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Hepatic UDP-Glucuronosyltransferase in Rainbow Trout (*Oncorhynchus mykiss*) and Preliminary Assessment of Response to Pulp Mill Cooking Liquor

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Glucuronidation, accomplished by UDP-glucuronosyltransferase (UDPGT), represents a major pathway of conjugative biotransformation metabolism in fish. Among the xenobiotics ultimately excreted as glucoronides are PAHs, PCBs, pesticides, and the major pulp mill waste constituents chlorophenols and resin acids (Clarke et al. 1991). In addition, different hormones and the heme breakdown product, bilirubin, are excreted as glucuronides (Clarke et al. 1991). UDPGT in fish exposed to environmental pollutants, especially to those released by the pulp and paper industry, can show both induction (Andersson et al. 1988; Oikari & Kunnamo-Ojala 1987) and inhibition (George et al. 1992; Oikari et al. 1985). Furthermore, metabolic disorders in fish, characterized by impaired glucuronidation of bilirubin (George et al. 1992) and steroid hormones (Munkittrick et al. 1992), have been reported from water systems receiving pulp and paper effluents. Therefore, the study of UDPGT-dependent conjugation may contribute to a mechanistic understanding of effluent toxicity to fish.

When we established an assay for UDPGT in our laboratory, we found quite a different kinetic behaviour of the enzyme than observed in a earlier report characterizing hepatic rainbow trout UDPGT (Castrén & Oikari 1983). Therefore, the first aim of this study was a brief re-characterization of UDPGT in rainbow trout liver, including its activation by detergents. Secondly, we report a screening experiment concerning the inducibility of trout UDPGT by effluents from the pulp and paper industry. Juvenile trout were exposed to spent pulp cooking liquid ('black liquor') and the levels of UDPGT and the cytochrome P450-dependent enzyme EROD (7-ethoxyresorufin-*O*-deethylase) determined. EROD represents the physiological marker most consistently affected by pulp and paper effluents.

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MATERIALS AND METHODS

Rainbow trout, *Oncorhynchus mykiss,* were kept in flow-through tanks containing dechlorinated city water at a temperature of 14°C. The light-dark rhythm was 16:8 (L:D). Dissolved oxygen concentrations in water were close to saturation, the pH was around 7.5. The fish were fed daily with commercial trout food following the manufacturer's recommendations. To serve as a source of UDPGT during the optimization of the assay, microsomal material was prepared from pooled livers (n = 10) of trout (120 g average weight) in February 1994. In June 1994, fish of an average weight of 25 g were exposed to black liquor. Equal volumes of four black liquor samples that showed a high EROD induction potential (Hodson et al. 1997) were mixed. Fish were exposed in 40 l tanks, stocked with 10 fish per tank. A flow-through of 180 ml/min/tank of water and 1.8 ml/mm/tank of 1% black liquor was applied to exposed groups, resulting in a final concentration of 0.01% black liquor. Control groups received water only. Flow rates were checked daily and readjusted if necessary. Black liquor concentrations were monitored daily by absorbance measurements (272 nm) and maximally deviated from nominal values by 20%. Water quality parameters (dissolved oxygen, temperature, pH, conductivity) were checked daily and were in the same range as indicated above, except for conductivity that was elevated from 0.25 ± 0.078 mS/cm in controls to 0.29 ± 0.053 mS/cm (mean ± s.d.) in black liquor treatments.

For the preparation of liver tissue fractions, fish were killed by decapitation and their livers excised, taking care that gall bladders were not ruptured. All subsequent steps were conducted at 2 - 4°C. Livers were ground with four volumes of buffer (0.1 M phosphate, 0.15 M KCl, pH 7.4) in a Potter-Elvehjem device. The homogenate was centrifuged at 10,000 g for 20 min. The supernatant, representing the postmitochondrial supernatant (PMS), was ultracentrifuged at 105,000 g for 60 min. The resulting pellet was diluted in buffer containing 20 % glycerol (v/v) to get microsomal preparations corresponding to 1 g liver per ml. Microsomal preparations were quick-frozen in liquid nitrogen and stored at -80 °C for up to 3 months. The processing of liver samples from fish of the exposure experiment was similar, except that excised livers were shock-frozen in liquid nitrogen and stored at -80°C. Microsomes were prepared from thawed livers as described above and UDPGT was measured the same day. An aliquot of the PMS was withdrawn before ultracentrifugation, shock-frozen for later EROD measurement.

