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SPECTRAL PROPERTIES OF SUBSTRATE–CYTOCHROME P-450 INTERACTION AND CATALYTIC ACTIVITY OF XENOBIOTIC METABOLIZING ENZYMES IN ISOLATED RAINBOW TROUT LIVER CELLS

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Abstract—A method for the isolation of intact viable rainbow trout liver cells in high numbers is described. The technique involves perfusion of collagenase through the liver. A major part of the cytochrome P-450 in isolated liver cells was present in the oxidized non-substrate bound form. It was observed that 7-ethoxycoumarin was rapidly taken up by the liver cells and bound to cellular cytochrome P-450. The substrate binding spectrum for isolated trout liver cells was slightly modified compared with that obtained with trout liver microsomes. The microsomal affinity of 7-ethoxycoumarin, calculated as the apparent spectral dissociation constant (k_s), was elevated 11-fold after fish were treated with β-naphthoflavone, indicating a qualitative alteration in the nature of the constitutive cytochrome P-450.

The metabolism of 7-ethoxycoumarin in isolated liver cells was found to be of a comparable rate to that obtained in liver microsomes. Pretreatment of fish with Clophen A50 or β-naphthoflavone significantly increased the content of cytochrome P-450 and elevated the rate of 7-ethoxycoumarin deethylation in isolated liver cells. Furthermore, the rate of conjugation of 7-hydroxycoumarin was significantly elevated in liver cells isolated from β-naphthoflavone treated fish when compared with the control rate.

In isolated liver cells, 90% of the 7-hydroxycoumarin formed from deethylation of 7-ethoxycoumarin was further metabolized to conjugated products. However, in β-naphthoflavone of Clophen A50 treated fish the fraction of conjugated metabolites was markedly decreased, indicating a changed balance between cytochrome P-450 dependent reactions and conjugation reactions in the cell.

The presence of a xenobiotic biotransformation enzyme system in fish is now well established and has been the subject of several recent reviews [1–4]. Research in this area has been focused on the *in vitro* activities of various liver microsomal cytochrome P-450 monooxygenases (phase I). An important characteristic of fish liver cytochrome P-450 enzymes is their ability to be induced by aquatic pollutants, such as polychlorinated biphenyls (PCB) and polyaromatic hydrocarbons (PAH), which results in 10- to 50-fold elevation of several monooxygenase activities [5–10].

Analyses of tissue from fish exposed to xenobiotics have shown that foreign compounds after monooxygenation are, to a large extent, further metabolized by epoxide hydrolase and/or are conjugated with glucuronide, sulfate or mercapturic acid (phase II) [11–14]. However, little attention has been paid to the postoxidation enzymes in fish. For rainbow trout, *in vivo* studies and studies on isolated perfused livers have shown that the metabolites of several aromatic hydrocarbons excreted into the bile are hydroxylated and glucuronidated products whereas sulfates are present in very low amount or absent [13–16]. These reports indicate that foreign compounds in fish are metabolized via sequential enzyme systems in the liver and that conjugated xenobiotics are endproducts of xenobiotic biotransformation.

Many of the features of xenobiotic metabolism in fish appear to be qualitatively similarly to the better known systems in mammals. However, the metabolic properties which an organism can express, may be regulated differently in poikilothermic organisms such as fish compared with homeothermic animals. In mammals, isolated liver cells have been extensively used and shown to be a suitable system for studies on factors regulating xenobiotic metabolism [17, 18]. The present study was therefore undertaken in order to characterize the sequence of reactions involved in xenobiotic metabolism in isolated rainbow trout liver cells. The rate of uptake and binding of 7-ethoxycoumarin (EC) to cytochrome P-450 and the subsequent conjugation of its product 7-hydroxycoumarin (HC) were investigated. The study was performed on liver cells isolated from control trout or trout treated with Clophen A50 (Cl A50) or β-naphthoflavone (BNF), potent inducers of cytochrome P-450.

