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Dehydroabietic Acid (DHAA) Does Not Inhibit Bilirubin Conjugation in the Liver of Rainbow Trout

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Pulp and paper mill effluents contain a group of acutely toxic chemicals, resin acids, which cause jaundice to exposed fishes in high concentrations (Kruzynski 1979; Nikinmaa and Oikari 1982; Mattsoff and Oikari 1987). In jaundice the metabolism of bilirubin, which is the end product of hemoglobin catabolism (Ganong 1981), is disturbed and as a consequence, bilirubin accumulates in plasma, interstitial fluids and tissues (Robbins and Angell 1976). Bilirubin is a toxic compound and is normally eliminated from the body by secretion to the bile (Dutton 1966). Jaundice can therefore result either from accelerated red cell hemolysis or from disturbances in the detoxification of bilirubin in the liver (Robbins and Angell 1976).

In vertebrates, liver is the main organ in detoxification of endo- and exogenous toxic compounds. These detoxification reactions reduce the toxicity of the compounds to be excreted and render them more polar and water soluble (Dutton 1966). This is usually achieved by conjugation reactions, of which glucuronidation is the most common. Glucuronic acid is attached to toxic compounds by the enzyme UDP-glucuronyltransferase (UDP-GT), which is a multiform enzyme with a broad substrate spectrum (Dutton 1966). As both bilirubin (Dutton 1966) and resin acids (Oikari et al. 1984) are excreted to the bile as glucuronic acid conjugates, we tested, whether the competition between resin acids and bilirubin for the conjugation is partly responsible for the development of jaundice in resin-acid-toxicated fish by measuring the activity of bilirubin UDP-GT in vitro in the presence of a resin acid, dehydroabietic acid (DHAA).

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MATERIALS AND METHODS

The effect of resin acids on the conjugation activity of bilirubin-UDP-GT was measured using rainbow trout liver homogenate. Rainbow trout (Salmo gairdneri, in experiment I weighing 800-1000 g and 40-45 cm in length and in experiment II and III weighing 200-500 g and 26-32 cm in length) were obtained from a commercial fish farm, Karkkilan Lohi. They were maintained in dechlorinated Helsinki tap water (8-10 °C) for at least four weeks before the experiments.

The fishes were first anesthetized (1 g 3-aminobenzoic acid ethyl ester, methane sulfonate salt/10 l, 4 min), then killed with a blow on the head and subsequently their abdominal cavity opened. The bile was collected to prevent its harmful effect on liver (Hendricks et al. 1976). Thereafter the liver was removed, rinsed in cold 0.25 M sucrose, blotted with a tissue paper and frozen and stored in liquid nitrogen until measurements.

For measuring the UDP-GT activity of the liver, tissue pieces were homogenized in cold 0.25 M sucrose solution yielding a 17 % (w/v) mixture. UDP-GT activity was assayed using the mitochondrial supernatant (10 000 g, 4-13 °C) at 25 °C. The incubation time was 20 min. The protein concentration of the supernatant was measured by the method of Lowry et al. (1951) using bovine serum albumin (Sigma) as a standard. All enzyme activities are given as units (U = µmol reacted substrate/min/g protein in assay sample).

Experiment I: The bilirubin-UDP-GT activity was measured as described by Castren (1984). The incubation mixtures were prepared at 0 °C in dark test-tubes to prevent the light-induced breakdown of bilirubin (Dutton 1966). The mixtures contained (final concentrations are in parenthesis) 100 µl $MgCl_2$ (7.5 mM), 100 µl of homogenized liver tissue, 100 µl of bilirubin-albumin substrate (0.012, 0.06, 0.12, 0.2 and 0.6 mM) and 200 µl Tris-HCl buffer of pH 7.7 (50 mM). The bilirubin-albumin solution was prepared as follows: bilirubin (BDH Chemicals Ltd, 18 mg) was first dissolved to 1 ml of 0.1 M NaOH and then diluted to 9 ml of 40 mM Tris-HCl buffer (pH 7.4) containing 20 g/l of bovine albumin (Sigma). This bilirubin-albumin substrate solution was first lyophilized (Phywe) and then stored in deepfreezer (-20 °C). 10 µl of DHAA dissolved in dimethyl sulfoxide (DMSO, Merck) was added to DHAA tubes, in test series I the DHAA concentration was 0.012 mM and in test series II 0.06 mM. The same amount of DMSO was added to control tubes. 10 µl of D-saccharic acid 1,4-lactone (1.5 mM, Sigma) was also added to all tubes to inhibit the β-glucuronidase acti-

vity of liver tissue (Merck Index 1968).

