Ann. Zool. Fennici 19: 61-64. 1982

Uptake of resin acids into tissues of trout (Salmo gairdneri Richardson)

Aimo Oikari, Bjarne Holmbom & Hannu Bister

Oikari, A., Holmbom, B. & Bister, H. 1982: Uptake of resin acids into tissues of trout (Salmo gairdneri Richardson). — Ann. Zool. Fennici 19: 61—64.

Uptake and distribution properties of resin acids, dehydroabietic acid (DHAA) and the eight acids occurring in a wood rosin, were studied by measuring amounts accumulated into different organs of rainbow trout after four and two days' semistatic exposures in approximate concentrations of 1.2 and 1.4 mg/l of water, respectively. In both experiments, liver and posterior kidney were among the organs showing highest uptake. Compared to these, however, even more DHAA was found in blood plasma. In addition, anterior (head) kidney, skin, heart and brain all ranked high in one or both experiments. On the other hand, skeletal muscle tissues and gonads displayed the lowest tendency for bioconcentration. The different resin acids of rosin occurred in liver (the only tissue studied) very close to their ratios in external water.

Aimo Oikari and Hannu Bister, Division of Physiology, Department of Zoology, University of Helsinki, Arkadiankatu 7, SF-00100 Helsinki 10, Finland.
Bjarne Holmbom, Institute of Wood Chemistry and Pulp and Paper Technology, Åbo Akademi, Porthaninkatu 3, SF-20500 Turku 50, Finland.

1. Introduction

Various resin acids are the most important single category of toxic compounds acutely lethal for salmonid fishes in waste waters from pulp and paper mills (Mäenpää et al. 1968, Rogers 1973, Leach & Thakore 1973, 1976, 1977). Although first quantified in Canada (Rogers 1973), recent results clearly show that the toxicity impact of resin acids is essentially similar in Scandinavian mills, too (Holmbom & Lehtinen 1980). Information on the distribution and the fate of any toxic substance inside an animal body provides an important starting-point for more detailed studies on its physiological and toxicological effects.

Uptake of dehydroabietic acid (DHAA) into fish exposed to DHAA-containing effluent was first shown qualitatively by Mahood & Rogers (1975). In 1977 Fox et al. reported, on the whole body basis, up to twenty-fold accumulation of DHAA into rainbow trout exposed for two days to kraft mill effluents containing 0.1—0.7 mg DHAA/l. Kruzynski (1979) published data from sockeye salmon *(Oncorhynchus nerka)* and rainbow trout exposed in flow-through aquaria for 5 days to 0.65 mg DHAA/l. Because only one pooled or a single sample, respectively, was analysed, no data on the variation in the amounts accumulated into different organs is yet available. The two short-term experiments reported here were conducted because of the general inadequacy of knowledge on the uptake of resin acids. The first experiment was carried out with DHAA, one of the most persistent and abundant resin acids (Brownlee et al. 1977), and the second with a mixture of various resin acids. The latter experiment describes the total uptake into liver with simultaneous measurements on uptake of different acids from a known resin acids mixture.

2. Material and methods

Dehydroabietic acid (DHAA) was purified from disproportionated rosin (Hercules 731D) according to Halbrook & Lawrence (1966). The purity of 98 % was checked, as described in Holmbom (1977), by GC/MS. Polish wood rosin (R), analysed in a similar way, had the percentage composition given in Table 3 (palustric acid 27.1 and levopimaric acid 9.0 % w/w). Stock solutions containing 50 mg DHAA or R/ml and applied to the test aquaria were prepared by dissolving the substance in a minimal amount of ethyl alcohol and further diluting it with 0.05 N sodium hydroxide. For determination of actual concentration in the test aquarium, 1.2 mg/l margarinic acid (17:0) was added as an internal standard into 100 ml of acidified (pH 3) water sample. The mixture was extracted with three 20 ml aliquots of redistilled diethyl ether,

combined extracts evaporated under nitrogen, and stored at —20 °C for later GC analysis (Holmbom 1977).

