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DIFFERENTIAL INDUCTION OF CYTOCHROME P-450-DEPENDENT MONOOXYGENASE, EPOXIDE HYDROLASE, GLUTATHIONE TRANSFERASE AND UDP GLUCURONOSYL TRANSFERASE ACTIVITIES IN THE LIVER OF THE RAINBOW TROUT BY β-NAPHTHOFLAVONE OR CLOPHEN A50

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Abstract—After administration of β-naphthoflavone and Clophen A50 to juvenile rainbow trout, activities of hepatic cytochrome P-450-dependent deethylation of 7-ethoxyresorufin was increased 172- and 49-fold, respectively. Glutathione transferase activity towards 1-chloro 2,4 dinitrobenzene and UDP glucuronosyltransferase activities towards *p*-nitrophenol, 1-naphthol and testosterone were increased 1.4 to 3.0-fold by β-naphthoflavone or Clophen A50. However, significant increases of the rate of glucuronidation of 1-naphthol by Clophen A50 and of testosterone by both Clophen A50 and β-naphthoflavone were only determined when the activities were measured in digitonin activated microsomes. Epoxide hydrolase activity was not affected by β-naphthoflavone or Clophen A50.

The time course of induction of the various xenobiotic metabolizing enzymes exhibited different patterns. 7-Ethoxyresorufin-*O*-deethylase activity reached peak values 3 and 7 days after the administration of β-naphthoflavone and Clophen A50, respectively. The rate of induction of glutathione transferase activity and UDP glucuronosyltransferase activities towards *p*-nitrophenol and 1-naphthol were relatively slow and did not reach distinct peak levels. These activities were still on maximum levels 4–6 weeks after the treatment. Glucuronidation of testosterone reached peak values 1 week after treatment with both β-naphthoflavone and Clophen A50.

The dissimilar patterns of induction of the cytochrome P-450-dependent activities and the various conjugation activities may indicate that these xenobiotic metabolizing enzymes are differently regulated in the rainbow trout liver.

The biotransformation of organic xenobiotics is one of the prime factors determining their distribution and retention in fish [1]. Xenobiotic biotransformation end-products are frequently metabolites which are combined with endogenous molecules to form conjugates. These conjugates are less toxic and more water-soluble than their precursors. In fish the major route by which xenobiotics, e.g. naphthalene, benzo(*a*)pyrene, polychlorinated biphenyls (PCB), are conjugated appears to be through the formation of glucuronides, which have been detected in both bile and urine [2, 3].

Many xenobiotics in fish are not directly conjugated but must first be metabolically transformed to yield a group suitable for combination with the conjugating agent. These reactions are carried out primarily by microsomal cytochrome P-450-dependent reactions. In several species of fish the existence of liver microsomal cytochrome P-450 capable of metabolizing lipid-soluble xenobiotics has been demonstrated [4, 5]. Many xenobiotics are metabolized to less biologically active compounds; however, cytochrome P-450 may also convert certain xenobiotics to intermediates which are more toxic than the parent compounds [6, 7]. Metabolic activation of polyaromatic hydrocarbons (PAH), e.g. formation of reactive areneoxides from benzo(*a*)pyrene, have been extensively studied in mammals [8]. If these reactive intermediates are not covalently bound to macromolecules, they are deactivated either by epoxide hydrolase to dihydrodiols or by glutathione transferase to glutathione conjugates. Glutathione transferase and epoxide hydrolase have been detected in some fish species [5] but the activities of these enzymes have been poorly characterized.

Several reports have demonstrated that cytochrome P-450 is a highly inducible enzyme system in fish [9–13]. Treatment of fish with PCB or polyaromatic hydrocarbons (PAH) results in up to a 50-fold elevation in certain *in vitro* monooxygenase activities. However, little attention has been directed towards the induction process regulating post-oxidation enzymes. In this study we sought to characterize the effects of Clophen A50 (Cl A50) and β-naphthoflavone (BNF), both potent inducers of cytochrome P-450, on UDP glucuronosyltransferase, glutathione transferase and epoxide hydrolase activities in the rainbow trout liver.

MATERIALS AND METHODS

Fish. Cultured immature rainbow trout, *Salmo gairdneri*, ranging in weight between 100 and 200 g,

were obtained from a local hatchery near Göteborg. The trout were kept in basins with aerated filtered and recirculating fresh water at 10°.