Incubations were started by adding the glucuronic acid donor, the UDP-glucuronic acid (UDPGA, 4 mM), dissolved in the Tris-HCl buffer (pH 7.7), to control and DHAA tubes. Blank tubes, not containing UDPGA, were incubated in parallel to both control and DHAA tubes. Incubations were performed at 25 °C for 20 min. Thereafter the tubes were quickly cooled on crushed ice and 2 ml of glycine-HCl buffer (0.4 M glycine in 0.4 M NaCl adjusted to pH 2.7 with HCl) was added. Usually the conjugated bile pigments synthetised, in vitro, are determined after their conversion into azopigment derivatives (Heirwegh et al. 1972). This was done using the method of Van Roy and Heirwegh (1968). One ml of diazotized ethyl anthranilate was added into the tubes at pH 2.7, and the diazocoupling was allowed to proceed in dark at room temperature for 30 min. The reaction was stopped with 10 % ascorbic acid (0.2 ml). After 5-10 min the tubes were cooled again on crushed ice and the azopigments, which develop color at pH 2.7, were extracted to 1.6 ml of butanol-butylacetate (17:3 v/v). After centrifugation (+4 °C, 4 000 g, 10 min) the absorbance of the upper organic layer was measured in microcuvettes (Hitachi spectrophotometer 100-10) at wavelength of 546 nm against the organic solvent.

The activity of bilirubin-UDP-GT was calculated from the following equation:

$$A = \frac{\Delta E \times V_{tot}}{\epsilon \times V_{sample} \times \text{time} \times \text{cyvette width}}$$

$$A = \Delta E \times 43.657 \text{ nmol/ml/min},$$

where

ΔE = absorbance of sample - absorbance of blank tube

ϵ = the molar absorption coefficient of bilirubin, taken as 60.7×10^3 $cm^2/mmol$ (Merck Index 1968).

V_{tot} = the total volume of the incubation and

V_{sample} = the sample volume

Experiment II: The bilirubin-UDP-GT activity was measured as in experiment I, but 20 µl of bilirubin (dissolved in DMSO) solution in the same final concentrations (0.012, 0.06, 0.12, 0.2 and 0.6 mM) and 100 µl of 40 mM Tris-HCl-buffer (pH 7.4) was used instead of bilirubin-albumin solution.

Experiment III: The liver UDP-GT activity was also measured using p-nitrophenol as a substrate (final conc. 0.12 mM, Sigma) according to Hänninen (1966) and Castren and Oikari (1979). DHAA concentrations, used in this experiment, were 0.06 mM and 0.12 mM of which the former one equals the DHAA concentration used in

