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THE INFLUENCE OF SALINITY ACCLIMATION ON THE TEMPERATURE SENSITIVITY OF OXYGEN BINDING TO THE HAEMOCYANIN OF THE PROSOBRANCH *NEPTUNEA ANTIQUA*

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Summary

The thermal sensitivity of oxygen binding has been studied at 10, 15, 20 and 25 °C in whole blood from specimens of *Neptunea antiqua* acclimated to ambient salinities of 24 and 35 ‰. The O_2 affinity is strongly pH-dependent, demonstrating a large reversed Bohr shift below pH 8.0. The magnitude of the Bohr shift is not significantly influenced by temperature or ionic concentration. At 35 ‰, the blood O_2-affinity is strongly influenced by temperature ($\Delta H_{app} \approx -58.6\,kJ\,mol^{-1}$), while at 24 ‰ there is almost no temperature sensitivity ($\Delta H_{app} < -18.8\,kJ\,mol^{-1}$).

Introduction

It has been established that inorganic ions, such as heterotropic allosteric ligands, may control the oxygen-binding properties of haemocyanin-containing blood from marine gastropods and arthropods (Truchot, 1975; Mangum *et al.* 1976; Mangum and Lykkeboe, 1979; Mangum, 1981; Brix, 1983; Torensma and Brix, 1981; Diefenbach and Mangum, 1983; Burnett *et al.* 1988). In contrast to this general scheme, and in particular to the study of Mangum and Lykkeboe (1979) on the blood of the prosobranch gastropod *Busycon canaliculatum*, Brix (1982) demonstrated no effects of salts on the O_2-affinity of the blood from the related marine gastropod *Buccinum undatum*. The two studies, however, were carried out at 20 °C and 10 °C, respectively.

The temperature sensitivity of a number of arthropod and molluscan haemocyanins has been studied (Redfield, 1934; Mauro and Mangum, 1982*a*,*b*; Bridges,

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1986; Morris and Bridges, 1986; Burnett *et al.* 1988; Brix *et al.* 1989*a*,*b*). While the variation in temperature sensitivity is very large, most haemocyanins show an exothermic heat of reaction, although an endothermic reaction has been reported for a few species (Morris *et al.* 1985; Sanders and Childress, 1985; Brix *et al.* 1989*b*). None of these studies, however, has focused on the interrelationship between ambient salinity and temperature sensitivity.

To analyse this interrelationship we studied the effect of temperature on the O_2-binding properties of haemolymph from the marine gastropod *Neptunea antiqua* acclimated to 24 or 35 ‰ salinity sea water at 10°C.

Materials and methods

The 29 specimens of *Neptunea antiqua* used in the present investigation were supplied by fishermen from the vicinity of Aarhus, Denmark. Fifteen animals were kept in aerated, recirculating natural sea water of 24 ‰ salinity at 10°C, while the rest were held in recirculating sea water of 35 ‰, prepared by addition of commercial sea salt to natural low-salinity water, also at 10°C. The animals were allowed to acclimate for several months before they were used in the experiments.

Blood samples were obtained by needle puncture of the foot sinus. In each experiment the blood was pooled from about five specimens and centrifuged to sediment cells and debris. The supernatant fluid was stored in iced water before experimentation.

The salinity acclimation was checked by measuring and comparing the Cl^- concentration and the freezing-point depression of blood and ambient water using a Radiometer chloride titrator and a Knauer osmometer, respectively (see Table 1).

O_2 dissociation curves for whole blood were constructed from 15–30 point values measured photometrically (366 nm) at 10, 15, 20 and 25°C (Brix *et al.* 1979). The blood pH was adjusted for each O_2-binding curve by changing the percentage of CO_2 to a constant value between 0.01 and 1.0 % of the equilibrating gas mixture by means of Wösthoff gas-mixing pumps. pH was measured with a microelectrode mounted in a thermostatted microtonometer system (BMS 2, Radiometer). The total pressure in the chamber was 101 kPa and P_{CO_2} never exceeded 1.0 kPa.