MATERIALS AND METHODS

Fish. Cultured immature rainbow trout, *Salmo gairdneri*, of both sexes and with an average weight of 200–300 g were obtained from a local hatchery near Göteborg. The trout were kept in basins with aerated, filtered and recirculated fresh water at a temperature of 10°. A 12 hr light–12 hr dark daily cycle was used. All fish were acclimated to these conditions for 7 days and then starved for the duration of this and the experimental period.

Treatment of fish. When induction of bio-

transformation was studied, the trout received a single intraperitoneal (i.p.) injection of Cl A50 (500 mg/kg body weight in peanut oil) or BNF (100 mg/kg body weight in peanut oil). Control trout received i.p. injections of peanut oil only (500 mg/kg body weight). Sampling was performed 7 days after BNF injection and 14 days after Cl A50 injection. These doses of inducers and treatment periods have been shown to assure maximum induction of cytochrome P-450 dependent activities [5, 7].

Isolation of liver cells. Liver cells were isolated by the perfusion method described by Berry and Friend [19] and modified by Seglen [20]. The operative technique was performed according to Förlin and Andersson [21]. The perfusion system consisted of a peristaltic pump which pumped a Salmo buffer (NaCl, 7.41 g/l; $CaCl_2$, 0.17 g/l; KCl, 0.36 g/l; $MgSO_4$, 0.15 g/l; Na_2HPO_4, 1.6 g/l; NaH_2PO_4, 0.4 g/l; and $NaHCO_3$, 0.31 g/l) [22] from a reservoir through an oxygen lung [23]. The lung provided the liver with a thermostated (20°) and oxygen-equilibrium medium which perfused the organ at a constant pressure and a flow-rate of 1–1.5 ml per min per g liver. The perfusion started *in situ* with a Ca^{2+} free Salmo buffer which contained 10 mM ethyleneglycol-bis-(β-amino ethyl ether) *N,N'*-tetraacetic acid (EGTA). The liver was removed from the fish, placed in an organ chamber and after 10 min of perfusion with Ca^{2+} free medium the perfusion solution was changed to a Salmo buffer with Ca^{2+}, which contained collagenase (1500 U) and hyaluronidase (6000 U) in a total volume of 50 ml. This perfusion fluid was allowed to circulate through the liver for 30 min. The liver was dispersed gently using a Teflon rod and then shaken for 5 min in the Salmo buffer containing the digestive enzymes. After filtration through a nylon mesh, the cells were centrifuged for 45 sec at 30 *g* and then washed twice with Salmo buffer. The number of cells was estimated by counting the cell suspension in a Salmo buffer containing 0.4% Trypan blue. Each cell preparation, which was a mixture of parenchymal and non-parenchymal cells, yielded about 100–200 × 10^6 cells per g wet weight of liver.

Transmission electron microscopy. Cell suspensions (0.5 ml aliquots) were fixed for 2 hr in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, postfixed in 1% OsO_4 in sodium cacodylate buffer, dehydrated and then embedded in Epon 812. Sections were stained with uranyl acetate and lead citrate and examined using a Zeiss EM 109 instrument.

Isolation of microsomes. The liver was isolated, weighed and placed in ice-cooled 0.15 M KCl. The preparation of microsomes was performed as described by Förlin [8]. The microsomal pellet was finally suspended in 0.1 M phosphate buffer pH 7.4 containing 0.15 M KCl, 1 mM EDTA and 20% glycerol.

Spectral measurements. Aminco DW2a uv/vis spectrophotometer was used for all spectral measurements. The amounts of cytochrome P-450 in liver cells and microsomes were measured using the method of Estabrook *et al.* [24]. Substrate binding spectra with ethylmorphine or EC were measured according to Schenkman *et al.* [25]. 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 rate of formation of EC induced spectral change was recorded as the increase in the absorbance difference between 415–433 and 408–428 nm for microsomes and liver cells, respectively, when 2 μl of 0.1 M EC in methanol was plunged into the cuvette.