Experimental treatment and sampling. In a time course study the trout received a single intraperitoneal injection of Cl A50 (500 mg per kg body weight) or BNF (100 mg per kg body weight) in peanut oil. Control trout received a single injection of peanut oil only. At the indicated times (see Figs 1 and 2) five fish from each group were sampled. In a subsequent single time study the trout received a single intraperitoneal injection of Cl A50 (50 or 500 mg per kg body weight) or BNF (50, 100 or 200 mg per body weight). The period of treatments were scheduled so as to obtain maximum induction of the various enzyme activities as indicated in the time course study. In fish injected with BNF the activities were measured after 3 days (7-ethoxycoumarin-*O*-deethylase), 1 week (UDP glucuronosyltransferase towards testosterone), 3 weeks (UDP glucuronosyltransferase towards *p*-nitrophenol and 1-naphthol and glutathione transferase). In fish injected with Cl A50 enzyme activities were measured after 1 week (7-ethoxyresorufin-*O*-deethylase, UDP glucuronosyltransferase towards testosterone), 4 weeks (UDP glucuronosyltransferase towards *p*-nitrophenol and 1-naphthol and glutathione transferase). Epoxide hydrolase activity did not vary during the study over time and the enzyme activity was measured 1 week after treatment with BNF or Cl A50. After the treatment the fish were held in separate aquaria filled with 50 l. circulating filtered water at 12°.

Sampled fish were killed by a blow on the head and their livers were removed and washed with ice-cooled 0.1 M phosphate buffer (pH 7.4). Microsomes were prepared according to Förlin [12] and the microsomal pellets were suspended in 0.1 M phosphate buffer (pH 7.4) containing 0.15 M KCl and 20% glycerol and they were then stored at −80°.

Assays. Protein content was determined according to Lowry *et al.* [14] using bovine albumin as a standard. The total amount of microsomal cytochrome P-450 was measured as described by Estabrook *et al.* [15]. 7-Ethoxyresorufin-*O*-deethylase activity was measured as described by Burke and Mayer [16]. The assay prepared in the fluorometric cuvette was carried out in 2 ml of solution consisting of 0.1 M Tris–HCl buffer (pH 7.8) and 0.2–0.4 mg microsomal protein. The reaction was initiated by adding 10 μl NADPH (10 mM). The progressive increase in fluorescence (excitation wavelength 530 nm, emission wavelength 585 nm) was measured at 20° for 1 min. The fluorometer was calibrated using known amounts of resorufin.

Glutathione transferase activity was measured according to Habig *et al.* [17] using 1-chloro 2,4 dinitrobenzene as substrate. The assay, prepared in the cuvette, was carried out in a 2.5 ml mixture of 0.1 M phosphate buffer (pH 7.4) and 20 mM 1-chloro 2,4 dinitrobenzene. The reaction was initiated by addition of 5 μl 100,000 *g* supernatant (75 μg protein). The increase in absorbance at 344 nm was recorded at 20° for 1 min.

UDP glucuronosyltransferase activity towards *p*-nitrophenol was determined as described by Koivusaari [18]. The incubation was carried out in 0.125 ml mixture containing 0.5 M potassium phosphate buffer (pH 7.0) and 7 mM UDP glucuronic acid. The incubation was started by adding microsomes (0.4–0.6 mg of protein) and lasted for 30 min at 10°. UDP glucuronosyltransferase activity towards 1-naphthol was measured using a modified version of Bock's method [19]. The incubation was carried out in 0.2 ml solution containing 0.5 mM 1-naphthol, 7 mM UDP glucuronic acid, 0.1 M Tris–HCl buffer (pH 7.4) and 5 mM $MgCl_2$. The reaction was started by adding microsomes (1.6–2.5 mg of protein) and performed for 30 min at 10°. The fluorescence of naphthol glucuronide was determined at pH 10 using an excitation wavelength of 290 nm and an emission wavelength of 330 nm. Known amounts of 1-naphthol-glucuronide were used to calibrate the spectrofluorometer. UDP glucuronosyltransferase activity towards testosterone was assayed according to Rao *et al.* [20]. The incubation was carried out in 1 ml solution containing 0.1 M Tris–HCl buffer (pH 8.3), 2 mM UDP glucuronic acid, 10 mM $MgCl_2$ and 0.6 μmol [4-^{14}C]testosterone (100,000 cpm). The incubation was started by adding microsomes (1.6–2.5 mg of protein) and lasted for 30 min at 10°. The incubated solution was extracted with 15 ml dichloromethane. Radioactivity counts were made in both water and organic phases. When assaying UDP glucuronosyltransferase in activated microsomes, the incubations contained 0.2% digitonin for *p*-nitrophenol assays and 0.8% digitonin for the testosterone and 1-naphthol assays. These concentrations of digitonin were found to maximally activate the conjugation enzymes (data not shown).

