

METABOLIC EFFECTS OF DEHYDROABIETIC ACID ON RAINBOW TROUT ERYTHROCYTES

PETER G. BUSHNELL,* MIKKO NIKINMAA† and AIMO OIKARI‡

Division of Physiology, Department of Zoology, University of Helsinki, Arkadiankatu 7, SF-00100 Helsinki 10, Finland

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Abstract—1. Oxygen consumption and ATP concentration were measured in rainbow trout erythrocytes incubated in a physiological saline containing 0, 5, 15, 30 or 60 mg/l dehydroabietic acid.

2. DHAA caused a decrease in cellular ATP level and oxygen consumption at concentrations above 15 mg/l. Haemolysis increased markedly, when the cellular ATP concentration decreased below 1 mM.

3. These data suggest that increased breakdown of red cells may be the primary reason for jaundice which is observed in resin acid-toxicated fish.

INTRODUCTION

Resin acids, among them dehydroabietic acid (DHAA), are an important toxicant group in the effluents of the pulp and paper industry. During exposures to either kraft mill effluents (Oikari *et al.*, 1980), simulated kraft mill effluents (Oikari *et al.*, 1985) or pure dehydroabietic acid (Oikari *et al.*, 1983) the plasma concentration of these toxicants increases to a level higher than in any other tissue (Oikari *et al.*, 1982). The bioconcentration factor (BCF = concentration in tissue/concentration in water) of DHAA in plasma was ca 200 in rainbow trout exposed to 1.2 mg/l DHAA for 4 days at water of pH 7.4 (Oikari *et al.*, 1982).

Although the red cell membrane seems to be relatively impermeable to resin acids, they do influence red cell function. Nikinmaa and Oikari (1982) observed that during a 24-hr exposure to DHAA (2 mg/l) the red cells initially shrank, their pH decreased and later the ATP/Hb ratio increased. In a 30-day exposure of rainbow trout to a resin acid mixture (toxic component 0.08×96 hr LC_{50}) the magnesium concentration of red cells doubled (Oikari *et al.*, 1985). McLeay (1973) observed that after a 25 day exposure to kraft mill effluent (KME) the haematocrit value of *Oncorhynchus kisutch* blood decreased, and the proportion of immature erythrocytes in the blood increased.

The above results indicate that the effects on red cell function may play a role in the toxic action of resin acids in fish. In the present paper we report on the direct effects of resin acids on red cell using *in vitro* incubations. We have investigated how dehydroabietic acid influences the oxygen consumption and ATP production of rainbow trout red cells incubated *in vitro* in physiological saline.

MATERIALS AND METHODS

Rainbow trout (*Salmo gairdneri*), 200–500 g in weight and 20–30 cm in length were obtained from the Laukaa Fish Culture Research Station. They were maintained in dechlorinated Helsinki tap water ($T 8-12^{\circ}\text{C}$, $P_{\text{O}_2} > 100$ mmHg, pH 7.2–7.5) for at least 2 weeks before experiments.

A blood sample (2–5 ml) was taken into a heparinized syringe from stunned fish using caudal puncture. The blood sample was then washed twice in physiological saline as described by Nikinmaa (1983). The composition of the physiological saline was 128 mM NaCl, 3 mM KCl, 1.5 mM CaCl_2 , 1.5 mM MgCl_2 and 20 mM Tris-HCl buffer. The pH of the buffer was initially adjusted to 7.7. Following the final rinse, the washed, packed cells were suspended in the above buffer to a haematocrit value of ca 18% and haemoglobin concentration of ca 40 g/l. The suspension was then divided into two portions (control and experimental) of 800 μl each and placed in a tonometer. While the blood was being equilibrated, 100 μl of 20 mM glucose solution was added to both samples, 100 μl dehydroabietic acid (concentrations 5, 15, 30 and 60 mg/l) was added to the experimental samples and 100 μl of physiological saline to the control sample. Stock solution of dehydroabietic acid (3000 mg/l) was prepared by dissolving the substance in small amount of ethanol and further diluting it with 0.05 M NaOH.

The blood was allowed to equilibrate for 30–45 min at room temperature (21–23°C) before the onset of oxygen consumption measurements. The pH of the red cell suspension, measured before every oxygen consumption determination, varied between 7.5 and 7.6, except in cells treated with NaOH and ethanol (solvent for DHAA) which had an extracellular pH of 7.67.