Before experiments, the rainbow trout (*Salmo gairdneri* Richardson; DHAA: 1+ y old, weight 150—250 g, R: 0+ y, 40—55 g) were kept for at least two weeks in dechlorinated Helsinki City tap water at temperatures close (within ±1 °C) to those during the tests (DHAA: 10.5 °C; R: 6 °C). A light:dark cycle of 12:12 (08:00—20:00) was maintained in the aquarium room, and the fish were daily fed *ad libitum* with pelleted food (Ewos, Sweden), except during the tests. Exposures, conducted in October-November, were made semistatically, replacing about 50 % of the water volume twice daily. The lowest and highest toxicant concentrations prevailed just before and after the replacements, respectively. Fish loadings were 3.5 and 5.5 g fish/l of water in the DHAA and R experiments, respectively. No mortality was observed. The tanks were gently aerated (just enough to keep the water 0_2 concentration above 8 mh/l). The toxicant concentrations (DHAA or total resin acids) ranged in the 4 day DHAA experiment from 0.5 to 2.0 mg/l (average 1.2 mg/l; $n = 8$ determinations) and in the 2 day R experiment from 0.8 to 2.2 mg/l (average 1.4 mg/l; $n = 4$). Other water quality variables were: pH 7.2—7.3, conductivity 24 mS/m, alkalinity 0.66 mval/l, chloride 21 mg/l and zinc 25 µg/l.

For tissue sampling, the fish were stunned with a blow on the head and blood was drawn from the heart into a heparinized syringe. Plasma was immediately separated by centrifuging the blood (1 min) with a Beckman Microfuge B. In this paper red blood cells are defined as the packed cells on the bottom of the tube. After blood sampling, pieces from different tissues were taken, quickly frozen in liquid nitrogen and stored there until extraction. The same part of each organ was always collected from the fish. As far as brain, heart, anterior (head) kidney and spleen were concerned, either the whole organ or half of it was sampled. The gill samples consisted of the filaments from the first and the second arches on both sides, the skin samples of a part on the midline on the left-hand side close to the anal fin, and the liver samples of the central 1/5—1/3 of the whole mass of the organ. Samples of red muscle tissue were prepared from along both sides of the left lateral line beneath the anterior dorsal fin, and of white muscle just dorsally to this area. The most caudal 1/6—1/4 were removed from the posterior kidney and gonads (fish were juveniles). The intestine, as referred to in this paper, comprises the whole alimentary canal caudal to the caeca.

Resin acids were extracted from tissues (300—800 mg) as follows: single or pooled (equal amounts from two fish) pieces of thawed gill, skin, intestine and muscles were immediately homogenized in distilled water for 1 min with an Ultraturrax^R device. These homogenates (as other tissues directly) were homogenized for 3 min/10 strokes (1500 rpm) with a Potter-Evehjelm glass-teflon type homogenizer. The homogenate was acidified to pH 3—4 with dilute sulpuric acid, about 20 µg/g tissue of 17:0 internal standard was added, and the final volume adjusted to contain 1 g tissue per 5 ml. The mixture was then transferred into sealable glass tubes and extracted three times (by shaking vigorously for 10 min each time) with 2 ml of redistilled *n*-hexane. Checking of the recovery showed that 80 % of total resin acids were extracted by this procedure. The combined extracts were evaporated under a nitrogen flow, the tubes sealed and stored at —20 °C until analysis. Generally, for the fish tissues of the R experiment, only the total resin acids concentration is given, but for the liver, which was analysed fresh, the differences between the acids (Table 3) are also presented. For GC, the extract was dissolved in c. 0.5 ml *n*-hexane, methylated with diazomethane and concentrations determined directly with a Varian 2100 gas chromatograph according to the procedure described in detail by Holmbom (1977). The length of the glass capillary columns, coated with BDS or SE-30, varied from 30 to 50 m. In most cases parallel analyses were performed using both of these liquid phase films. If peak identification was doubtful, confirmation was made using and LKB 9000 GC-MS instrument (Holmbom et al. 1974).