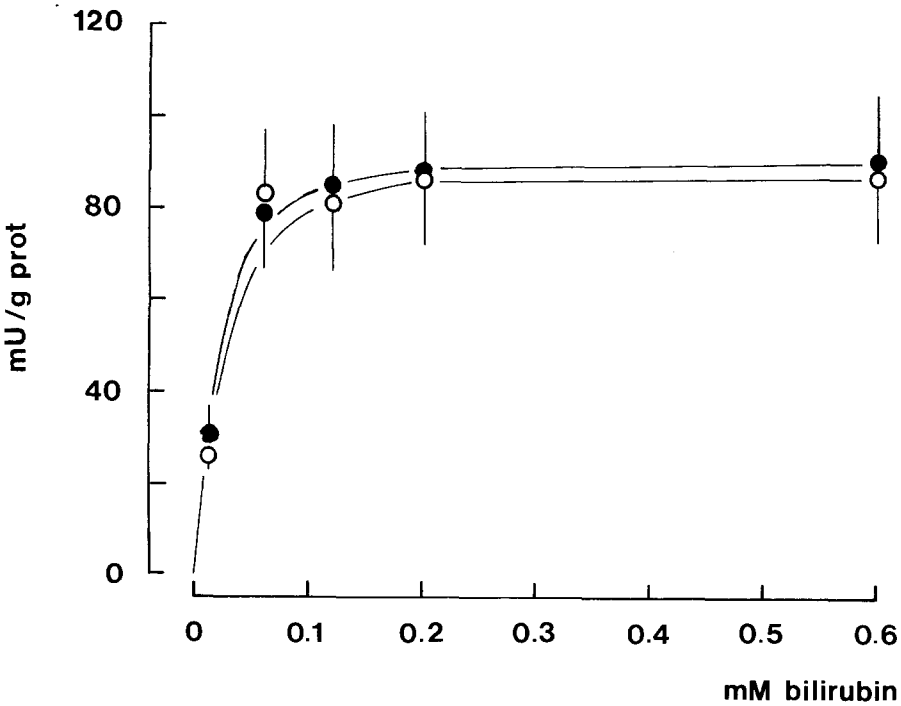

Figure 1. The liver bilirubin UDP-glucuronosyltransferase activity in the absence (○) and presence of 0.012 mM DHAA (●) in albumin containing medium. Means and S.E.M. are given (n=4). Statistical differences determined using Student's paired t-test are indicated by asterisks (* $P<0.05$, ** $P<0.01$).

experiments I and II.

Statistical differences were determined using Student's paired t-test.

RESULTS AND DISCUSSION

The bilirubin conjugation activity of the liver tissue was not affected by DHAA in the incubation mixture in either DHAA concentration in experiment I (Fig 1 and 2). This shows that bilirubin and DHAA do not compete for the conjugation in the liver in the conditions used. These results are in accordance with the earlier findings on jaundiced trout, in which the accumulated resin acids did not affect the bilirubin UDP-GT activity (Mattsoff and Oikari 1987).

Since albumin binds DHAA quite effectively (Mattsoff and Nikinmaa 1987) decreasing the free DHAA concentration in plasma approximately 20-fold, we tested if the lack of competition between DHAA and bilirubin were caused by the binding of DHAA to

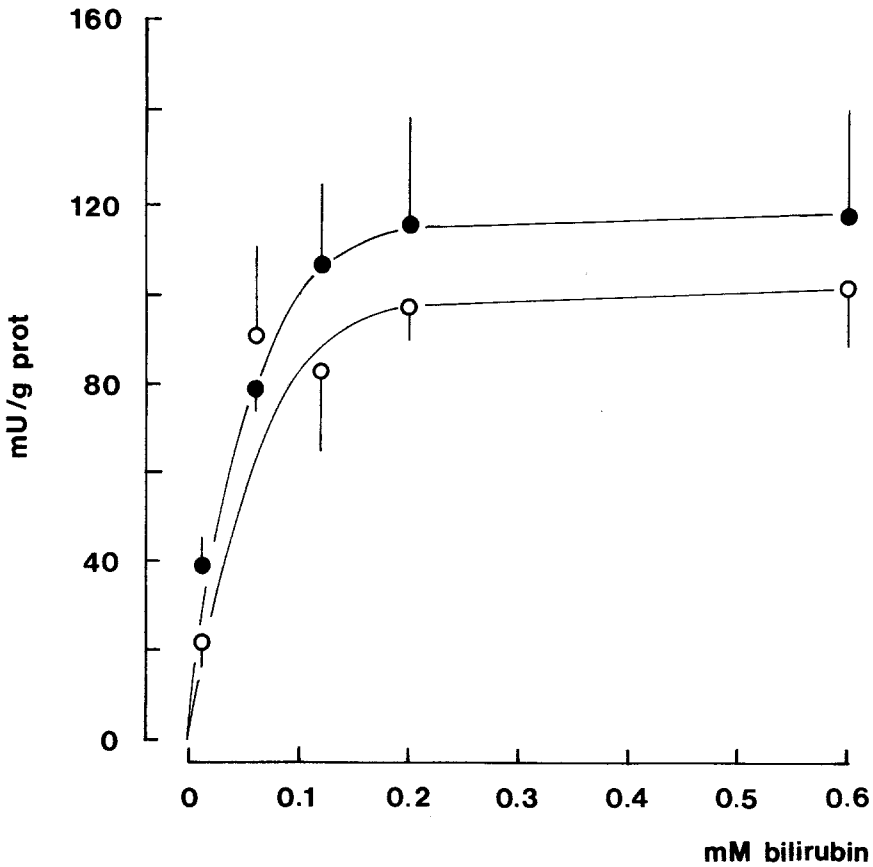

Fig. 2. The liver bilirubin UDP-glucuronosyltransferase activity in the absence (○) and presence of 0.06 mM DHAA (●). Legend as in Fig. 1.

Table 1. DHAA inhibits the liver UDP-glucuronosyltransferase activity when p-nitrophenol is used as a substrate (n=5). Legend as in Fig. 1.

DHAA conc.	control mU/g prot	P	DHAA mU/g prot
0.06 mM	218 ± 17	* *	178 ± 13
0.012 mM	199 ± 18	* *	152 ± 14

albumin. However, the conjugation activities of samples with and without DHAA were similar even in the absence of albumin (Fig. 3).