Assays. Liver cells were incubated in a total volume of 3.5 ml using 25 ml Erlenmeyer flasks which were gently shaken on a water bath at 16°. EC or HC was added to the incubation in 10 μl acetone or dimethylsulfoxide. Reactions were stopped by transferring 0.5 ml of the incubate into 0.25 ml 5% trichloroacetic acid. After centrifugation, the supernatant was used for metabolite analysis. Supernatant from EC incubation was adjusted to pH 5.0 and incubated with β-glucuronidase/arylsulfatase in order to hydrolase the HC-conjugates. The sample transferred at zero time was used as the blank. Acid hydrolase (2 M HCl at 100° for 3 hr) did not release any further HC. No attempts were made to separate HC glucuronide and sulfate conjugates. In a previous report on EC metabolism in an isolated perfused trout liver, it was shown that only a minor fraction (<5%) of HC, excreted in the perfusate was sulfate conjugates [16]. The activity of conjugation enzymes was measured by determining the disappearance of free HC from the incubation. The amount of HC in the supernatant was measured, as previously described [16], by determining the fluorescence (excitation wavelength 370 nm and emission wavelength 460 nm) of a 50 μl sample in 3 ml phosphate–borate buffer (pH 10.5).

EC-*O*-deethylase activity in microsomes was assayed at 16° in an incubation medium containing 0.5–1 mg microsomal protein as described by Förlin and Hansson [26]. Microsomal protein content was determined by the method of Lowry *et al.* [27] using bovine serum albumin as standard. Latency of lactate dehydrogenase in cell incubations was measured as described by Moldéus *et al.* [28].

Chemicals. 7-Ethoxycoumarin and 7-hydroxycoumarin were purchased from Aldrich-Europe (Bersee, Belgium). Collagenase (type V), hyaluronidase (type 1-S), β-glucuronidase containing arylsulfatase activity, NADPH, NADH and β-naphthoflavone were purchased from Sigma Chemical Co. (St Louis, MO). Clophen A50 was a gift from Bayer Chemical, Germany. All other chemicals were of analytical grade.

Statistics. All values represent means ± S.D. Statistical analysis was performed using two-tailed Mann–Whitney U-test and the significance level was set at 0.05.

RESULTS

Cell viability

The isolated cell fraction consisted mainly of morphological intact and free hepatocytes (Fig. 1). Cell organelles and plasma membranes appeared normal and showed the usual arrangements. Only small amounts of cell debris could be detected. The yield of cells (100–200 × 10^6 cells per g liver) was comparable to that obtained from rainbow trout livers by Parker *et al.* [29] (80–380 × 10^6 cells per g liver)

and slightly higher than the yield from rat samples (70–100 × 10^6 cells per g liver) [26]. The viability which was always estimated by Trypan blue exclusion test prior to other experiments, yielded a high percentage (>90%) of unstained cells. All experiments were performed within 2 hr after the cell isolation. During this time no changes in viability were detected. Before the cell isolation procedure was routinely used, isolated liver cells were tested for latency of cytosolic enzymes (lactate dehydrogenase) and a leakage of less than 15% was recorded. The viability was further confirmed by the retention of integrated xenobiotic metabolism.

Spectral studies of cytochrome P-450

Introduction of EC into the sample cuvette containing microsomes produced a binding spectrum with a peak at 415 nm and a trough at 432 nm (Fig. 2A). When the reciprocal increase in absorbance was plotted against the reciprocal concentration of EC, apparent spectral dissociation constant (k_s) values of 0.25 and 0.022 mM were obtained for microsomes from control and BNF treated trout, respectively (data not shown). Introduction of EC to the sample cuvette containing isolated liver cells caused a spectral shift with a peak at about 408 nm and a trough