3. Results and discussion

The highest accumulation of DHAA was observed in the blood plasma. Compared to this (237 µg/g wet weight) the suspension of packed red cells (RBC) contained only about 16 % of the plasma concentration. Therefore, when the extracellular volume of the RBC suspension (inulin space about 5 % vol/vol; H. Virtanen unpubl.) is taken into consideration, the concentration of DHAA in RBC itself was as low as about 28 µg/g. Hence the permeability of RBC for DHAA, despite efficient plasma perfusion, seems to be much lower e.g. than in liver cells. On the other hand, an unknown portion of plasma DHAA might be reversibly bound to protein. If this is the case and DHAA is released during extraction, our figures will represent too high plasma activities of this substance. Table 1 further implies that tissues possess different abilities to keep DHAA outside the cells. However, this may well be a function of time. It is interesting to speculate that the lowest uptake of DHAA into gonads (Table 1) may protect the fish from direct reproductive failures, at least as far as short-term spill exposures are concerned. However, fairly strong accumulation of resin acids into the brain (Tables 1 and 2) may give rise to impaired fish behaviour in various stages of reproduction. This assumption can be supported by observing a poisoned trout (see below), as well as by our previous findings on perch (*Perca fluviatilis*) in the rotary-flow test (Lehtinen & Oikari 1980).

In the present study, as well as in that of Kruzynski (1979), liver and posterior (urinary) kidney accumulated DHAA most effectively, and bioconcentration factors (BCF = conc. in tissue/conc. in water) of approximately 80 and 70, respectively, were now noted. However, in the single fish studied by Kruzynski the BCFs as well as the absolute concentrations in these organs of trout were more than twice as high as in the present

Table 1. Concentration (μg/g wet weight) of dehydroabietic acid (DHAA) in tissues of trout exposed to an average concentration of 1.2 mg DHAA/l of water for 4 d. Mean values with ranges are given. Number of pooled samples (each pool from two fish) in parentheses (*N*).

Tissue	Mean	Range	(*N*)
Blood plasma	237	155—318	(2)
Liver	101	98—103	(3)
Anterior kidney	88	47—114	(3)
Posterior kidney	83	75—99	(3)
Heart	76	35—109	(3)
Intestine	49	20—90	(3)
Gill	44	12—74	(3)
Skin	39	15—49	(3)
Red blood cells	38	37—39	(2)
Brain	37	13—50	(3)
Spleen	35	20—46	(3)
Red muscle	17	8—24	(3)
White muscle	15	5—28	(3)
Gonads	6	2—9	(3)

Table 2. Concentration (μg/g wet weight) of total resin acids in tissues of trout exposed to an average concentration of 1.4 mg rosin/l of water for 2 d. Mean values with ranges are given. Number of samples (each from a single fish or a pool from two) analysed in parentheses (*N*).

Tissue	Mean	Range	(*N*)
Liver	273	202—351	(4)
Skin	94	61—137	(7)
Posterior kidney	88	72—115	(3)
Brain	82	77—86	(3)
Gill	42	34—53	(6)
White muscle	24	17—29	(4)

study. The reason for this discrepancy is, besides the mode of toxicant application (constant-flow vs periodic replacement), most probably the difference in test water pH (McLeay et al. 1979). When trout were exposed to resin acid mixture R (Table 2), liver again revealed the greatest tendency to bioaccumulation (BCF approx. 200), thus potentially serving the purposes of elimination and detoxication mechanisms through this organ. However, almost nothing is known of whether resin acids are excreted as free or as conjugated derivatives or even about the relative roles of the most probable excretory organs: the liver, kidney and gills (cf. Oikari et al. 1980). The DHAA concentration in the intestinal tissue was also fairly high (Table 1), which may also reflect hepatic excretion or enterohepatic cycling.