Resin acids inhibit the glucuronidation of p-nitrophenol in exposed rainbow trout (Oikari et al. 1983; Mattsoff and Oikari 1987). The in vitro measurements in experiment III show that DHAA decreases the amount of

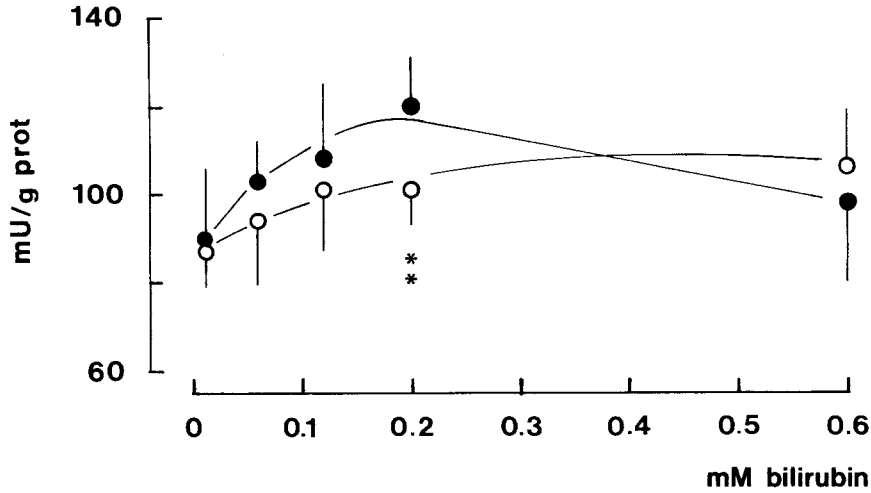

Figure 3. The liver bilirubin UDP-glucuronosyltransferase activity in protein free incubation in the absence (○) and presence of 0.06 mM DHAA (●), (n=5). Legend as in Fig. 1.

conjugated p-nitrophenol with almost 20 % even at a concentration half of the p-nitrophenol concentration used (Table 1).

These results suggest that different forms of UDP-GT enzyme conjugate bilirubin and dehydroabietic acid. This is probably also true for other resin acids, since the bilirubin UDP-GT activity from rainbow trout, exposed to water containing a mixture of eight different resin acids, did not change (Mattsoff and Oikari 1987). It is, however, also possible that bilirubin, p-nitrophenol and resin acids are conjugated by the same enzyme, but at different pH values. This is possible, because the optimal incubation conditions for UDP-GT assays using the different substrates, p-nitrophenol and bilirubin are different. If this were the case, then effects of pulp and paper mill effluents on the pH of liver cells might affect conjugation activity.

Despite the above possibility our results strongly suggest that inhibition of bilirubin conjugation does not play a role in the development of jaundice. This conclusion is supported by findings on jaundiced, resin acid- exposed fish in which the accumulation of conjugated bilirubin in plasma far exceeded the

accumulation of unconjugated bilirubin (Kruzynski 1979; Mattsoff and Oikari 1987). However, since unconjugated bilirubin was also accumulated in plasma (Mattsoff and Oikari 1987), the bilirubin conjugation capacity of liver is exceeded in jaundice. Our measurements show that the bilirubin conjugation capacity of liver is indeed saturable.

As conjugated bilirubin accounted for over 50 % of the plasma bilirubin concentration (Mattsoff and Oikari 1987) jaundice is in this case diagnostized as conjugated hyperbilirubinaemia. Conjugated hyperbilirubinaemia points to the insufficient function of transcanalicular secretion or to a cholestasis as the primary reason for the development of jaundice (Ostrow 1987). In humans, the secretion to bile canaliculi is normally the rate-limiting step in overall clearance of bilirubin from blood to bile (Ostrow 1987) and therefore any disturbances to bilirubin secretion would lead to conjugated hyperbilirubinaemia. There is, at present, no data on the effects of resin acids on the canalicular secretion of bilirubin or other organic anions in fish. However, as the secretion route of bilirubin is shared by many other organic anions (Ostrow 1987) it is possible that bilirubin and resin acid glucuronides compete with each other for transportation.

Thus, the resin acid-induced jaundice in rainbow trout result partly from accelerated red cell hemolysis, (Bushnell et al. 1985, Mattsoff and Nikinmaa 1987), and partly because the secretion of bilirubin from blood to bile is inhibited.

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