The Monod–Wyman–Changeux model (Monod *et al.* 1965) was adapted to fit the oxygen-binding data (Torensma and Brix, 1981). The quality of the fitting procedure was estimated from the *r*-index, which did not exceed 1 % for any of the fitted curves. The temperature sensitivity of oxygen affinity was calculated from the slope of van't Hoff plots. The data used in Fig. 2 were obtained at constant pH from plots of $\log P_{50}$ *vs* pH at 15 and 25°C. The reported values of the apparent heat of oxygenation:

$$\Delta H = \frac{-19.14 T_1 T_2 \times \Delta \log P_{50}}{(T_2 - T_1) \times 1000} \quad (\text{kJ mol}^{-1}),$$

include the heat solution of oxygen ($\approx -12.6\,\text{kJ mol}^{-1}$).

Results

Table 1 shows very good agreement between the blood and water osmolality and Cl^- concentration for all the gastropods at both 24‰ and 35‰ salinity. Fig. 1 shows the oxygen affinity expressed as P_{50} (kPa) as a function of pH at 10 and 20°C. The results indicate the presence of a marked reversed Bohr shift within the pH range 7.2–8.0, as previously reported (Torensma and Brix, 1981). Below pH 7.2 there was no Bohr effect. At 10°C there was no effect of salinity on the pH

Table 1. *Osmolarity and Cl^- concentration of* Neptunea antiqua *blood, in 15 animals acclimated to 24‰ salinity and 14 animals acclimated to 35‰ salinity for several months, and of the ambient water*

Salinity of water (‰)	Water		Blood		
	Osmolarity (mosmol l^{-1})	$[Cl^-]$ (mmol l^{-1})	Osmolarity (mosmol l^{-1})	$[Cl^-]$ (mmol l^{-1})	*N*
24	710	381	700	351	5
24	776	385	750	375	6
24	749	384	735	368	4
35	1090	566	1070	555	5
35	1086	559	1056	544	4
35	1070	560	1070	555	5

The blood was pooled, and the number of specimens used for each pool (*N*) is given. S.D. is less than 1%.

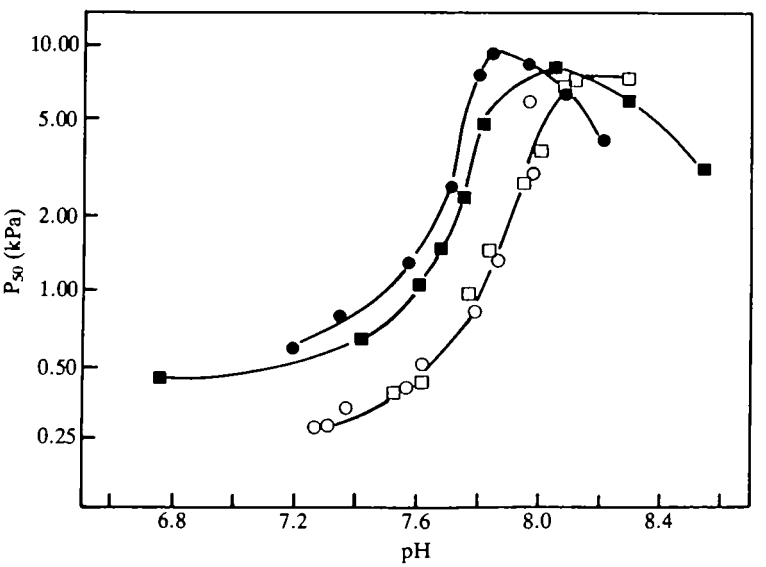

Fig. 1. The pH dependence of oxygen affinity expressed as P_{50} (kPa) for low-salinity (squares) and high-salinity blood (circles) at 10°C (open symbols) and 20°C (closed symbols). The points represent single measurements.