UDPGT-activity was determined using p-nitrophenol (p-NP, Sigma) as an aglycon and uridine 5'-diphospho-glucuronic acid (UDPGA, Sigma, ammonium salt) as the glucuronyl donor (Castrén & Oikari 1983). Final concentrations were 0.12 mg microsomal protein, 10 mM K_2EDTA, 40 µM p-NP and 2.3 mM UDPGA in 0.5 M phosphate buffer (pH 7.0). A higher p-NP concentration (80 µM) was used in the assay of UDPGT from fish of the exposure experiment. When the nonionic detergents Triton X-100 (Sigma) or Lubrol (ICN) were used as enzyme activators,

microsomes were incubated with the detergent on ice for 20 min before the enzyme assay. The reaction was started by addition of substrates and allowed to proceed for 20 min at 25°C before ice-cold 3% (w/v) trichloroacetic acid (TCA) was added to stop the reaction. After removal of precipitated protein by centrifugation, the concentration of non-conjugated p-NP was determined calorimetrically (λ = 400 nm, ε = 18.2 nM^{-1} c m^{-1}) in an aliquot of the supernatant after alkalinization with NaOH. Activities were corrected for the change in absorbance in reference blanks (UDPGA omitted). In the experiments to optimize the assay system, single parameters in the protocol were modified while keeping the others constant. Unless otherwise noted, assay conditions were as described above involving no detergent treatment of microsomes. EROD activity in PMS and protein concentrations of subcellular fractions were measured as described previously (Hodson et al. 1997).

Michaelis constants were estimated from initial reaction rates observed at different substrate concentrations by linear regression of data in double reciprocal plots (Lineweaver-Burk plots). Enzyme activities (EROD and UDPGT) were compared in t-tests. EROD data were log-transformed before comparison to increase the homogeneity of variances.

RESULTS AND DISCUSSION

The influence of the pH during the reaction was investigated by variation of the pH of the phosphate buffer in the range of 6.0 and 8.0 (steps of 0.25). Between pH 6 and 7.25, there was no effect of pH on the enzyme activity, whereas activities were approximately 20% lower between pH 7.5 and 8.0 (not shown). From an incubation temperature of 5°C to 20°C UDPGT-activities rose and remained about constant in the range from 20°C to 40°C (Fig. 1). A pH of 7.0 and a reaction temperature of 25°C were chosen as standard conditions. The relationship between concentration and effect on UDPGT of the non-ionic detergent Lubrol was characterized by a bell-shaped curve (Fig. 2). When applied at an optimal concentration of 0.24 mg/mg microsomal protein, Lubrol increased enzyme activities about 2.5-fold. Concentrations below and above the optimum were less effective (Fig. 2). The nonionic detergent Triton X-100 effected only slight activation at a low concentration and inhibited UDPGT at concentrations of 0.5 mg/mg microsomal protein and above (Fig. 2). With the Lubrol-treated enzyme, activities followed Michaelis-Menten kinetics with respect to both the acceptor substrate p-NP (Fig. 3) and the glucuronyl donor UDPGA (Fig. 4). From the Lineweaver-Burk plot of data from experiments with varying p-NP concentrations (2.3 mM UDPGA fixed), a K_m(p-NP) of 21.6 (16.0; 27.2) µM (95% confidence limits) and a V_{max} of 1.22 nmol/mg/min was obtained (Fig. 3). When the concentration of UDPGA was varied (75 µM p-NP fixed), the Lineweaver-Burk plot yielded a K_m of 0.27 (0.24; 0.31) mM (95% confidence limits) and a V_{max} of 1.59 nmol/mg/min (Fig. 4). From these kinetic parameters, standard substrate

