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Free and Conjugated Resin Acids in the Bile of Rainbow Trout, *Salmo gairdneri*

A. Oikari,[1]* E. Ånäs,[2] G. Kruzynski,[1] and B. Holmbom[2]

[1]Department of Zoology, Division of Physiology, University of Helsinki, SF-00100 Helsinki 10, Finland, and [2]Department of Wood Chemistry, Åbo Akademi, SF-20500 Turku 50, Finland

It is well known that resin acids, altogether about ten different compounds, contribute the major part of the acute lethal toxicity of pulp and paper mill effluents to fish (Leach and Thakore 1977; Holmbom and Lehtinen 1980). Fish stocks living in the vicinity of pulp mills may be acutely exposed to resin acids through occasional spills or chronically via deliberate discharges from the mill. Recent work indicates that the function of the liver, a key organ in detoxication and elimination of xenobiotics in teleost fishes (Chambers and Yarbrough 1976; Lech and Bend 1980), is seriously affected in an acutely poisoned rainbow trout (Oikari and Nakari 1982; Nikinmaa and Oikari 1982). Subacute exposures to water containing resin acids led to fairly similar changes in trout liver, e.g. to partial inhibition of the enzyme UDP-glucuronosyltransferase (Oikari et al. 1984). Although the relative contributions of the liver, kidney, gills and skin in the metabolism of resin acids by fish are not yet known, the results presented here demonstrate, for the first time, the existence of resin acid conjugates in fish bile. This suggest that the hepato-biliary route is significant in the excretion of these toxicants.Maintenance of the ability of the liver to conjugate resin acids may be fundamental to the survival of fish populations in waters receiving continuous discharges of waters from the pulp and paper industry.

MATERIALS AND METHODS

Juvenile 1+ year old rainbow trout (Salmo gairdneri Richardson; Savon Taimen Inc.) were used, if not otherwise specified, throughout the experiments. The aquarium conditions varied to some degree because some of the experiments were conducted in the laboratory while others were done at an "on plant" exposure station. At least three days' recovery was allowed after transport of fish from the hatchery to the test aquaria where the water temperature (T_W) was within $\pm$ 1.5 °C of that in the hatchery. Water O_2 concentration was 7.5-9 mg/L, and a seasonal photoperiod was maintained through partially covered windows. Two sets of experiments

* Reprint requests: University of Joensuu, Department of Biology, SF-80130 Joensuu 13, Finland

can be distinquished; Experiments I (Tables 1 and 2), where only free resin acids (= hexane extractable) were measured, and Experiments II (Tables 3-5), where bile samples were also analysed for resin acid conjugates. In most experiments the concentrations of these toxicants were also determined in blood plasma samples.

Five trout (180-225 g; Table 1) were exposed for three days to a wood rosin containing 95.5 % the following eight resin acids: pimaric 7.7 %, sandaracopimaric 1.8 %, isopimaric 3.8 %, palustric 27.1 %, levopimaric 9.0 %, dehydroabietic 5.1 %, abietic 24.9 % and neoabietic 16.1 % (w/w). T_w was 10 ± 1 °C (range), pH 7.3-7.4 and the total nominal resin acid concentration 2.5 mg/L. Procedures in the application of toxicants into test water were those described in Oikari et al. (1982); part of the water volume was replaced twice daily (loading density about 1.5 g/L per day).

Four trout (155-190 g; Table 2) were exposed for 20 days to 2 % (v/v) solution of untreated total effluent from a Finnish mechanical (debarking, groundwood, thermomechanical) pulp mill. During the flow-through-type (20 L/h) exposure, T_w was 14-16 °C, pH 6.5-6.7 and O_2 concentration 60-80 % of the air saturation value (ASV). Samples from four fish were pooled for one (triplicate) analysis.

Three 4 y old trout (700-950 g; Table 3) were exposed for three days to the nominal 1 mg/L dehydroabietic acid (> 98 % pure). The exposure type was semistatic, water pH 7.3 and T_w 12 °C. Each fish was analysed separately.

