The Journal of Experimental Biology 205, 253–263 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
JEB3731

The archaeogastropod mollusc *Haliotis iris*: tissue and blood metabolites and allosteric regulation of haemocyanin function

Jane W. Behrens[1,3], John P. Elias[2,3], H. Harry Taylor[3] and Roy E. Weber[1,*]

[1]*Department of Zoophysiology, Institute Biological Sciences, University of Aarhus, DK 8000 Aarhus, Denmark,* [2]*School of Biological Sciences, Monash University, Clayton, Victoria 3800, Australia* and [3]*Department of Zoology, University of Canterbury, Private Bag 4800, Christchurch, New Zealand*

*Author for correspondence (e-mail: roy.weber@biology.au.dk)

Accepted 30 October 2001

Summary

We investigated divalent cation and anaerobic end-product concentrations and the interactive effects of these substances and pH on haemocyanin oxygen-binding (Hc-O_2) in the New Zealand abalone *Haliotis iris*. During 24 h of environmental hypoxia (emersion), D-lactate and tauropine accumulated in the foot and shell adductor muscles and in the haemolymph of the aorta, the pedal sinus and adductor muscle lacunae, whereas L-lactate was not detected. Intramuscular and haemolymph D-lactate concentrations were similar, but tauropine accumulated to much higher levels in muscle tissues. Repeated disturbance and short-term exposure to air over 3 h induced no accumulation of D- or L-lactate and no change in [Ca^{2+}], [Mg^{2+}], pH and O_2-binding properties of the native haemolymph.

The haemolymph showed a low Hc-O_2 affinity, a large reverse Bohr effect and marked cooperativity. Dialysis increased Hc-O_2 affinity, obliterated cooperativity and decreased the pH-sensitivity of O_2 binding. Replacing Mg^{2+} and Ca^{2+} restored the native O_2-binding properties and the reverse Bohr shift. L- and D-lactate exerted minor modulatory effects on O_2-affinity. At *in vivo* concentrations of Mg^{2+} and Ca^{2+}, the cooperativity is dependent largely on Mg^{2+}, which modulates the O_2 association equilibrium constants of both the high-affinity (K_R) and the low-affinity (K_T) states (increasing and decreasing, respectively). This allosteric mechanism contrasts with that encountered in other haemocyanins and haemoglobins. The functional properties of *H. iris* haemocyanin suggest that high rates of O_2 delivery to the tissues are not a priority but are consistent with the provision of a large O_2 reserve for facultatively anaerobic tissues during internal hypoxia associated with clamping to the substratum.

Key words: gastropod, mollusc, *Haliotis iris*, haemocyanin, oxygen-binding, Bohr effect, ionic effector, association constant, calcium, magnesium, lactate, tauropine.

Introduction

The New Zealand blackfoot abalone (*Haliotis iris*; also known by the Maori name, paua) belongs to an ancient morphologically conservative group, the Archaeogastropoda (Andrews, 1988). Blood concentrations of the oxygen-transporting protein haemocyanin (Hc) show remarkably high individual variability in *Haliotis* species (Pilson, 1965; Ainslie, 1980a) (H. H. Taylor and J. E. Taylor, unpublished data for *H. iris*). Nevertheless, oxygen-binding studies suggest that the pigment plays a quantitatively important role, transporting more than 80 % of the O_2 delivered to the tissues (Ainslie, 1980b). Ultrastructural, immunocytochemical and cDNA hybridisation studies strongly implicate rhogocytes (pore cells), located in connective tissues of the mantle, digestive gland and foot, as the site of Hc biosynthesis in *Haliotis* spp. and other gastropods (Sminia and Boer, 1973; Sminia and Vlugt van Dalen, 1977; Hazprunar, 1996; Taylor and Anstiss, 1999; Albrecht et al., 2001).