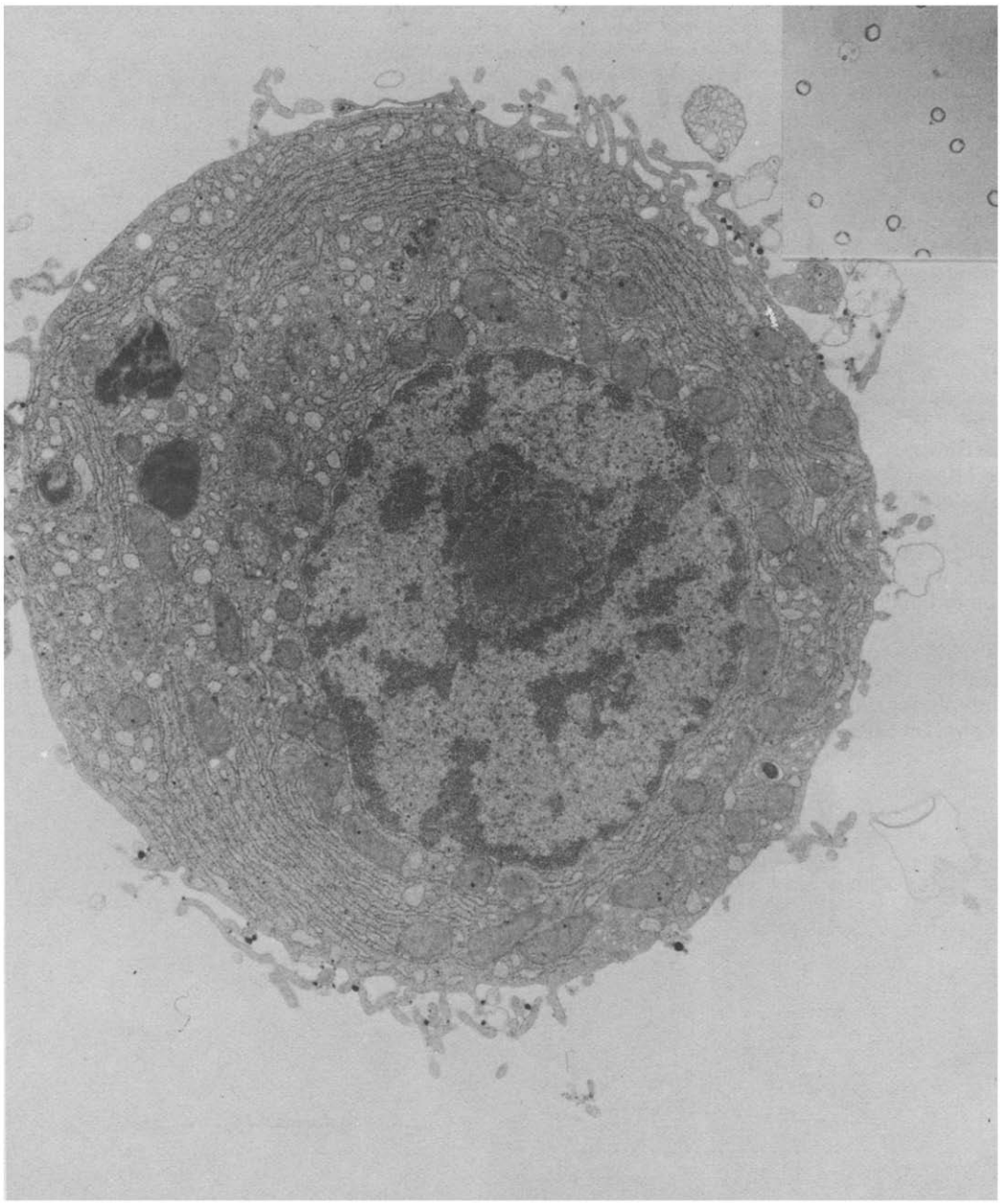

Fig. 1. Electron micrograph showing isolated hepatocyte from control rainbow trout (magnification: 16,300×). Inserted light microscopic photograph of freshly isolated liver cells suspended in Salmo buffer containing 4% Trypan blue (magnification: 250×).