Epoxide hydrolase activity was measured according to Jerina *et al.* [21] using styrene oxide as a substrate. The incubation solution contained 25 μl 0.5 Tris–HCl buffer (pH 8.7) and 50 μl water containing resuspended microsomes (120 μg of protein). The reaction was initiated by adding 5 μl substrate (80 nmol, 10^6 DPM dissolved in acetonitrile containing 0.1% concentrated ammonia) and performed for 10 min at 10°. It was terminated by adding 1 ml hexane. After one more extraction with hexane, 25 μl of tetrahydrofuran containing 10% styrene oxide and 10% styrene glycole was added to the water phase. A 35 μl portion of the water phase was then applied to the loading zone of a 19 channel LK5DF silica gel plate (Whatman) and developed in ethylacetate/chloroform (2:8 v/v). After localization of the styrene glycole band by uv irridation, the material in this zone was scraped from the plate into a scintillation vial, extracted with 2 ml methanol and its radioactivity level determined.

All enzyme activities were measured at acclimation temperature except for 7-ethoxyresorufin-*O*-deethylase and glutathione transferase activities which were measured at 20°. The pH levels for the enzyme reactions used were found to be optimal in all cases and enzyme activities showed linear correlation with time and protein concentration at the temperatures used.

Spectrophotometric and spectrofluorometric measurements were carried out using an Aminco DW 2a uv/vis and a Farrand spectrofluorometer, respectively. Scintillation counting was performed

using a LKB Wallac Rackbeta scintillation counter.

Chemicals. NADPH, UDP glucuronic acid, 1-chloro 2,4 dintrobenzene, 1-naphthol-glucuronide, testosterone and BNF were purchased from Sigma Chemical Co. (St Louis, MO) ER and resorufin from Pierce Eurochemie B.V. (Rotterdam, Netherlands), *p*-nitrophenol and 1-naphthol from Fluka AG (Buchs, Switzerland), [^{14}C]testosterone and [^{14}C] styreneoxide from Amersham International (Amersham, U.K.). Cl A50 was a gift from Bayer Chemical, F.R.G. All other chemicals were of analytical grade.

Statistics. Statistical analysis was performed using a two-tailed Mann–Whitney *U*-test.

RESULTS

The level of cytochrome P-450-dependent 7-ethoxyresorufin-*O*-deethylase activity increased markedly following the BNF and Cl A50 treatments and reached peak values on day 3 (BNF) and day 7 (Cl A50) (Fig. 1A). Glutathione transferase activity reached maximum values 3 and 4 weeks after treatment with BNF and Cl A50, respectively, and was never observed to decline during the experimental period (Fig. 1B). In contrast, epoxide hydrolase activity was not apparently affected by the inducing agents during the 4-weeks long experimental period (Fig. 1C).

UDP glucuronosyltransferase activity towards *p*-nitrophenol was markedly increased 1–2 weeks following a single injection of BNF (Fig. 2A). The UDP glucuronosyltransferase activity towards 1-naphthol was increased 2–3 weeks after a single injection of BNF. The elevated rates of *p*-nitrophenol and 1-naphthol conjugation following BNF injection did not reach distinct peak values and were still on maximum levels 4–6 weeks after the treatment. Treatment of fish with Cl A50 increased the UDP glucuronosyltransferase activity towards *p*-nitrophenol 4 weeks after the single injection whereas the activity towards 1-naphthol was not apparently affected by Cl A50 (Figs 2A, 2B). The UDP glucuronysltransferase towards testosterone was equally affected by treatment with Cl A50 and BNF; a slight increase was observed 1 week after the injection with these inducers (Fig. 2C).