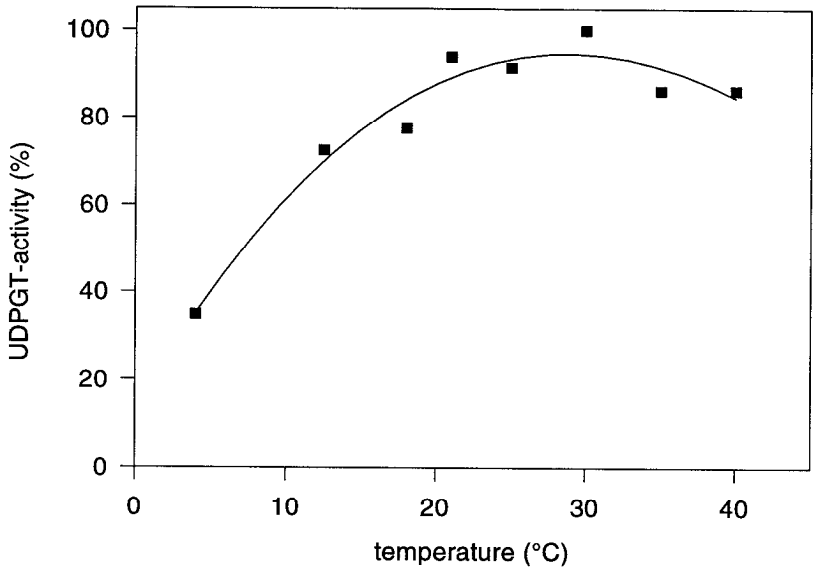

Figure 1. Effect of temperature on UDPGT in rainbow trout liver. Values represent triplicate observations on pools of ten livers.

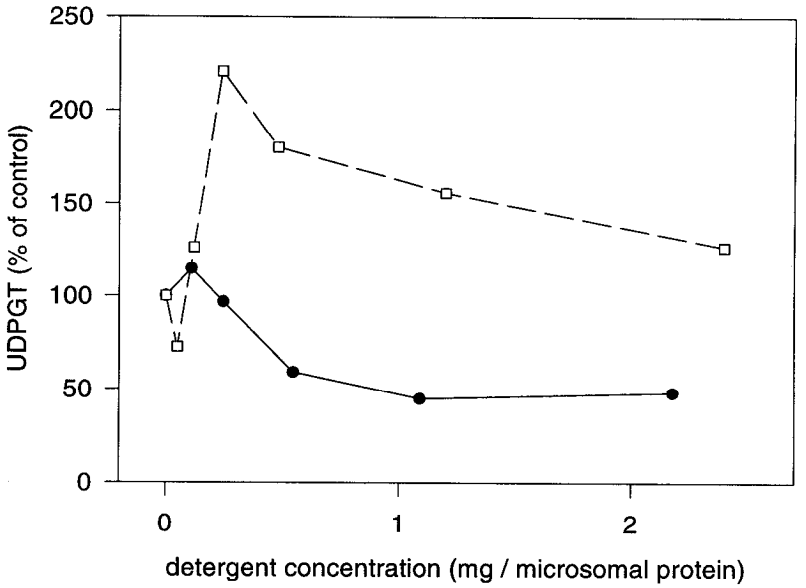

Figure 2. Effect of the non-ionic detergents lubrol (open squares) and triton X-100 (filled circles) on UDPGT from rainbow trout liver. Values represent dublicate observations on pools of ten livers