Results on the uptake of different resin acids from R into trout liver are presented in Table 3. Most importantly, the acids occur in the liver tissue in similar ratios to those found in external water, i.e. different acids are fairly similar in their accumulation characteristics. In terms of water treatment practice, this means that the reduction of any single resin acid from pulp mill effluents is toxicologically beneficial to fishes. This observation is, of course, related to the additive acute toxicity of resin acids in pulp mill effluents observed earlier (Leach & Thakore 1973; Holmbom & Lehtinen 1980). The results in Table 3 also show that alterations in resin acid composition of the test water can take place within five hours. Decreases in the concentrations of palustric and neoabietic, and increases in abietic and dehydroabietic acids were especially noteworthy. It is plausible that similar changes also occur during stagnant standard bioassays of pulp mill effluents.

In fresh-water fish the most probable route of resin acids into the circulation is the integument, especially the gill epithelium. At the moment, however, this conclusion is based only on the relative extent of gill surface capillaries (c. 90 % of total integument area in the carp; Randall 1970), but not on direct flux data. In fact, the present results point to the possible importance of skin. At least the resin acid concentration in the skin is clearly higher than that in the gills (Table 2). Obviously the apparent dominance of gills in the resin acid uptake should be confirmed e.g. by applying some suitable divided chamber technique.

The visual observations on fish, repeatedly made in our laboratory during exposures to resin acids very similar to the present ones, point to toxicological effects on the central nervous system (CNS). Irritability towards external stimuli (noise, light, netting, touch etc.) and upright or slanting head-down position in water were the most striking behavioural symptoms. These consequences fit well with the considerable uptake of resin acids into brain tissue (Table 2), also giving clues to the relatively easy transfer of these poisons across the blood-brain barrier (see also Kruzynski 1979).

In striated muscles, i.e. the red and white parts of the great lateral muscle, the levels of resin acids were among the lowest measured in this study (Tables 1 and 2). These results agree well with those of Kruzynski (1979) on rainbow trout and sockeye salmon. On the other hand, the concentration of DHAA in heart muscle was almost five times that in the lateral muscle (Table 1), and in fact ranked among the "highest" (over 75 μg/g) group of tissues. In studies on the effects of DHAA on circulating leucocytes (Iwama et al. 1976), it is important to remember its strong bioaccumulation into the most important lymphatic organ of teleosts, the anterior (head) kidney. Compared to this, the level of DHAA in spleen was clearly lower, falling into the "intermediate" (35—50 μg/g) group of organs studied (Table 1).

Table 3. Relative amounts (% of total resin acids) of various resin acids accumulated in 2 d into trout liver. Approximate concentration of rosin in water was 1.4 mg/l. Means of three fish with ranges are given.

Sample/time	Pimaric	Sandaraco-pimaric	Iso-pimaric	Palustric + Levopimaric	Dehydro-abietic	Abietic	Neo-abietic
Rosin/0 h	7.7	1.8	3.8	36.1	5.1	24.9	16.1
Water/5 h	9.4	1.8	4.2	31.4	11.9	30.0	11.4
Water/46 h	8.5	1.5	5.3	30.2	11.6	30.2	10.4
Liver/48 h	9.4	2.0	5.7	20.6	17.6	36.0	8.7
	7.8—12.0	1.4—2.7	4.4—7.1	15.2—25.6	12.1—24.7	30.4—41.4	7.3—11.1

Pathological examination of an animal often includes efforts to correlate observations of "chemical autopsy" with "histopathological findings". At the moment, no published information is available on the structural consequences of DHAA or other resin acid poisoning. The present results will therefore also help in finding the most probable organs affected histopathologically. Histopathological studies are now underway in our laboratory.