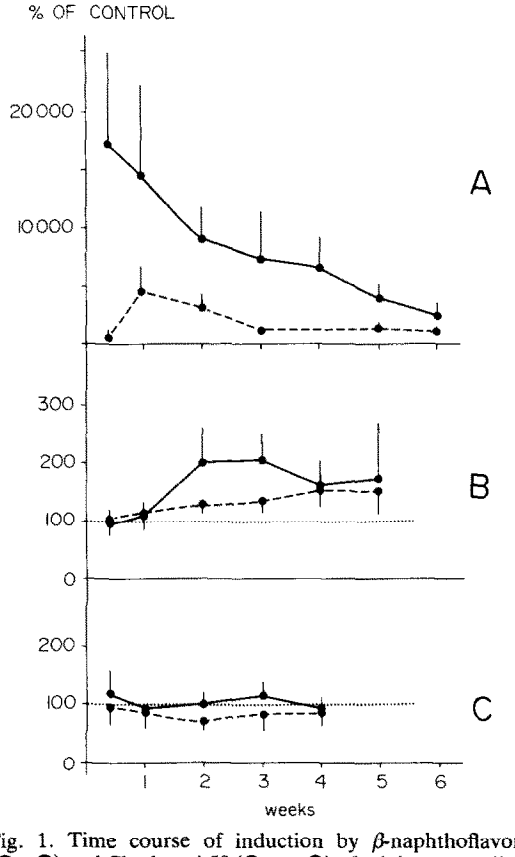

Fig. 1. Time course of induction by β-naphthoflavone (●—●) and Clophen A50 (●– – –●) of rainbow trout liver microsomal 7-ethoxyresorufin-*O*-deethylase (A), glutathione transferase (B) and epoxide hydrolase (C). β-Naphthoflavone (100 mg/kg body wt) or Clophen A50 (500 mg/kg body wt) was administered once i.p. at zero time. At each time of sampling activities are expressed as a percentage of control group activities which were set at 100. Values are means ± S.D. for five animals. Enzyme activities were measured as nmoles or μmoles per min per mg protein. The values for all control fish in the study over time are 0.022 ± 0.006 nmole per min per mg protein (A), 0.56 ± 0.16 μmole per min per mg protein (B), 1.16 ± 0.39 nmole per min per mg protein (C).

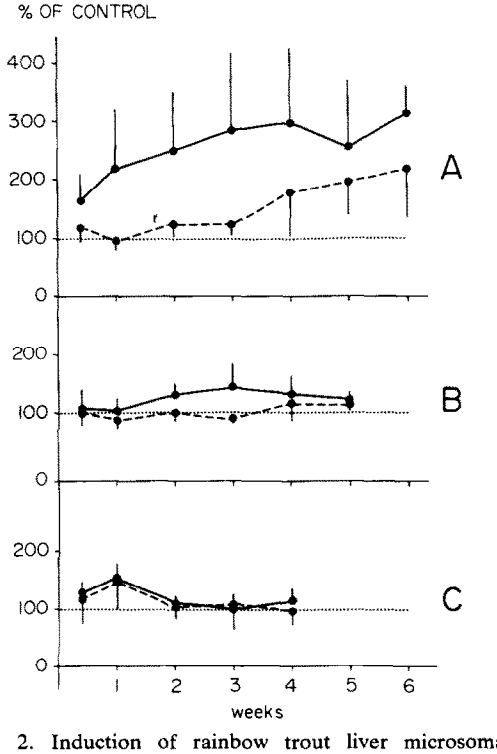

Fig. 2. Induction of rainbow trout liver microsomal UDP glucuronosyltransferase towards *p*-nitrophenol (A), 1-naphthol (B) or testosterone (C) by β-naphthoflavone (●—●) or Clophen A50 (●– – –●), shown over time. Fish were treated as shown in Fig. 1. At each time of sampling activities are expressed as a percentage of control group activities which were set at 100. Values are means ± S.D. for five animals. Enzyme activities were measured as nmoles per min per mg protein. The values for all control fish in the study over time are 0.22 ± 0.06 (A), 0.20 ± 0.05 (B) and 0.061 ± 0.013 (C).