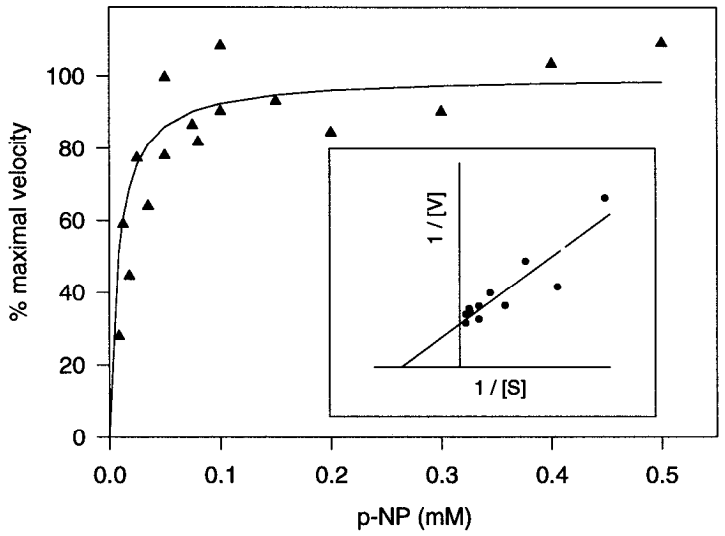

Figure 3. Substrate kinetics of UDPGT in rainbow trout liver with respect to the acceptor substrate p-nitrophenol (p-NP). Values are from pooled liver microsomes from 10 fish. The insert picture represents the Lineweaver-Burk-plot of the data. The intersection gives an K_m (p-NP) of 21.6 µM.

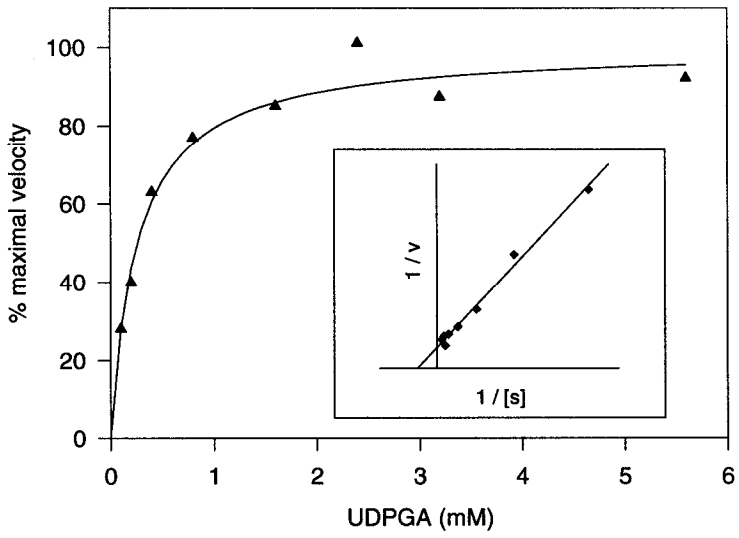

Figure 4. Substrate kinetics of UDPGT in rainbow trout liver with respect to the donor substrate UDPGA. Values are from pooled liver microsomes from 10 fish. The insert pictures represent the Lineweaver-Burk-plot of the data. The intersection gives an K_m (UDPGA) of 0.27 mM.

Table 1. Effects of black liquor on biotransformation enzymes in rainbow trout.

	exposure (days)	control[3]	black liquor (0.01%)	p
EROD[1]	4	0.82 ± 0.12 (9)	1.90 ± 0.34 (10)	0.015
	8	0.78 ± 0.20 (9)	1.68 ± 0.35 (8)	0.015
UDPGT[2]	8	3.13 ± 0.38 (9)	2.90 ± 0.51 (8)	0.708

[1](pmol . min^{-1} . mg^{-1} protein)
[2](nmol . min^{-1} . mg^{-1} protein)
[3]average ± sem (n)

concentrations of 250 µM p-NP and 0.24 mM UDPGA were calculated, at which more than 90% of the maximal velocity is achieved.