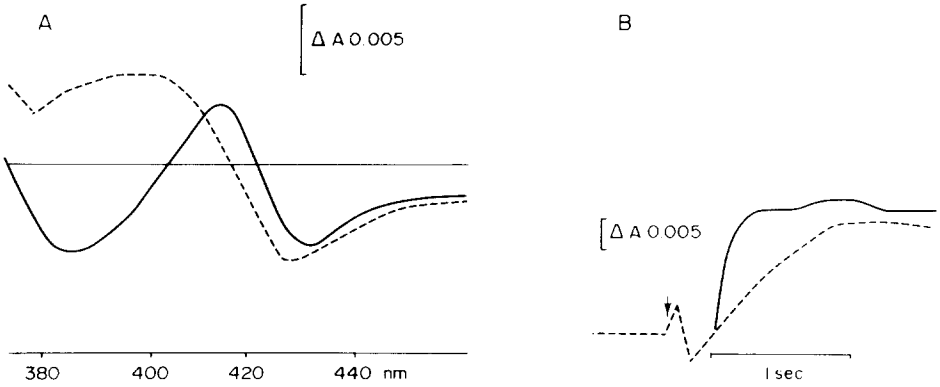

Fig. 2. Spectra produced by the addition of 7-ethoxycoumarin (0.1 mM final concentration) to microsomes (———) (2 mg microsomal protein per ml) or liver cells (– – – –) (1×10^7 cells per ml) (A). The rate of formation of 7-ethoxycoumarin-cytochrome P-450 complex was recorded as the interaction of the absorbance between 415 and 433 nm in microsomes (———) or between 408 and 428 nm in hepatocytes (– – – –) (B). 7-Ethoxycoumarin addition is indicated by the arrow.

at 428 nm (Fig. 2A). The uptake and subsequent binding of EC to cytochrome P-450 in isolated liver cells, estimated by the rate of formation of spectral change after EC addition into the cuvette, was very rapid and took place within seconds however was slower than that observed for microsomes (Fig. 2B).

The introduction of CO and the subsequent addition of ethylmorphine to the sample cuvette, sample and reference cuvette containing control trout liver cells, did not produce any different spectrum. However, in liver cells isolated from BNF-treated trout, the CO-bubbling caused a slight increase in absorption at about 450 nm. Subsequent addition of ethylmorphine caused a further increase in absorption. The maximum spectral change was obtained when the sample cuvette was reduced with sodium dithionite (Fig. 3).

Metabolism of EC and HC

The metabolism of EC in liver cells was measured for different concentrations of the substrate. Saturated concentration was reached when 0.2 mM of EC was used. The EC metabolism showed a linear correlation between time and cell concentrations for at least 90 min for the range $3–7 \times 10^6$ cells per ml (Fig. 4). The conjugation of HC for different concentrations of the substrate is shown in Fig. 5. A slight increase in activity was found only above 2.5 μM. Formation of conjugated products was linear for 10 min for the range $1–3 \times 10^6$ cells per ml.

Pretreatment of fish with BNF or Cl A50 resulted in a significantly elevated content of cytochrome P-450 and an increased rate of metabolism of EC in isolated liver cells (Table 1). Furthermore, the rate of conjugation of HC was found to be significantly higher in liver cells isolated from BNF pretreated fish than in control. In isolated liver cells 90% of the HC formed from deethylation of EC was further metabolized to conjugated products. However, the conjugated metabolite fractions in liver cells from Cl A50 and BNF pretreated fish were 31% and 73%, respectively. The extent of induction of EC metabolism in liver cells was similar to the increase of EC-*O*-deethylase activity in liver microsomes following

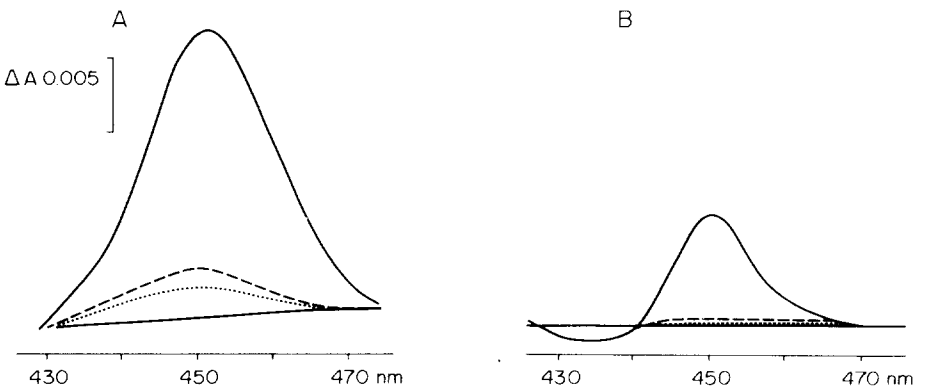

Fig. 3. Spectra of isolated liver cells from β-naphthoflavone treated (A) and control (B) trout. Each cuvette contained 4×10^6 cells per ml. Spectra were recorded after CO bubbling for 30 sec (.....), addition of ethylmorphine (– – – –) (1 mM final concentration) and sodium dithionite (———).