As in other gastropods, the Hc of *Haliotis* exists primarily as a didecamer of approximately 8 MDa with a hollow cylindrical quaternary structure, comprising 20 subunits of approximately 400 kDa each, arranged as two end-to-end ring-shaped decamers. However, variable proportions of decamers and multidecamers are also present (van Holde and Miller, 1995; Söhngen et al., 1997; Harris et al., 2000). Each subunit is folded into eight functional units of approximately 50 kDa (Ellerton and Lankovsky, 1983; Ellerton et al., 1983; Keller et al., 1999), each reversibly binding one O_2 molecule at a binuclear copper site (van Holde and Miller, 1995). *H. tuberculata* Hc exists in two isoforms (HtH1 and HtH2) (Keller et al., 1999; Lieb et al., 1999, 2000; Harris et al., 2000; Meissner et al., 2000), which correspond immunologically to the much-studied KLH1 and KLH2 isoforms of another archaeogastropod, the keyhole limpet *Megathuria crenulata* (Gebauer et al., 1994; Söhngen et al., 1997). Considerable progress has been made in elucidating

the structure and sequence of molluscan Hcs and their genes, including those of *H. tuberculata*, and the evolutionary implications of these studies are currently of great interest (e.g. Gebauer et al., 1994; Cuff et al., 1998; Keller et al., 1999; Meissner et al., 2000; Decker and Terwilliger, 2000; Lieb et al., 2001; Van Holde et al., 2001).

The Hcs of marine gastropods, including *Haliotis* spp., commonly exhibit a pronounced reverse Bohr shift (O_2 affinity increases with falling pH) in the physiological pH range (Brix et al., 1979, 1990; Ainslie, 1980b; Petrovich et al., 1990; Wells et al., 1998). A specific adaptive advantage of the reversed Bohr shift has been proposed in relation to salinity acclimation (*Buccinum undatum*) (Brix and Lomholt, 1981) and to dormancy (*Otala lactea*) (Barnhart, 1986), but it is unclear what factors led to the appearance of this property in early gastropods. In the highly aerated sea water inhabited by these animals, the shift would tend to compromise both O_2 loading at the body surface and unloading in the tissues. Intriguingly, the reverse Bohr effect is present in shelled snails but not in other gastropods, and Redmond (1968) hypothesised that it may be related to internal conditions during their defensive withdrawal into the shell. As yet, such conditions have been poorly documented. In *Haliotis* spp., withdrawal consists of clamping to the substratum by contraction of the massive right adductor muscle of the shell. Both the adductor muscle and the large foot muscle are facultatively anaerobic tissues, and during functional and environmental hypoxia they accumulate D-lactate and the uncommon opine tauropine (Gäde, 1988; Baldwin et al., 1992; Wells and Baldwin, 1995). L-Lactate is an important modulator of oxygen binding to crustacean Hc (Truchot, 1980; Bridges et al., 1984; Morris and Bridges, 1986; Lallier and Truchot, 1989), but it is not known whether lactate or tauropine plays a role in oxygen delivery in *Haliotis* spp. Indeed, it is unclear whether these products enter the haemolymph or are retained intracellularly.

Besides pH effects, inorganic ions and CO_2 may modulate the Hc-O_2 affinity and cooperativity of O_2 binding (expressed, respectively, as the half-saturation O_2 tension, P_{50}, and Hill's cooperativity coefficient, n_{50}), often in a temperature-dependent manner (Spoek et al., 1964; Mangum and Lykkeboe, 1979; Brix et al., 1990; Mikkelsen and Weber, 1992; Wells et al., 1998). However, compared with haemoglobins (Hbs), much less is known about the allosteric control of O_2 affinity of Hcs, and such information is unavailable for Hcs that show a reverse Bohr shift. In vertebrate Hbs, anionic organic phosphates and protons lower O_2 affinity by decreasing the O_2 association equilibrium constant of the Hb in the low-affinity, tense state (K_T) (Tyuma et al., 1971, 1973; Weber et al., 1987). In contrast, in the giant extracellular Hbs of annelids, inorganic cations and protons modulate the O_2 association equilibrium constant of the high-affinity, relaxed state (K_R) (Weber, 1981), and in the extracellular Hb of the pulmonate snail *Biomphalaria glabrata* protons bind preferentially to the oxygenated Hb, decreasing K_R, whereas cations bind preferentially to the deoxygenated Hb, increasing K_T (Bugge and Weber, 1999). In the Hc of the shrimp *Callianassa californiensis*, Mg^{2+} increases O_2 affinity by increasing both K_R and K_T, exerting a more pronounced effect on the latter (Miller and van Holde, 1974).