Exposure of juvenile rainbow trout to 0.01% black liquor effected EROD induction after 4 and 8 days, but had no effect on UDPGT activity (Table 1). After 8 days of exposure, 20% mortality occured in the exposed group, indicating the toxicity of black liquor at the used concentration. Mortalities in the control group were below 10%.

The effects of different pH and temperature conditions on UDPGT found in this study are very similar to those reported by Castrén & Oikari (1983), i.e. no profound effect of pH in the range from 6.0 to 8.0, and a linear increase of UPDGT with increasing temperature between 5 and 20 °C with little additional effects between 20 °C and 40 °C. As observed with the mammalian enzyme (Winsnes 1969), UDPGT-activity in fish shows a characteristic 'latency' *in vitro*, i.e. maximal activity is expressed after membrane disruption by freezing/thawing (Lindström-Seppä & Hänninen 1988) or treatment with detergents (Ankley & Agosin 1987). In this study, the optimal activation of the enzyme by the non-ionic detergent Lubrol was about 2.5-fold, which is similar to other reports of activation of the teleost UDPGT (Ankley & Agosin 1987; Clarke et al 1991). In contrast, activities of mammalian UDPGT can be activated up to 20-fold (Burchell & Coughtrie 1989). The activation of UDPGT by various methods is generally not additive, i.e. after optimal activation by one kind of treatment, other methods of activation fail to exert effects (Winsnes 1969). However, control fish from the experimental exposure showed a higher activity than found in the microcomal preparations used in the optimization of the assay. As confirmed after the experiment (not shown), this was due the different sampling protocol in the experiment (storage of livers at -80 °C) compared to the pooled microsomal preparations (storage of microsomes at -80 °C). This suggests that treatment of microsomes by Lubrol does not achieve optimal activation of hepatic trout UDPGT, as freezing of livers exerts additional effects. Substrate kinetics of UDPGT found in this study differ from that described

by Castrén & Oikari (1983) mainly in that no substrate inhibition by p-NP was found in this study for a range of p-NP concentrations between 8.6 µM and 500 µM. By contrast, Castrén & Oikari (1983) observed substrate inhibition of UDPGT by p-NP at concentrations exceeding 25 µM. Inhibition was about 95% at 0.25 µM. The Michaelis constants, K_m(p-NP) of 26 µM and K_m(UDPGA) of 0.27 mM, found in this study for hepatic trout UDPGT are roughly in the same range as those reported by Castrén & Oikari (1983) (K_m(p-NP) of 8.3 µM and K_m(UDPGA) of 0.20 mM).

Exposure of juvenile trout to 0.01% black liquor for 4 to 8 days resulted in an about 2-fold induction of EROD activity, but had no effects on UDPGT. In a complete concentration-response curve, EROD induction by black liquor commonly rises linearly with black liquor concentrations to reach levels of 20-50 times the control value, before a further increase of concentration effects a decrease of the response curve, coinciding with lethality (Hodson et al. 1997). In previous 4 day semistatic exposures with fingerling trout, 0.01 % of the black liquor samples used in this study exerted close-to-maximal EROD induction, but no toxic effects. The low EROD induction by 0.01% black liquor and the lethal effects beginning after 8 days in the present study was unexpected and reflects the difficulty to extrapolate between experiments using different life-stages and exposure conditions. In pulp mills, black liquor is usually collected and combusted so that the final effluent contains black liquor only from losses. It was estimated that the final concentration of black liquor in effluent is 0.045% (Hodson et al. 1997). Black liquor represents one of the major sources of EROD inducers present in final effluent. In addition to EROD inducers it contains, however, also various toxic compounds that may be removed upon effluent treatment. UDPGT and EROD can be coinduced in trout by i.p. administration of strong model inducers as β-naphthoflavone (Andersson et al. 1985; Zhang et al. 1990). The lack of effects on UDPGT in this study does not preclude potential effects of pulp mill effluents on trout UDPGT at a higher concentration of EROD inducers. Consequently, further research is needed to elucidate whether pulp and paper effluents exert relevant effects on glucuronidation in fsh.

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