Table 1. Effects of β-naphthoflavone and Clophen A50 on hepatic 7-ethoxyresorufin-*O*-deethylase, glutathione transferase and epoxide hydrolase activities in rainbow trout*

In vivo treatment	7-Ethoxyresorufin-*O*-deethylase†	Glutathione transferase‡	Epoxide hydrolase†
Control	0.022 ± 0.010	0.43 ± 0.06	1.16 ± 0.29
β-Naphthoflavone	3.78 ± 1.74§	0.85 ± 0.11§	0.95 ± 0.27
Control	0.018 ± 0.011	0.48 ± 0.06	0.90 ± 0.18
Clophen A50	0.88 ± 0.47§	0.71 ± 0.15§	0.91 ± 0.24

* In fish treated (single intraperitoneal injection) with β-naphthoflavone enzyme activities were measured after 3 days (7-ethoxyresorufin-*O*-deethylase) and 3 weeks (glutathione transferase). In fish treated (single intraperitoneal injection) with Clophen A50 the activities were measured after 7 days (7-ethoxyresorufinin-*O*-deethylase) and 4 weeks (glutathione transferase). The epoxide hydrolase activity was measured 1 week after the treatment with either β-naphthoflavone or Clophen A50. Values represent the mean ± S.D. for five animals.

† nmol per mg microsomal protein per min.

‡ μmol per mg cytosolic protein per min.

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

In a subsequent single time study, fish were treated with several doses of BNF (50, 100 or 200 mg per kg body wt) or Cl A50 (50 or 500 mg per kg body wt) and sampling was performed based on the times at which maximum responses were observed for the xenobiotic biotransformation enzymes in the previous study over time. However, the activities of the various enzymes did not vary when the different doses of inducers were used; therefore, only results from the experiments using 100 mg BNF or 500 mg Cl A50 per kg body wt are included in Tables 1 and 2. The extent to which UDP glucuronosyltransferase and glutathione transferase activities increased in response to treatments with BNF or Cl A50 varied between 1.4 and 3-fold which is considerably lower than the 50-to 170-fold increase in 7-ethoxyresorufin-*O*-deethylase activity by these inducing agents.

In the single time study the UDP glucuronosyltransferase activities were measured in non-activated and in maximum digitonin-activated states. UDP glucuronosyltransferase activities towards *p*-nitrophenol, 1-naphthol and testosterone were activated 2-, 3- and 1.6-fold, respectively, in both control and induced fish by digitonin (Table 2). When non-activated microsomes were used the UDP glucuronosyltransferase activities towards *p*-nitrophenol and 1-naphthol were significantly elevated by BNF. In digitonin-activated microsomes, however, the UDP glucuronosyltransferase activities towards *p*-nitrophenol, 1-naphthol and testosterone were all significantly increased by both Cl A50 and BNF (Table 2).

DISCUSSION

It is well known that certain hepatic microsomal cytochrome P-450-dependent reactions in fish can be induced by xenobiotics classified as PAH type inducers (3-methycholanthrene, BNF, certain BCPs) [9–13]. In addition to the prominent increase in cytochrome P-450-dependent 7-ethoxyresorufin-*O*-deethylase activity by Cl A50 and BNF, we also observed that these inducing agents increased the hepatic UDP glucuronosyltransferase and glutathione transferase activities. However, the effects of BNF and Cl A50 on the conjugation reactions were not as pronounced as were their effects on the cytochrome P-450-dependent reaction. In contrast, epoxide hydrolase activity was not influenced by these two inducing agents. This is in line with previous studies on fish where the hepatic epoxide hydrolase was found to be refractive to 3-methylcholanthrene [22, 23].

Sivarajah *et al.* [24] observed that the hepatic UDP

Table 2. Effects of β-naphthoflavone and Clophen A50 on hepatic UDP glucuronosyltransferase activities towards *p*-nitrophenol 1-naphthol and testosterone*

In vivo treatment	*In vitro* treatment	*p*-Nitrophenol†	1-Naphthol†	Testosterone†
Control	None	0.36 ± 0.02	0.22 ± 0.02	0.084 ± 0.010
	Digitonin	0.66 ± 0.04	0.66 ± 0.04	0.133 ± 0.025
β-Naphthoflavone	None	0.79 ± 0.25‡	0.44 ± 0.16‡	0.101 ± 0.030
	Digitonin	1.97 ± 0.23‡	1.36 ± 0.16‡	0.184 ± 0.024‡
Control	None	0.23 ± 0.16	0.26 ± 0.05	0.084 ± 0.010
	Digitonin	0.94 ± 0.18	0.72 ± 0.17	0.133 ± 0.023
Clophen A50	None	0.38 ± 0.18	0.33 ± 0.10	0.122 ± 0.008
	Digitonin	1.72 ± 0.52‡	1.20 ± 0.41‡	0.202 ± 0.049‡

* UDP glucuronosyltransferase activities towards *p*-nitrophenol and 1-naphthol were measured 3 weeks after treatment (single intraperitoneal injection) with β-naphthoflavone and 4 weeks after treatment with Clophen A50. UDP glucuronosyltransferase activity towards testosteron was measured 1 week after treatment with either β-naphthoflavone or Clophen A50. Values represent the mean ± S.D. for five animals.