In this paper, we report changes in the composition of the arterial, venous and intramuscular haemolymph of *H. iris* during environmental hypoxia associated with prolonged emersion and modest functional hypoxia associated with repeated disturbance. To elucidate further the respiratory function of *H. iris* Hc and the underlying allosteric mechanisms, we measured the effects of divalent cations and of lactate in relation to the Bohr shift and cooperativity.

Materials and methods

Animals

Male and female specimens of *Haliotis iris* Gmelin (approximately 200–400 g including shell) were collected around Akaroa and Lyttelton Harbours, Banks Peninsula, New Zealand, maintained in a recirculated seawater system at 15 °C, on a 12 h:12 h photoperiod, and fed with commercial abalone food (AbFeed, Sea Plant Production Ltd, South Africa). Animals were acclimated to the system for at least 1 week and deprived of food for 2–3 days before experiments.

Effects of emersion (environmental hypoxia) on haemolymph and muscle metabolite levels

Two series of 14 animals were cannulated for sampling of either the aortic haemolymph or the interstitial haemolymph from the lacunae of the main (right) adductor muscle and transferred to 1 l containers supplied with flowing sea water from the recirculated system. After 24 h, one series (control) was left undisturbed and the other series (emersed) was drained of water without disturbing the animals. After a further 24 h, haemolymph samples (1 ml) were removed from the aortic cannula and from the pedal sinus (using a 23 gauge needle and syringe, accessed from the ventral surface of the foot in the anterior midline), followed quickly by foot and adductor muscle samples (0.5–1.0 g), from both groups. Haemolymph samples were treated immediately with 0.1 ml (1:10 v:v) of ice-cold 6 mol l^{-1} perchloric acid (PCA) and centrifuged at 13 000 ***g*** for 5 min; the supernatant was frozen and stored until assayed for lactate and tauropine.

Muscle tissue samples were immediately freeze-clamped in liquid nitrogen and stored under liquid nitrogen until processed. Aortic cannulae, placed through a window in the shell, consisted of a short length of 23-gauge needle attached to several centimetres of plugged PE tubing. Adductor muscle haemolymph was aspirated from fluid which accumulated in a well (5 mm diameter and 10 mm deep) accessed *via* a glass tube glued into a hole in the shell drilled through the centre of the muscle insertion (N. L. C. Ragg and H. H. Taylor, unpublished method). The well was cleared 1 h before collection commenced and, in some cases, gentle suction was applied to obtain sufficient sample.

Thirteen animals in each series were sampled for both foot and adductor muscle tissues. Six of these in each series were

also matched with adductor muscle haemolymph samples. Six animals in each series were sampled for both aortic and pedal blood, of which five were matched with simultaneous muscle tissue samples.

Effects of handling disturbance on haemolymph composition and oxygen binding

Two series of seven animals (control, disturbed) were acclimated as above (without cannulation). Control animals were removed from their containers once to quickly take a single blood sample (4–5 ml) from the cephalic arterial sinus (CAS; accessed anteriorly at the angle between the foot and the head), blotted dry and weighed. Disturbed animals were blotted and weighed (involving 2–3 min of handling and air exposure) at 30 min intervals, with a final blood sampling (4–5 ml) from the CAS at 3 h. Individual samples were centrifuged, a small sub-sample was removed for lactate determination and to measure Hc absorbance at 346 nm, and the remainder was frozen at –80 °C and air-freighted on dry ice to Denmark for O_2-binding studies. For both groups, a second blood sample (0.5–1.0 ml) was taken from the CAS for P_{O_2} and pH determination.