The oxygen consumption of both the control and experimental samples were determined simultaneously in two Tucker chambers (Vol. 350 and 343 μl) thermostatted at 23°C. The partial pressure of oxygen in the chambers was monitored by Radiometer D546 electrodes, connected to Radiometer PHM72 MkII oxygen analysers and a two-channel chart recorder. The determination of oxygen consumption (V_{O_2}) was a three-step process. Firstly, the oxygen content of the two blood samples was determined. Secondly, the oxygen consumption of the electrodes was determined by measuring the oxygen consumption of air saturated physiological saline for 15 min. Finally, the oxygen consumption of the blood sample + the electrode was measured for 25 min. Between each of these steps the chambers were thoroughly rinsed with physiological saline and distilled water. Between each portion of the cycle the electrodes were

*Present address: Department of Physiology, John A. Burns School of Medicine, University of Hawaii, 1960 East-West Road, Honolulu, Hawaii 96822, USA.

†Author to whom correspondence and reprint requests should be addressed.

‡Present address: Department of Biology, University of Joensuu, Box 111, SF-80101 Joensuu 10, Finland.

recalibrated with air-saturated water and allowed to stabilize before the next step of the determinations. At least two determinations of oxygen consumption were carried out, generally after 1 and 3 hr incubations. Since two chambers were in use, the oxygen consumption of both the control and the experimental samples were measured simultaneously to ensure that the incubation time of each sample was equal.

The O_2 consumption of red cells was calculated from the decrease in the partial pressure of oxygen in the measuring chamber. In simple terms, the O_2 consumption is given by the formula

$$V_{O_2} = \alpha(\text{fluid}) \times \text{slope}$$

in which α = the capacity coefficient of oxygen in the fluid ($\text{ml } O_2 \cdot \text{l}^{-1} \cdot \text{torr}^{-1}$) and slope = the slope of decrease in oxygen tension ($\text{torr} \cdot \text{min}^{-1}$).

The capacity coefficient of oxygen in blood was calculated from the oxygen content of the blood sample, measured using Tucker's (1967) method, and simultaneous measurement of the oxygen tension of the sample. The slope of the decrease of P_{O_2} was calculated from the linear regression P_{O_2} vs time (given by the chart record). The regression was based on the oxygen partial pressure values taken every 2.5 min, with the first 5 min period being discarded to ensure that the system had stabilized.

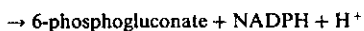
The apparent oxygen consumption of blood included the oxygen consumption of the electrode, which had to be subtracted to get the true oxygen consumption. The oxygen consumption of the electrode was taken to be the oxygen consumption of air saturated Ringer. The value for human plasma (Altman and Dittmer, 1971) was used as the solubility coefficient of oxygen in the Ringer. Additionally, the haematocrit value of the red cell suspension had to be taken into account in calculating the oxygen consumption of red cells. Thus, the following formula was used for calculations

$$V_{O_2} = (V_{O_2}(\text{blood}) - V_{O_2}(\text{electr.})) \times 100/\text{Hct} (\%)$$

ATP concentration was measured fluorometrically by following the formation of $\text{NADPH} + \text{H}^+$ in the reactions



and



using a Transcon 102 FN (Orion Analytica, Finland) analyser. The ATP concentration was determined after 1 and 3 hr incubations in the different DHAA concentrations. The samples were deproteinized with 0.6 M HClO_4 and stored in liquid nitrogen until measurements. The haematocrit value was determined by centrifugation, and haemoglobin concentration using the cyanmethaemoglobin method. The mean cellular haemoglobin concentration was calculated from the haemoglobin concentration and haematocrit value, and the ATP/haemoglobin molar ratio from the molar concentrations of ATP and haemoglobin in the cells (molecular weight of haemoglobin was taken as 67,000). For microscopical examination the cells were treated as described by Ferrell and Huestis (1982).

Statistical differences between the means of the control and the experimental group were determined by paired Student's *t* tests. All values in the text, tables and figures are given as mean \pm SEM.