† nmol per mg microsomal protein per min.

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

glucuronosyltransferase towards *p*-nitrophenol in rainbow trout was elevated (3.5-fold) following Arochlor treatment (weekly injections for 4 weeks) whereas Elcombe *et al.* [25] found no changes in this enzyme activity 2 days after one dose of 3-methylcholanthrene or BNF. The observation in the present study that the rate of induction of UDP glucuronosyltransferase activity towards *p*-nitrophenol was relatively slow indicates that the treatment time used by Elcombe *et al.* [25] may be too short to elicit any change of this enzyme activity.

James and Bend [22] and Balk *et al.* [23] reported that the hepatic glutathione transferase activities in the southern flounder and the northern pike were unresponsive to treatment with 3-methylcholanthrene for 8 and 5 days, respectively. Hepatic glutathione transferase in rainbow trout, however, was observed in the present study to be elevated 2-fold 2 and 3 weeks after treatment with BNF and Cl A50, respectively.

The different rates of induction of the various xenobiotic metabolizing enzymes observed in the present study emphasize the need to carefully characterize the time courses of induction in studies on the response of these enzymes to xenobiotics. Another time-related phenomenon associated with the above-mentioned enzyme induction was the plateau reached in maximum elevated activity. Thus, UDP glucuronosyltransferase activities towards *p*-nitrophenol and 1-naphthol and glutathione transferase activity were still at maximum levels 4–6 weeks after the single injection of BNF. This observation is noteworthy and in sharp contrast to the rapidly declining activities of the induced 7-ethoxyresorufin-*O*-deethylase and UDP glucuronosyltransferase towards testosterone after reaching peak values. These dissimilar responses to the effects of BNF may reflect differences in the regulation of the enzyme activities, owing, for example, to independent genetic control and/or differences in the turn-over of the enzyme protein.

In rats, multiple forms of hepatic UDP glucuronosyltansferases exist and glucuronidation of *p*-nitrophenol and 1-naphthol are catalyzed mainly by one enzyme form while testosterone UDP glucuronosyltransferase has been identified as a separate entity. Furthermore, testosterone UDP glucuronosyltransferase activity is not affected by PAH-type inducers whereas *p*-nitrophenol and 1-naphthol UDP glucuronosyltransferase activities are increased 3 to 4-fold by these chemicals [26]. In the present study the time course of induction of UDP glucuronosyltransferase activities towards *p*-nitrophenol, 1-naphthol and testosterone showed different patterns. These results may indicate that the substrates are conjugated by functionally distinct enzyme systems in the rainbow trout liver. In view of this it seems important to investigate whether trout liver microsomes contain one or more forms of UDP glucuronosyltransferase.

The effects of xenobiotics on UDP glucuronosyltransferase activities are complicated and difficult to interpret because of the enzyme latency. Xenobiotic treatment may either affect the activity of the existing UDP glucuronosyltransferase within the microsomal membrane or alter the amounts of enzymes available. To reduce problems with activation caused by treatments, UDP glucoronosyltransferase activities should be assayed in maximally activated states which probably best reflect the amounts of enzyme available [27]. The importance of using activated trout liver microsomes was shown when measuring UDP glucuronosyltransferase activities towards 1-naphthol and testosterone; no distinct elevations of these activities were observed following BNF or Cl A50 treatments when non-activated microsomes were used. In the present study the UDP glucuronosyltransferase activities were activated about 2-fold by digitonin in accordance with the report on rainbow trout by Hänninen *et al.* [28] but lower than the 12-fold activation of UDP glucuronosyltransferase activities obtained by surfactants in rat liver microsomes [29].

In summary, the present study shows that glutathione transferase and UDP glucuronosyltransferase activities in rainbow trout liver are responsive to cytochrome P-450 inducers. The dissimilar patterns of induction of the cytochrome P-450-dependent activity and the various conjugation activities may indicate that these xenobiotic metabolizing enzymes are differently regulated in the rainbow trout liver.

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