Measurements of Hc concentration, pH, P_{O_2}, and concentrations of D- and L-lactate, tauropine, Ca^{2+} and Mg^{2+}

Haemocyanin concentrations were estimated from absorbances at 346 nm (Uvikon 860 spectrophotometer) of fresh, centrifuged haemolymph diluted 10-fold in aerated buffer (glycine 50 mmol l^{-1}, EDTA 10 mmol l^{-1}, pH 8.8). These values were converted to mmol l^{-1} HcO_2 functional units using a practical extinction coefficient ($E_{mM,1cm}$=11.42±0.17, mean ± S.E.M., N=90), uncorrected for residual scattering, derived from copper analysis (H. H. Taylor, J. W. Behrens and N. Fawzi, unpublished data).

D- and L-lactate and tauropine concentrations in the haemolymph and muscle samples were measured enzymatically. PCA-treated hemolymph samples were thawed and assayed directly. Approximately 0.5 g of the freeze-clamped muscle tissue was weighed, crushed under liquid nitrogen, then homogenised in 5 ml of 0.6 mol l^{-1} PCA. The PCA extracts were centrifuged as above, and the supernatants were neutralised with 5 mol l^{-1} K_2CO_3. After standing on ice for 1 h, the precipitated potassium perchlorate was removed by centrifugation, and the supernatants were stored frozen until assayed for lactate and tauropine. Metabolites were assayed spectrophotometrically following modification of the methods of Gutmann and Wahlfield (1974) and Engel and Jones (1978). Assay mixtures contained 875 µl of buffer (glycine, 333 mmol l^{-1}; hydrazine sulphate, 133 mmol l^{-1}; EDTA, 10 mmol l^{-1}; pH 9.0), 100 µl of 50 mmol l^{-1} NAD^+, 25 µl of PCA extract and approximately 10 i.u. of D-lactate dehydrogenase (Sigma), L-lactate dehydrogenase (Sigma) or tauropine dehydrogenase. The latter was purified from abalone adductor muscle by ion-exchange chromatography (Gäde, 1987) as modified by Baldwin et al. (1992).

In the disturbance trials, D- and L-lactate were measured in neutralized deproteinized haemolymph samples (100 µl of sample plus 200 µl of ice-cold 1 mol l^{-1} PCA and 67 µl of 3 mol l^{-1} KOH) using a test kit (Boehringer Mannheim no. 1 112 821). The pH of the glycyl-glycine buffer was reduced from 10 to 9, and 0 mmol l^{-1} EDTA was added to reduce interference by trace metals, as above (Engel and Jones, 1978). After centrifugation, 200 µl sub-samples of the supernatants were used in the assay. All determinations were made in duplicate, and appropriate controls were run for non-specific activity. The detection limit for lactate and tauropine in blood and tissue samples was approximately 0.1 mmol l^{-1}.

P_{O_2} was measured using a Clarke-type O_2 electrode (model 1302, Strathkelvin) housed in a microcell (MC100), thermostatted at 15 °C and calibrated using room air and sodium sulphite solution (for zero P_{O_2}). pH was measured using a flat-tipped pH electrode (Activon AEP332) in a specially constructed thermostatted microcell calibrated with BDH Colorkey Buffers. Measurements were performed within 2 min of sampling.

In vivo concentrations of Ca^{2+} and Mg^{2+} were assayed on individual blood samples, diluted 20-fold, using ICP emission spectrometry (Perkin-Elmer).

O_2-binding measurements of native haemolymph

Samples of 130 µl of haemolymph from individual animals were stored at –80 °C and freshly thawed. Samples with varying pH were prepared by adding 1 mol l^{-1} Bis-Tris buffers to a final buffer concentration of 0.1 mol l^{-1}. O_2 equilibria of 4–6 µl haemolymph samples were recorded using a modified gas diffusion chamber fed by cascaded Wösthoff gas-mixing pumps (Bochum, Germany) that produce stepwise increases in O_2 tension by mixing air with ultrapure (>99.998 %) N_2 (Weber, 1981; Weber et al., 1987) while absorbance was recorded continuously. Measurements of pH were carried out in parallel on 100 µl sub-samples using a BMS 2 Mk 2 microelectrode coupled to a PHM 64 Research pH meter (Radiometer, Copenhagen). All measurements were carried out at 15 °C. For each O_2-binding curve, at least four equilibrium steps between 20 and 80 % saturation were recorded, and P_{50} and n_{50} values were interpolated from Hill plots {$\log[S/(1–S)]$ *versus* $\log P_{O_2}$, where S is the fractional O_2 saturation and P is the O_2 tension}.