RESULTS

The red cell ATP concentration and ATP/Hb molar ratio decreased significantly within 1 hr when the DHAA concentration in the incubation medium was either 30 or 60 mg/l (Figs 1 and 2). After 3 hr incubation the ATP/Hb ratio tended to decrease also

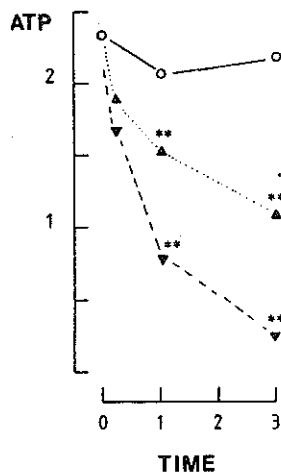


Fig. 1. The red cell ATP concentration (ATP; mmol/l red cells) as a function of incubation time (TIME; hr) in controls (O), in cells treated with 30 mg/l dehydroabietic acid (Δ), and in cells treated with 60 mg/l dehydroabietic acid (∇). Means of five experiments are given. The statistical significance of the difference between DHAA-treated cells and controls was tested using paired *t* tests (** $P < 0.01$).

in 15 mg/l DHAA concentration. It is notable that the sample started to haemolyse each time the ATP concentration decreased below 1 mM.

Microscopical examination of the cells showed that they lost the smooth ellipsoidal appearance within 3 hr owing to DHAA treatment (30 or 60 mg/l), and lost membrane fragments so that after 24 hr incubation (after which complete haemolysis had occurred in 15, 30 and 60 mg/l DHAA concentration) only nuclei and membrane fragments were visible under the microscope.

The red cell volume did not seem to be affected by the DHAA treatment, since the MCHC remained unchanged. However, at the lower concentrations (5 and 15 mg/l) the erythrocytes tended to shrink after 3 hr incubation; the MCHC increased from $255 \pm 11(5)$ g/l in controls to $291 \pm 23(5)$ g/l in 5 mg/l DHAA and to $321 \pm 30(5)$ g/l in 15 mg/l DHAA ($P < 0.1$ in both cases).

The oxygen consumption of control red cells was $3-4 \text{ ml } O_2 \cdot \text{l}^{-1} \cdot \text{min}^{-1}$ (Table 1). In 1-hr samples, treat-

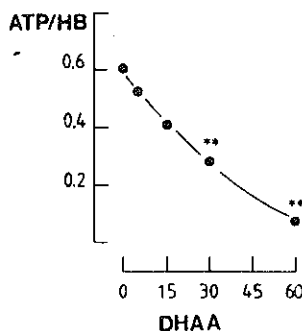


Fig. 2. The ATP/Hb molar ratio (ATP/Hb) of rainbow trout red cells as a function of dehydroabietic acid concentration (DHAA; mg/l) after 3-hr incubation. See Fig. 1 for further explanations.

Table 1. Oxygen consumption of rainbow trout red cells treated with different concentrations of DHAA. Means \pm SEM are given. The statistical significance of the difference between the oxygen consumption of treated cells and control cells was tested using paired *t* tests. Each oxygen consumption measurement was carried out simultaneously with its control. Oxygen consumption is given in $\text{mlO}_2 \cdot \text{lRBC}^{-1} \cdot \text{min}^{-1}$

DHAA (mg/l)	1 hr		3 hr	
	Exp.	Cont.	Exp.	Cont.
5	3.76 \pm 0.23 (6)	NS 3.44 \pm 0.09 (6)	3.40 \pm 0.30 (6)	NS 3.13 \pm 0.26 (6)
15	3.67 \pm 0.23 (6)	NS 4.23 \pm 0.49 (6)	4.17 \pm 0.42 (6)	NS* 4.82 \pm 0.75 (6)
30	3.00 \pm 0.33 (6)	NS 2.82 \pm 0.18 (6)	2.15 \pm 0.10 (6)	0.05 2.84 \pm 0.12 (6)
60	3.06 \pm 0.19 (7)	NS 3.34 \pm 0.24 (7)	2.36 \pm 0.21 (7)	0.05 3.06 \pm 0.24 (7)

ment of cells with sodium hydroxide and ethanol (solvent for DHAA) caused an increase in pH (from 7.53 to 7.67) and an increase in the oxygen consumption of the cells from $3.4 \pm 0.29(6)$ to $4.3 \pm 0.37(5) \text{ mlO}_2 \cdot \text{l}^{-1} \cdot \text{min}^{-1}$, $P < 0.05$. In contrast, the oxygen consumption of cells treated with 30 and 60 mg/l DHAA was significantly lower than that of controls after 3 hr incubation (Table 1, Fig. 3), and tended to decrease also in 15 mg/l DHAA concentration. Thus, DHAA seems to have a direct effect on the energy metabolism of the cells.

