the sample cuvette.

The temperature optima for cytochrome P-450-dependent EC- and ER-*O*-deethylase activities were about 30°, and the pH optima were about 8. These activities were linearly related to protein amounts

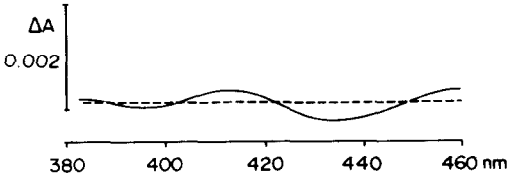

Fig. 5. Difference spectra caused by addition of 7-ethoxycoumarin (80 μM final concentration) to the sample cuvette. Each cuvette contained suspended rainbow trout kidney microsomes at a final concentration of 0.72 mg protein.

between 0.05 and 0.3 mg of microsomal protein. The temperature optimum for UDP glucuronosyltransferase activity was about 35° and the pH optimum 7.5. The activity was linearly related to protein amounts from 0.05 to 0.1 mg. The specific activities of EC- and ER-*O*-deethylase and UDP glucuronosyltransferase in kidney and liver microsomes are shown in Table 1.

DISCUSSION

Although it is known from mammalian studies that preparations of microsomes from extrahepatic organs often give low yields [29], the microsomal fraction from rainbow trout kidney prepared by the method described here contained about 52% of the total kidney NADPH-cytochrome *c* reductase activity. This value is higher than that found for the trunk kidney microsomes of Northern pike (24%) [30] and near to that found for rainbow trout liver microsomes (55%) [31]. Separation of microsomes from plasma membrane has been difficult to achieve in the liver of both rat [32] and rainbow trout [31] as well as in the trunk kidney of the Northern pike [30]. Similar contamination of the microsomal pellet by plasma membrane fragments was also observed in the present study on rainbow trout. On the basis of marker enzyme studies and electron microscopy we therefore suggest that the larger counterpart of the

Table 1. 7-Ethoxycoumarin-*O*-deethylase, 7-ethoxyresorufin-*O*-deethylase and UDP glucuronosyltransferase activities in kidney and liver microsomes from various fish species

	Rainbow trout* (20°)§	Carp† (29°)	Vendace‡ (18°)	Roach‡ (18°)
Kidney				
7-Ethoxyresorufin-*O*-deethylase	0.02 ± 0.01	0.004 ± 0.002	—	—
7-Ethoxycoumarin-*O*-deethylase	0.04 ± 0.01	0.011 ± 0.003	0.006	0.001
UDP-glucuronosyltransferase	0.15 ± 0.07	—	0.088	0.158
Liver				
7-Ethoxyresorufin-*O*-deethylase	0.03 ± 0.01	0.046 ± 0.009	—	—
7-Ethoxycoumarin-*O*-deethylase	0.04 ± 0.01	0.265 ± 0.008	0.011	0.019
UDP-glucuronosyltransferase	0.36 ± 0.02	—	0.066	0.066

Values are expressed as nmoles per mg protein and min and are expressed as means ± SE.
* From present study.
† From ref. 35.
‡ From ref. 6.
§ Incubation temperature.

kidney microsomal fraction isolated by this method was derived from endoplasmic reticulum. The contamination of kidney microsomes with mitochondria was nearly as low (7% of the total homogenate activity of cytochrome oxidase) as the corresponding values for liver microsomes from rainbow trout (0% of the total homogenate activity of succinic dehydrogenase [31]) and trunk kidney microsomes from the Northern pike (6.5% of the total homogenate activity of cytochrome oxidase) [30]. The microsomes from the rainbow trout kidney in our study were also relatively free of lysosomes (14% of the total homogenate activity of β-glycerophosphatase). This contamination of trout kidney microsomes is lower than the corresponding contamination of the microsomal fraction from rainbow trout liver (46% of the total homogenate activity of acid phosphatase) [31] and Northern pike trunk kidney (26% of the total homogenate activity of β-glycerophosphatase) [30].

However, the microsomal fraction was contaminated with a large amount of hemoglobin. The presence of hemoglobin was indicated by absorbance maximum at about 418–419 nm and a minimum at about 434 nm when the carbon monoxide-difference spectrum (400 nm–500 nm) was measured for air-oxidized microsomes (Fig. 4). The presence of hemoglobin in kidney microsomes obscured the peak at 450 nm when both sample and reference cuvettes were reduced and the sample cuvette was bubbled with carbon monoxide. However, the contamination by reduced hemoglobin was compensated for when both cuvettes were bubbled with carbon monoxide and when dithionite was added to the sample cuvette only [26]. By using this method the difference spectrum for the complex between carbon monoxide and rainbow trout kidney microsomal cytochrome P-450 can be measured. The absorption peak at about 427 nm, which was found in the difference spectrum for reduced microsomes bubbled with carbon monoxide from kidney (Fig. 3A) but not from liver (Fig. 3B) was probably due to oxyhemoglobin contamination of the microsomes, i.e. oxyhemoglobin is converted to carbonoxyhemoglobin [27]. Part of this peak may also be attributed to cytochrome b_5, and it is obvious that hemoglobin interferes with the spectral measurement of cytochrome b_5 in this assay. When the influence of the contaminating hemoglobin and cytochrome b_5 were excluded according to the method described by Estabrook *et al.* [27], the peak in the carbon monoxide-difference spectrum for reduced cytochrome P-450 was shifted from 449 nm to 452 nm (Fig. 5). This shift in wavelength has also been reported for renal microsomal cytochrome P-450 from rat after correcting for all known contaminants in the microsomal fraction [29].

The addition of various substrates microsomes produce characteristic absorbance changes in the difference spectrum. The addition of EC to the sample cuvette containing kidney microsomes produced a spectral change with a peak at 415 nm and a trough at about 435 nm. This EC-induced spectral change in rainbow trout kidney microsomes was similar to the substrate binding spectrum obtained by adding EC to liver microsomes from rainbow trout [33] and rat [34].

Table 1 compares the levels of these enzymes in rainbow trout kidney and liver found in this study with the corresponding levels in teleost fish reported by other investigators. The specific activities of EC- and ER-*O*-deethylase in the microsomal fraction of the kidney in the present study were notable because these values were in the same order of magnitude as those found in rainbow trout liver microsomes and much higher than the activities reported for the renal microsomes of carp, vendace, and roach [6, 35]. UDP glucuronosyl transferase activity in the trout kidney microsomes was about half of that found in liver microsomes.

Although marked variations have been reported, the activities of xenobiotic biotransformation enzymes in mammalian extrahepatic tissue are generally much lower than the corresponding activities in the liver. However, in the adrenal gland the specific activity of the cytochrome P-450-dependent monooxygenases, such as benzo(*a*)pyrene hydroxylase and EC-*O*-deethylase, have been found at about the same level or higher compared with those found in the liver [36]. In rainbow trout the adrenal tissue is located in the anterior part of the kidney [37]. Thus, significant amount of the enzyme activity measured in the kidney in our study may be derived from the adrenal cells. It therefore seems essential to investigate the distribution of cytochrome P-450 dependent activities in different parts of the kidney.

In conclusion, this study shows that the biotransformation enzyme systems in the rainbow trout kidney effectively metabolize certain xenobiotic substrates. Future studies are needed to further characterize kidney xenobiotic metabolizing enzymes, including their responsiveness to xenobiotics which are well-known inducers of biotransformation enzymes in the trout liver [38]. Kidney microsomes prepared by the method described here should be suitable for such studies.

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