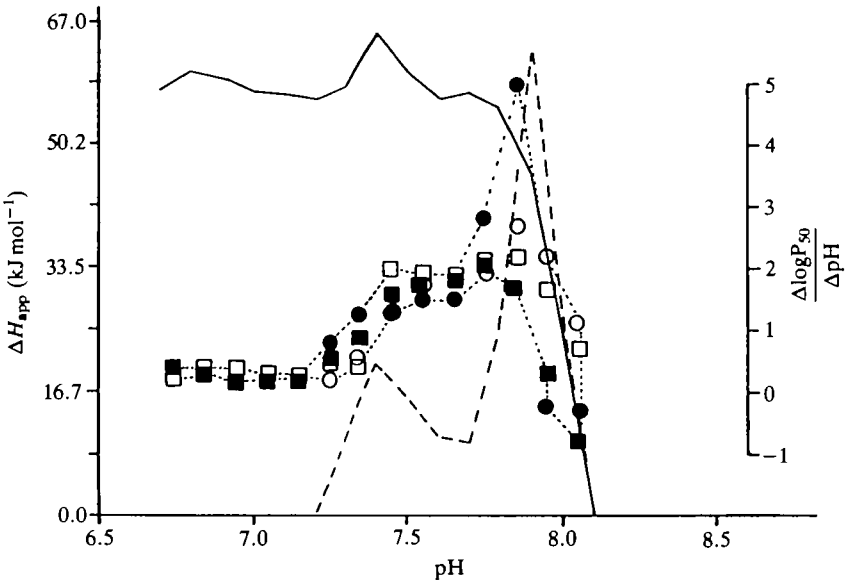

Fig. 2. Apparent heat of oxygenation, ΔH_{app} at 35‰ salinity (solid line) and 24‰ salinity (broken line), and the Bohr factor expressed by $\Delta \log P_{50}/\Delta pH$ (symbols) as a function of pH. (●) 24‰ salinity and 25°C; (○) 24‰ salinity and 15°C; (■) 35‰ salinity and 25°C; (□) 35‰ salinity and 15°C. The points represent single measurements.

dependence of oxygen affinity, while at the higher temperature increased salinity decreased the O_2 affinity. Neither temperature nor salinity seemed to change the magnitude of the Bohr shift except for one point at about pH 7.8, where a marked Bohr shift was observed in the low-salinity blood at 25°C (Fig. 2). Fig. 2 also shows a very low temperature sensitivity in the low-salinity blood. When the heat of solution of oxygen was subtracted, the overall heat of oxygenation (ΔH) approached zero. At pH 7.8, where there was a large increase in the magnitude of the Bohr effect, there was a marked increase in ΔH. In the high-salinity blood, the ΔH value was relatively constant up to just below pH 8.0. Subtracting the heat of solution of oxygen, ΔH is about $-46.0\,kJ\,mol^{-1}$. In the present analysis we focus on the O_2-binding behaviour of the whole blood in the pH range of the reversed Bohr shift.

Fig. 3 shows some of the fitted curves for both low- and high-salinity blood at 15 (solid lines) and 25°C (broken lines). For both low- and high-salinity blood we found a marked shift in the low-affinity state (T-state, represented by the lower asymptote) with pH (Fig. 3). Temperature had very little influence on the T-states of blood at 24‰ (at constant pH values) but for 35‰ blood a significant rightward shift was seen in the middle pH range. The minimum and maximum P_{50} values of the T-state were fairly constant in all the blood analysed (Fig. 4).