DISCUSSION

Adequate ATP levels are required for structural integrity of both anucleated (e.g. Ferrell and Huestis, 1982) and nucleated (Nikinmaa and Huestis, 1984) red cells. Treatment of fish red cells with DHAA caused a rapid ATP depletion which resulted in cell haemolysis. Such a decrease in red cell ATP concentration was not observed in the previous *in vivo* experiment of Nikinmaa and Oikari (1982). Perhaps the cells in which the ATP concentration had decreased had already broken down. As a result, the measured ATP concentration was that of the remaining, more resistant (possibly younger) red cells, in which appreciable ATP depletion did not occur.

Two actions of DHAA may cause the depletion of ATP. Firstly, DHAA probably acts as a detergent, dissolving the lipid portions of the erythrocyte membrane and making them increasingly permeable. In-

creasing amounts of energy are then required to maintain normal ionic balance within the cells. This may deplete the intracellular ATP pool. Also, the detergent action as such can cause breakdown of the cells. Secondly, DHAA seems to inhibit aerobic energy metabolism of the cells, as indicated by the simultaneous decrease of ATP concentration and oxygen consumption of the cells. In contrast to mammalian red cells, nucleated fish red cells contain mitochondria (Zapata and Carrato, 1981), and are capable of aerobic energy metabolism (e.g. Powers, 1980). Eddy (1977) determined the oxygen consumption of rainbow trout red cells obtaining a value of $2.4 \mu\text{l oxygen/ml RBC/min}$ at 20°C , and calculated that red cells consumed about 5% of the total oxygen consumption of the animals. The red cells thus metabolize quite actively, as their volume is only ca 1% of the total volume of the fish. Our values for oxygen consumption of the red cells are slightly higher than that of Eddy.

The initial effects of DHAA on the blood of fish are dependent on the speed of accumulation of resin acids into plasma. The acute experiment of Nikinmaa and Oikari (1982) reported that red cells shrank during the initial stages of exposure (0–3 hr). Afterwards the bilirubin concentration in plasma started to increase, and jaundice was observed. The initial changes in cell volume resemble the observation about increased MCHC at 5 and 15 mg/l DHAA after 3 hr in the present experiment.

The measured concentrations of DHAA in rainbow trout plasma after acute sublethal exposures (e.g. Oikari *et al.*, 1982) can be much higher, up to 300 mg/l, than the ones used in this study. When concentrations above 100 mg/l were tested in the physiological saline, complete haemolysis invariably occurred in less than an hour. Thus, some plasma components decrease the effect of DHAA on the red cells. Oikari (unpublished data) has observed that more than 1/2 of plasma DHAA is loosely bound to macromolecules. Despite this, it is probable that free dehydroabiatic acid concentration in plasma is often high enough to cause increased lysis of the red cells.

Increased breakdown of red cells may, then, be the primary reason for jaundice observed in some studies (Kruzynski, 1979; Nikinmaa and Oikari, 1982). The breakdown of cells results in increased formation of bilirubin. If the liver cannot handle all of the excess bilirubin, jaundice is observed. Additionally, the capability of liver to glucuronidate bilirubin may be limited if DHAA inhibits UDP-glucuronyl transferase enzyme in the liver (see Oikari and Nakari, 1982).

Haemolysis would explain some other findings observed in studies about the effects of pulp and paper mill effluents on the blood of fish. McLeay

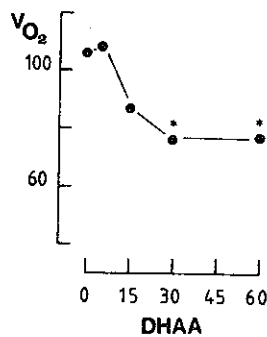


Fig. 3. The relative oxygen consumption (V_{O_2} ; in %) of rainbow trout red cells treated with different concentrations of dehydroabiatic acid (DHAA; mg/l) for 3 hr; 0 mg DHAA/l = sample treated with NaOH + ethanol (solvent for DHAA). The percentage oxygen consumption of cells at different DHAA concentrations was obtained by comparing the individual experimental values with their own controls. Asterisks give the statistical significance of the difference between the treatment and control. Paired *t* tests on the original oxygen consumption values were used for comparisons ($*P < 0.05$).

(1973) found that the proportion of immature erythrocytes increased in both a 12-hr and a 25-day exposure to kraft mill effluents. This cannot solely be due to increased erythropoiesis, since the absolute red cell count and absolute number of mature erythrocytes tended to decrease. A logical explanation would be that mature erythrocytes are more easily broken down than immature ones. In elasmobranch fish immature erythrocytes contain more numerous mitochondria than mature ones (Zapata and Carrato, 1981). Thus, they can probably produce energy more effectively than mature erythrocytes, and may therefore be more resistant to lysis.

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