Ten trout (115-145 g; Table 4) were exposed for 30 days to 1 % (v/v) solution of biologically treated (2-3 days retention time) bleached kraft mill effluent (BKME). During the flow-through-type (60 L/h) exposure, conducted in the fall, T_w gradually decreased from 15-16 °C to 11-12 °C. Water pH was 6.7 and the diluent water was taken from a location upstream relative to the mill (southern Lake Saimaa, SE Finland). Samples from 3-4 fish were pooled for one analysis. In a related experiment, another lot of ten trout (120-140 g; Table 5) were exposed for 10 days to 0.5 % (v/v) BKME. However, T_w was 14-16 °C and bile samples from all fish were pooled for one analysis.

In all experiments polyethene aquaria with volumes from 300 to 500 L were used. Water hardness was approximately 80 mg $CaCO_3$/L in the first and the third experiment, and 20 mg/L in the remaining exposures. At the end of exposure, the fish was stunned by a blow on the head, blood samples were collected from caudal vessels into heparinized syringes (and centrifuged immediately), and bile was collected directly from the gall bladder with a hypodermic needle. Plasma and bile samples were frozen in liquid nitrogen or at -20 °C until analysed.

For determination of the resin acid concentrations in samples (volume 0.5-1.5 mL) of Expts I, the technique outlined in previous papers was followed (Holmbom 1977; Holmbom and Lehtinen 1980; Oika-

ri et al. 1982). Freeze dried samples were finely ground and acidified with diluted H_2SO_4 (pH 2-3), an internal standard mixture was added (heptadecanoic and tricosanoic acids, 20 ug/g each), and then extracted with n-hexane in a small-scale Soxhlet apparatus for 5 h (corresponding to about 150 extraction cycles). The extract was methylated with diazomethane and further analysed on a Varian 1400 GC equipped with 32 m/0.3 mm i.d. glass capillary column coated with BDS (two or three runs each, isothermally at 190 °C) or with SE-30 (150 °C ——> 270 °C, 4 °C/min). Concentrations on the wet weight basis were calculated using the value of measured water content in the sample. Samples of Expts II were directly acidified with diluted H_2SO_4 and the internal standards were added (17:0 and 23:0, 10 ug/mL each). Extraction was performed with three 3 mL portions of n-hexane:acetone (3:1 v/v) yielding at least 80 % of the total resin acids extractable. The combined extract was methylated and analysed by GC as above.

For determination of the total conjugated resin acids in Expts II, after initial extraction, the sample was further treated by alkaline solution (0.5 M KOH in 90 % ethanol at 70 °C). Before hydrolysis the internal standard (0.2-2.0 mg of 17:0 per g d.w.) was added. Since increasing the hydrolysis time from 2 to 24 h produced no increase in resin acid recoveries, a base hydrolysis treatment of 3 h was chosen as routine. Finally, the sample was acidified to pH 2-3 and resin acids extracted and analysed as above.

To separate the glucuronic acid conjugates of resin acids from the total (= hydrolysed with KOH), another part of the first extraction residue was treated by beta-glucuronidase (Sigma L-II). The dry bile residue (50-100 mg) was resuspended in 1 mL distilled water, 2 mL acetate buffer (0.3 M, pH 3.8) and 1 mL enzyme solution (3000 units/mL distilled water) were added. After an incubation (24 h at 37 °C) the liberated resin acids were extracted, derivatized and analysed by GC as previously described. The control incubation medium, containing the inhibitor D-glucuronate (10 uM), yielded only negligible amounts of substractable resin acids.