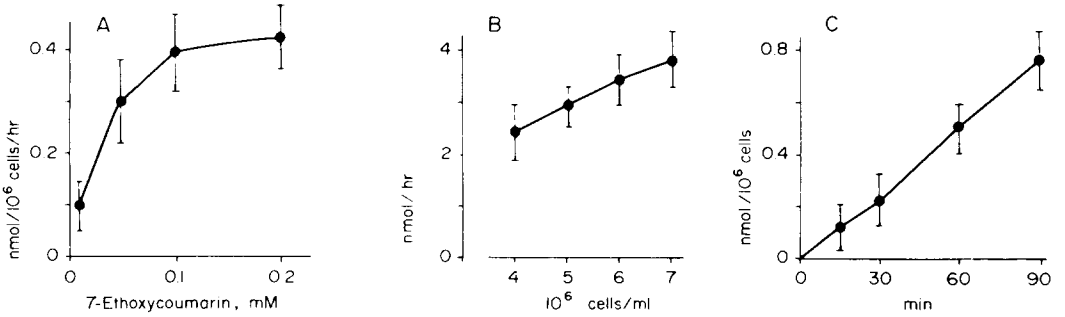

Fig. 4. Metabolism of 7-ethoxycoumarin in isolated liver cells from control rainbow trout: (A) incubation time 60 min, cell concentration 5×10^6 cells per ml; (B) incubation time 60 min, substrate concentration 0.2 mM; (C) cell concentration 5×10^6 cells per ml, substrate concentration 0.2 mM. Values represent means ± S.D. of four animals.

BNF treatment of rainbow trout. Furthermore, when the cytochrome P-450 linked metabolism of EC was expressed on a per cytochrome P-450 basis, it was seen that the activities in liver cells and microsomes were in the same order of magnitude both for control and BNF treated fish (Table 1).

DISCUSSION

In mammals it is well documented that cytochrome P-450 catalyses the monooxygenation of a high variety of exogenous as well as endogenous substrates [30, 31]. The catalytic cycle of cytochrome P-450 dependent hydroxylation reactions is a series of events which include binding of the substrate to the oxidized form of cytochrome P-450 and reduction by way of cytochrome P-450 reductase. The interaction between mammalian microsomal cytochrome P-450 and substrate evokes spectral changes classified as type I, type II or reversed type I, dependent on the character of the spectrum recorded [32]. Upon addition of EC to the sample cuvette containing trout liver microsomes, a substrate binding spectrum was recorded (peak at 415 nm and trough at 432 nm) similar to that found when EC was added to rat liver microsomes (peak at 410 nm and trough at 428 nm) [33]. However, the EC induced spectral change in the trout microsomes was different from the type I substrate binding spectrum (peak at 390 nm and trough at 420 nm) of ethylmorphine, hexobarbital or piperonylbutoxide and the type II substrate binding spectrum (peak at 430 nm and trough at 410 nm) of imidazole previously recorded in rainbow trout liver microsomes [7, 34]. The magnitude of spectral changes observed in the present study in trout microsomes was dependent on the concentration of EC in the sample cuvette in a similar fashion to the effect of substrate concentration on the EC-*O*-deethylase activity in trout microsomes [7]. This indicates that spectral changes induced by EC in trout liver microsomes are a manifestation of enzyme–substrate complex. The microsomal affinity of EC in BNF pretreated fish, calculated as the apparent spectral dissociation constant (k_s), was 11-fold higher than the control value, thus indicating a qualitative alteration in the nature of the constitutive cytochrome P-450. The slightly modified substrate binding spectrum of isolated liver cells, when compared with liver microsomes, may be due to intracellular components influencing the cytochrome P-450–substrate interaction. The appearance of a spectral change in isolated liver cells was used in the study of the rate of cellular uptake and the subsequent binding of EC to the cytochrome P-450. While this process was found to be very fast and took place within seconds, which is of a comparable rate to that found in isolated rat liver cells [35], it was slower than the rate found in isolated trout microsomes upon addition of EC. The