Moreover, a single high-affinity state (R-state, indicated by 1 in Figs 3 and 4) was found at 15°C for 24‰ blood. At 25°C, the R-state shifted to the right and

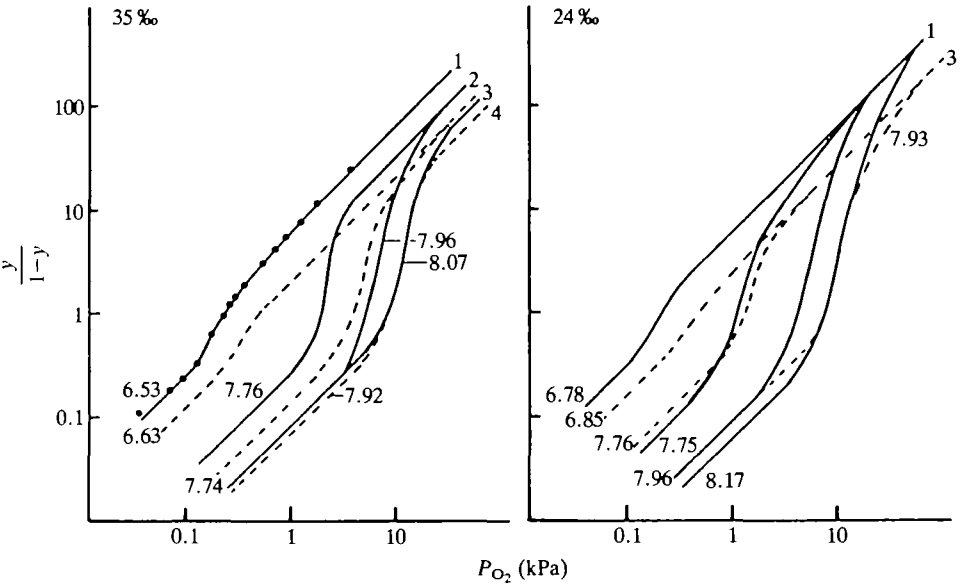

Fig. 3. Hill plots for low- and high-salinity blood from *Neptunea antiqua* at 15 °C (solid lines) and 25 °C (broken lines) at two salinities and constant carbon dioxide tensions. pH values are indicated for each curve. The data points are shown only in one curve for clarity. 1, 2, 3 and 4 indicate the four different R-states, which can be identified from the plot.

stabilized at a lower oxygen affinity (3 in Figs 3 and 4). The lack of effect of pH on the R-state implies that the Bohr shift was present mainly when the first molecules of O_2 were bound, as can also be seen in Fig. 4. In the high-salinity blood, however, the R-state was pH-dependent. Four different R-states are demonstrated in the Hill plots of Fig. 3. These are also indicated in Fig. 4, which outlines the very sharp transition induced by raising pH.

Discussion

The present investigation demonstrates a very large difference in thermal sensitivity between low- and high-salinity blood from the arctic marine prosobranch gastropod *Neptunea antiqua*. The effect of salinity on the O_2-binding behaviour decreases with decreasing temperature. Against this background it is possible to speculate on the possible ecophysiological relevance of these findings.

In the North Sea and the Baltic low-salinity water prevails in coastal regions characterized by shallow water and large inflows of fresh water from rivers. In this habitat, there are large diurnal variations in the ambient temperature which cause blood temperature in gastropods to vary in parallel. Therefore, an increase in ambient temperature would generally not result in a decrease of both blood pH (assuming that the blood pH changes with temperature according to the Rosenthal curve) and haemocyanin oxygen-affinity. However, in low-salinity water, owing to