RESULTS AND DISCUSSION

In the present context, the "free" resin acid is definied as the part of the total which is directly extractable (by n-hexane or n-hexane:acetone) from an acidified bile or plasma sample. Correspondingly, the "conjugated" resin acid is that part of total releasable, e.g. by base hydrolysis (Hunn and Allen 1975), from the residue of the first extraction. Results in Table 1 show that after a 3-day exposure to resin acids in water the concentrations of free acids are much lower in the bile than in the blood plasma. The bile/plasma (B/P) ratios varied from 0.15 for pimaric to 0.59 for dehydroabietic (DHAA) acid. In the other acute exposure (= Table 3), the B/P ratio of free DHAA was also low (B/P 0.18). Interestingly, the different resin acids seem to behave in a similar manner, i.e. the excretion takes place "downhill" and may be

Table 1. Bile concentration (ug/mL) and the bile/plasma ratio (B/P) of free resin acids in rainbow trout exposed acutely (3 d) to 2.5 mg/L of wood rosin.

Resin acid	Concentration in bile	B/P
Pimaric	9 ± 4	0.15 ± 0.06
Sandaracopimaric	2 ± 1	0.20 ± 0.08
Isopimaric	5 ± 2	0.20 ± 0.08
Abietic	27 ± 13	0.34 ± 0.14
Dehydroabietic	7 ± 3	0.59 ± 0.31
Total (5 acids)	49 ± 24	0.27 ± 0.11

Mean ± SD, n = 5

Table 2. Bile concentration (ug/mL) and the bile/plasma ratio (B/P) of free resin acids in rainbow trout* exposed subacutely (20 d) to 2 % v/v solution of untreated effluent from a mechanical pulp mill.

Resin acid	Concentration in bile	B/P
Pimaric	16	3.4
Isopimaric	64	5.3
Abietic	65	6.8
Dehydroabietic	141	10.4

*Pool of 4 fish

primarily passive. This conclusion presupposes that all resin acid molecules extractable by hexane are equally accessible to this transfer mechanism. When the exposure to resin acids lasts longer than a few days an "uphill", possibly active transcellular transfer of free acids seem to be developed (B/P > 1, Table 2). This result supports our previous observation (Oikari and Holmbom 1981) that this pattern of excretion may work at fairly low water resin acid concentrations (ca. 20 ug/L) and fairly soon (5-10 days) after start of exposure.

Table 3. Bile concentrations (ug/mL) of free and conjugated dehydroabietic acid (DHAA) in rainbow trout exposed acutely (? d) to 1 mg/L of DHAA.

Fish No.	Sex	Fish weight	Concentration in bile Free DHAA	Conjug.DHAA	Per cent conjug. DHAA in bile
1	♀	810 g	9	1490	99.4
2	♀	700 g	3	4560	99.9
3	♂	950 g	56	9260	99.4

In all exposure times (3-30 days; Tables 3-5) alkaline hydrolysis of bile samples yielded a conjugation degree more than 99% for all resin acids. It is important to realize that the conjugation mechanisms are functional within a few days of the start of exposure. However, separate consideration of the three mature trouts in Table 3 indicates more than a six-fold difference in conjugated amounts of DHAA (the data are too limited to conclude if it is sex- or size-related). This may well be characteristic of the immediate "crisis period" that the fish encounters in an acute exposure. The results in Table 4 indicate that other resin acids occurring in BKME are treated like DHAA. Concentrations of conjugated resin acids are, however, in the 30-day exposure much lower than in the short-term exposure to pure DHAA (Table 3). This is likely related to the much lower concentrations of these compounds (total ca. 5 ug/L) in the water. What the maximum excretion capacity through conjugation could be, remains unknown.

Table 4. Bile concentrations (ug/mL) of free and conjugated resin acids in rainbow trout exposed subacutely (30 d) to 1 % v/v solution of biologically treated kraft pulp mill effluent.