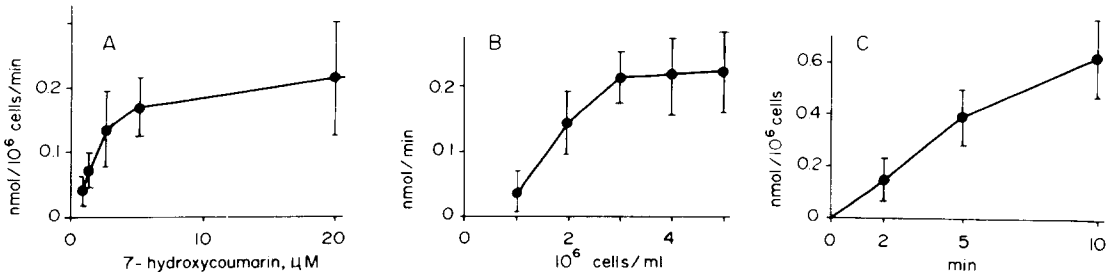

Fig. 5. Metabolism of 7-hydroxycoumarin in isolated liver cells from control rainbow trout: (A) incubation time 5 min, cell concentrations 3×10^6 cells per ml; (B) incubation time 5 min, substrate concentration 5 μM; (C) substrate concentration 5 μM cell concentration 3×10^6 cells per ml. Values represents means ± S.D. of four animals.

Table 1. Cytochrome P-450 content, 7-ethoxycoumarin* and 7-hydroxycoumarin† metabolism in isolated liver cells and 7-ethoxycoumarin metabolism‡ in liver microsomes from rainbow trout

	Control (6)§	β-naphthoflavone (4)	Clophen A50 (4)
Cells			
Cytochrome P-450 content			
nmoles per 10^6 cells	0.039 ± 0.003	0.094 ± 0.042	0.107 ± 0.048
7-Ethoxycoumarin metabolism			
nmoles 7-hydroxycoumarin per 10^6 cells per min	0.009 ± 0.003	0.100 ± 0.034‖	0.028 ± 0.021‖
% Conjugated products	90 ± 10	31 ± 12‖	73 ± 14‖
nmoles 7-hydroxycoumarin per nmoles cytochrome P-450 per min	0.23 ± 0.08	0.93 ± 0.78	n.d.
7-Hydroxycoumarin conjugation			
nmoles conjugate per 10^6 cells per min	0.078 ± 0.024	0.153 ± 0.026‖	0.096 ± 0.046
Microsomes			
7-Ethoxycoumarin metabolism¶			
nmoles 7-hydroxycoumarin per nmoles cytochrome P-450	0.135 ± 0.073	0.783 ± 0.158	n.d.

Values are means ± S.D.

* Incubations were performed at 16°, 60 min, using 5×10^6 cells per ml and 0.2 mM 7-ethoxycoumarin.

† Incubations were performed at 16°, 5 min, using 3×10^6 cells per ml and 5 μM 7-hydroxycoumarin.

‡ Incubations were performed at 16°, 15 min using 0.5–1 mg microsomal protein per assay

§ Number of animals used.

‖ Significantly different from control group ($P < 0.05$).

¶ Cytochrome P-450 content per nmol per mg microsomal protein: control 0.325 ± 0.073; β-naphthoflavone 0.544 ± 0.034. 7-Ethoxycoumarin-*O*-deethylase activity, nmoles per mg microsomal protein: control 0.044 ± 0.341; β-naphthoflavone 0.434 ± 0.293.

n.d. not determined.

fast appearance of the binding spectrum in liver cells indicates that the uptake of EC is not a rate limiting step for the metabolism in the cell.