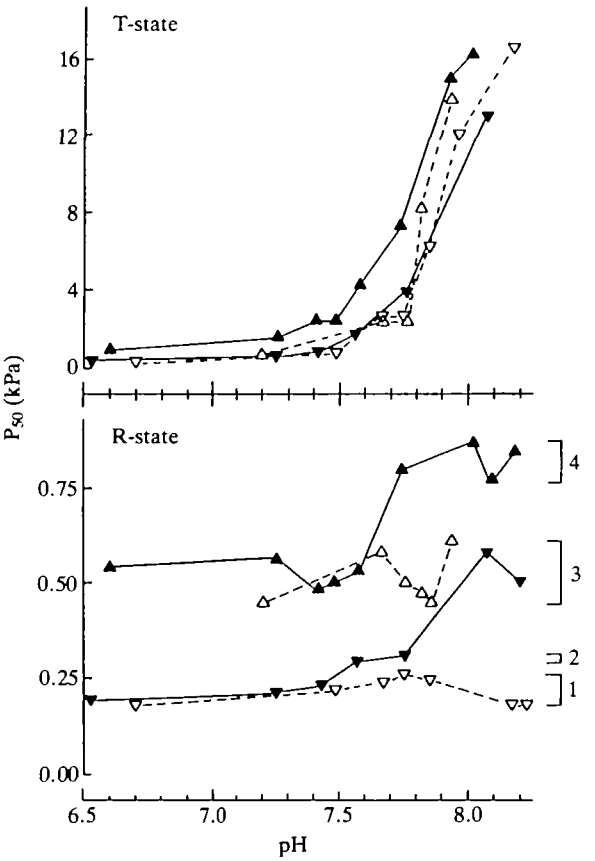

Fig. 4. The oxygen affinities of the T-state (A) and the R-states (B) in the blood of 35‰ (filled symbols) and 24‰ (open symbols) acclimated *Neptunea antiqua* expressed as the P_{50} values as a function of pH at 15°C (▽) and 25°C (△). The four different R-states referred to in the text are indicated in B. The points represent single measurements.

the thermodynamic properties displayed by the haemocyanin molecule under these conditions, the latter effect is almost negligible. Hence, from a physiological point of view, we have to consider only the first point, i.e. the temperature-induced decrease of blood pH. This will bring about the reverse Bohr shift, thereby increasing the O_2 affinity of haemocyanin. This feature may safeguard the postbranchial O_2 saturation in the blood and in conditions of reduced O_2, such as will occur when a rise in water temperature decreases O_2 solubility at the same time as metabolic activity (of organisms) in the surrounding water is depleting available oxygen. The higher salinity prevails at greater depths in more stable environments. Under these conditions the temperature sensitivity may act to minimize effects of environmental perturbations by stabilizing the oxygen-binding properties of the haemolymph. In other words, increased temperatures would decrease the O_2 affinity of haemocyanin, but the concomitant decrease in blood

pH would increase the O_2 affinity *via* the reversed Bohr shift, thus keeping it stable.

In the North Atlantic, however, where the gastropod experiences a limited range of temperatures, rarely exceeding 10°C, a combination of high salinity and shallow water often occurs. The low temperature may thus protect the gastropod from the effects of salinity on O_2 binding.

At present it must be clearly stressed that our knowledge of *in vivo* temperature-induced changes of blood pH is very limited and further investigation is called for. Some recent papers on crustacean haemocyanins may be very useful in elucidating the possible mechanisms underlying the findings of the present investigation. Anderson *et al.* (1982) demonstrated for crustacean haemocyanin that the number of available binding sites for the allosteric modulator Ca^{2+}, which is known to raise O_2 affinity in haemocyanins with a normal Bohr shift, increases with temperature. In this case, a greater interaction between calcium ions and haemocyanin at higher temperature would tend to increase O_2 affinity while temperature is acting to decrease it. This feature would thus tend to decrease the effect of higher temperatures (Burnett *et al.* 1988). However, in blood characterized by a reversed Bohr shift, the greater calcium ion activity would reduce O_2 affinity by increasing P_{50} (Diefenbach and Mangum, 1983). Therefore, one could expect a larger temperature-induced decrease in O_2 affinity at higher temperature, caused by an increase in haemocyanin calcium-binding in combination with higher Ca^{2+} activity. From previous results obtained on *N. antiqua* haemocyanin (Torensma and Brix, 1981), Cl^- would be expected to have the same effect. However, a definitive conclusion must await a direct demonstration of the effect of temperature on the binding of inorganic ions.

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