Resin acid	Concentration in bile		Percentage conjugated
	Free	Conjug.	
Pimaric	0.09 ± 0.06	48.1 ± 3.4	99.8
Sandaracopimaric	ND	11.2 ± 3.8	100
Isopimaric	0.11 ± 0.07	76.4 ± 10.6	99.9
Palustric + levopimaric	0.05 ± 0.02	27.2 ± 3.8	99.8
Abietic	0.14 ± 0.10	53.2 ± 9.3	99.7
Dehydroabietic	0.16 ± 0.10	76.4 ± 16.5	99.8
Neoabietic	0.06 ± 0.04	17.6 ± 5.4	99.7
Total (8 acids)	0.6 ± 0.3	310 ± 53	99.7-100

Mean ± SD, n = 3 sample pools from 10 fish
ND = not detectable (< 0.02 ug/mL)

The biological significance of the ability of an animal to dispose harmful substances through metabolic conjugation is well documented (Dutton 1966; Chambers and Yarbrough 1976). The origin of an undesirable compound may be endogenous (e.g. bilirubin, steroids) or exogenous. Elimination of substances in both categories generally requires metabolic energy, frequently more if the load is high. Several harmful compounds may be metabolized by the same metabolic pathway (e.g. glucuronidation) or transport route in the liver cell (Levine 1978). Overloading a mechanism adapted for elimination of endogenous compounds can lead to an unbalanced physiological status. This has been demonstrated in salmonids exposed acutely to resin acids in the water (Kruzynski 1979; Oikari and Nakari 1982; Nikinmaa and Oikari 1982). In cases of exposure of fish to lethal or high sublethal concen-

Table 5. Bile concentrations (ug/mL) of free and conjugated resin acids, and the percentage of total conjugates as glucuronides, in rainbow trout* exposed for 10 days to 0.5 % v/v solution of biologically treated kraft pulp mill effluent.

Resin acid	Conc. in bile Free	Conjug.		Percentage conjug.	Percentage glucuronides
Pimaric	ND	36		100	100
Sandaracopimaric	ND	20		100	100
Isopimaric	ND	36		100	100
Abietic	ND	50		100	84.0
Dehydroabietic	0.50	53		99.1	91.1
Total (5 acids)	0.50	195	Mean	99.8	95.0

* Pool of 10 fish
ND = not detectable (< 0.02 ug/mL)

trations of resin acids, the capacity of the liver to eliminate both bilirubin and the resin acid may be insufficient. However, in long-term exposures the metabolic capacity may even increase, enabling fish to resist chronic low level pollution. In such conditions, although individual fish can survive, they will have to utilize more energy for detoxication and may also suffer from related physiological dysfunctions, e.g. steroid imbalance or reproductive non-synchrony (Hansson 1981).

The results in Table 5 show, that the majority of resin acid conjugates are glucuronides i.e. the amounts hydrolysed by the KOH solution and by beta-glucuronidase are approximately equal. Some other conjugating agents may, however, account for a small part of the conjugations e.g. in the case of abietic acid.

The utility of fish bile for biomonitoring a variety of environmental xenobiotics was described by Lech et al. (1973) and Statham et al. (1976). Use of a bile sample, instead of e.g. the liver, as a biomonitoring tool for water resin acids has the analytical advantage that it does not contain fatty acids in amounts masking the resin acid peaks in GC analysis. Thus the bulk of fatty acids extracted by hexane or ether do not interfere with minor amounts of toxic resin acids (Oikari and Holmbom 1981). The present results provide support for and demonstrate some new aspects of the concept of "chemical bile bioassay", with which we want to detect and monitor the sublethal impact caused by the pulp and paper industry effluents on fish (Oikari and Holmbom 1981). Firstly, analysis of the total conjugated resin acids increase (when compared to direct hexane extraction) the sensitivity of the assay up to 500 times. It also appears to be possible to detect, from the B/P ratio of the free resin acid (Tables 1 and 2), whether the exposure has been transient or more permanent one. Finally, an advantage of this assay becomes apparent when dealing with partial fish kills; the bile of sur-

vivors or moribund fish can be analysed even after the resin acids have disappeared from the water. On the other hand, the usefulness of this bioassay in subacute and chronic exposures depends on how well we understand the meaning of simultaneous toxicological and physiological symptoms in fish. We should also learn more about the pharmacokinetics of resin acids in bile and tissues, especially during the recovery in uncontaminated water.

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