In the reduced form, cytochrome P-450 binds to CO which gives rise to a prominent absorption band at 450 nm [36]. In intact rat liver cells it has been suggested that only the substrate bound form of cytochrome P-450 can accept endogenous reducing equivalents and thus interact with CO [37]. Because the introduction of CO into the sample cuvette containing freshly isolated liver cells from control trout did not produce any peak at 450 nm, the cytochrome P-450 was present in the oxidized non-substrate-bound form, whereas a minor fraction of the cytochrome P-450 in isolated liver cells from BNF-treated trout was endogenously reduced.

The cytochrome P-450 content in trout liver cells shown in the present study (0.039 nmole/10^6 cells) was slightly higher than that reported by Parker *et al.* [29] (0.025 nmol/10^6 cells). However the level of cytochrome P-450 in trout liver cells is about 15% of the level in rat liver cells [28]. Furthermore, in trout liver cells incubated at 16°, the rates of EC-metabolism and HC-conjugation were about 11% and 46%, respectively, of the activities reported in rat liver cells incubated at 37° [38].

Several studies in fish have shown experimental induction of cytochrome P-450 dependent activity by compounds such as PCB and PAH [5–10]. In the present study BNF or Cl A50 treatment of rainbow trout increased the metabolism of EC in isolated liver cells to the same extent as in the liver microsomal fraction seen in the present and previous studies [7]. Conflicting results have been reported for the inducibility of UDP GT activity in rainbow trout, possibly due to variations in the dose and the treatment times used [39, 40]. The two-fold elevation of the conjugation of HC following BNF treatment found in the present study was in accordance with a previous study in which we found a 1.6-fold elevation by BNF of microsomal UDP GT activity towards *p*-nitrophenol as substrate (T. Andersson and U. Koivusaari, unpublished observations).

In rainbow trout, EC is metabolized in a sequence of reactions including deethylation by cytochrome P-450 to HC which is further conjugated mainly with glucuronic acid [16]. In the present study the 8.6-fold faster rate of conjugation when compared to the deethylation of EC in isolated liver cells supports the contention that the rate of conjugation proceeds faster than that of oxidation in fish [41]. However, in liver cells from BNF or Cl A50 treated fish, the difference in rate between cytochrome P-450 and conjugation activity was smaller than in non-treated fish because of the greater responsiveness of the cytochrome P-450 to the inducing agents. The shift in the balance between phase I and phase II reactions was further demonstrated by the formation in the incubation of a greater portion of non-conjugated EC-products for cells isolated from induced fish compared with cells from non-treated fish. The decreased portion of conjugated metabolites may reflect a change in the relative amount of xenobiotic metabolizing enzymes but may also be due to exhaustion of co-factors needed for the conjugation enzymes. The dramatic alteration of the balance between phase I and phase II reactions in induced fish may have effects on the fate and toxicity of organic chemicals *in vivo* in the organism. Induction of cytochrome P-450 dependent reactions may be particularly

important for the formation of toxic products of polyaromatic hydrocarbons such as benzo(*a*)pyrene. Recent reports on fish have demonstrated that benzo(*a*)pyrene is metabolized via cytochrome P-450 enzyme system to reactive intermediates in both subfractionated organ and cell culture assay systems as well as in fish exposed to benzo(*a*)pyrene *in vivo* [42–44].

Isolated mammalian liver cells have been used in studies on the regulation of cytochrome P-450 system. The enzyme system is dependent on a continuing supply of reducing equivalents for its function and the cytosolic NADPH/NADP ratio has been suggested to be an important factor controlling cytochrome P-450 activity in the cell (18). When cytochrome P-450 activity is measured in isolated microsomes, the reaction is assayed under optimal conditions with NADPH in excess, whereas the cytochrome P-450 enzyme system in isolated hepatocytes is intimately related to other cellular events which include the generation of reducing co-factors. In the present study, isolated trout liver cells were found to catalyse deethylation of EC at a comparable rate to that obtained with liver microsomes, when the rate of deethylation was expressed on a per cytochrome P-450 basis for control and BNF treated fish. These observations indicate that the metabolism of EC in the trout liver cell was not limited by NADPH supply but rather by factors such as the amount of cytochrome P-450. However, further studies are needed in order to improve the understanding of intracellular regulation of xenobiotic metabolism in fish. Isolated liver cells may be a valuable tool in such studies.

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