Acknowledgements. This study was supported by a grant from the Maj and Tor Nessling Foundation to A.O. and B.H.

References

Brownlee, B., Fox, M. E., Strachan, W. M. J. & Joshi, S. R. 1977: Distribution of dehydroabietic acid in sediments adjacent to a kraft pulp and paper mill. — J. Fish. Res. Board Canada 34: 838—843.

Fox, M. E., Whittle, D. M. & Kaiser, K. L. E. 1977: Dehydroabietic acid accumulation by rainbow trout (Salmo gairdneri) exposed to kraft mill effluent. — J. Great Lakes Res. 3: 155—158.

Halbrook, N. J. & Lawrence, R. V. 1966: The isolation of dehydroabietic acid from disproportionated rosin. — J. Org. Chem. 31: 4246—4247.

Holmbom, B. 1977: Improved gas chromatographic analysis of fatty and resin acid mixtures with special references to tall oil. — J. Amer. Oil Soc. 54: 289—293.

Holmbom, B., Avela, E. & Pekkala, S. 1974: Gas chromatography-mass spectrometry of resin acids in tall oil rosin. — J. Amer. Oil Soc. 51: 397—400.

Holmbom, B. & Lehtinen, K-J. 1980: Acute toxicity to fish of kraft pulp mill waste waters. — Paperi ja Puu — Papper och Trä 62: 673—684.

Iwama, G. K., Greer, G. L. & Larkin, P. A. 1976: Changes in some hematological characteristics of coho salmon (Oncorhynchus kisutch) in response to acute exposure to dehydroabietic acid (DHAA) at different exercise levels. — J. Fish. Res. Board Canada 33: 285—289.

Kruzynski, G. M. 1979: Some effects of dehydroabietic acid (DHA) on hydromineral balance and other physiological parameters in juvenile sockeye salmon Oncorhynchus nerka. — Ph.D. Thesis, University of British Columbia, 187 pp.

Leach, J. M. & Thakore, A. N. 1973: Identification of the constituents of kraft pulping effluent that are toxic to juvenile coho salmon (Oncorhynchus kisutch). — J. Fish. Res. Board Canada 30: 479—484.

—»— 1976: Toxic constituents in mechanical pulping effluents. — TAPPI 59: 129—134.

—»— 1977: Compounds toxic to fish in pulp mill waste streams. — Prog. Water Tech. 9: 787—798.

Lehtinen, K-J. & Oikari, A. 1980: Sublethal effects of kraft pulp mill waste water on the perch, Perca fluviatilis, studied by rotary-flow and histological techniques. — Ann. Zool. Fennici 17: 255—259.

Mahood, H. W. & Rogers, I. H. 1975: Separation of resin acids from fatty acids in relation to environmental studies. — J. Chromatogr. 109: 281—286.

McLeay, D. J., Walden, C. C. & Munro, J. R. 1979: Influence of dilution water on the toxicity of kraft pulp and paper mill effluent, including mechanisms of effect. — Water Res. 13: 151—158.

Mäenpää, R., Hynninen, P. & Tikka, J. 1968: On the occurrence of abietic and pimaric acid type resin acids in the effluents of sulphite and sulphate pulp mills. — Paperi ja Puu — Papper och Trä 50(4a): 143—150.

Oikari, A., Holmbom, B., Ånäs, E. & Bister, H. 1980: Distribution in a recipient lake and bioaccumulation in fish of resin acids from kraft pulp mill waste waters. — Paperi ja Puu — Papper och Trä 62(4a): 193—202.

Randall, D. J. 1970: Gas exchange in fish. — In: Hoar, W. S. & Randall, D. J. (eds.), Fish Physiology 4: 253—291. Academic Press, New York.

Rogers, I. H. 1973: Isolation and chemical identification of toxic components of kraft mill waters. — Pulp Paper Mag. Canada 74: 111—116.

Received 23. XI. 1981
Printed 1. III. 1982