Effects of Ca^{2+}, Mg^{2+}, D-lactate and L-lactate on O_2 binding

Unused fractions of native haemolymph samples taken from the 14 animals used to investigate the effects of handling were pooled and dialysed against Tris buffer to remove possible cofactors (‘stripped’ Hc). Dialysis was carried out using Spectra/Por 2.1 semi-permeable tubing (molecular mass cut-off 15 000 Da; Biotech regenerated cellulose dialysis membranes) at 4 °C for 24 h against three changes of 0.02 mol l^{-1} Tris buffer with 0.1 mol l^{-1} NaCl (pH 7.75). The individual and combined effects of Ca^{2+}, Mg^{2+} and D- and L-lactate on O_2 binding were examined by adding 1 mol l^{-1} $CaCl_2$, 1 mol l^{-1} $MgCl_2$ and 0.5 mol l^{-1} of either D- or L-lactate, singly or in combination, to obtain final concentrations of

Table 1. *Concentrations of the anaerobic products tauropine, D-lactate and L-lactate in the haemolymph and in muscle tissue in* Haliotis iris *settled in water or emersed in air for 24 h at 15 °C*

	Water					Air				
	Haemolymph (mmol l^{-1})			Tissue (mmol kg^{-1} fresh mass)		Haemolymph (mmol l^{-1})			Tissue (mmol kg^{-1} fresh mass)	
	Aorta	Pedal sinus	Adductor haemolymph	Adductor muscle	Foot muscle	Aorta	Pedal sinus	Adductor haemolymph	Foot muscle	Adductor muscle
Tauropine	0.25±0.09 (6)	0.33±0.05 (6)	0.38±0.05 (6)	0.78±0.24 (13)	0.42±0.19 (13)	0.82±0.27 (6)	1.58±0.47 (6)	0.58±0.13 (6)	5.69±0.76 (13)	5.45±0.71 (13)
D-Lactate	0.12±0.04 (6)	0.02±0.01 (6)	0.47±0.15 (6)	0.35±0.14 (13)	0.39±0.137 (13)	1.69±0.28 (6)	1.88±0.35 (6)	1.74±0.22 (6)	1.75±0.20 (13)	1.59±0.26 (13)
L-Lactate	0.11±0.04 (3)	0.08±0.02 (3)	0.06±0.01 (3)	–	–	0.11±0.06 (6)	0.05±0.01 (6)	0.31±0.12 (2)	0.01±0.01 (4)	0.02±0.01 (4)

Values are means ± S.E.M. (*N*).
The detection limit for these assays is approximately 0.1 mmol l^{-1}.

10 mmol l^{-1} Ca^{2+}, 50 mmol l^{-1} Mg^{2+} and 5 and 10 mmol l^{-1} of the respective lactate forms.

Precise O_2 equilibrium measurements for extended Hill plots

Precise measurements emphasizing extreme (low and high) O_2 saturations (Weber, 1981) were carried out using dialysed haemolymph that had been concentrated 10-fold by centrifugation at 2600 ***g*** and 4 °C for 15 h in Ultrafree-4 Millipore tubes (with 30 kDa molecular mass cut-off membranes). The data were analysed in terms of the two-state Monod–Wyman–Changeux (MWC) model, according to the equation:

$$S = [LK_TP(1 + K_TP)^{q-1} + K_RP(1 + K_RP)^{q-1}]/[L(1 + K_TP)^q + (1 + K_RP)^q],$$

where S denotes O_2 saturation, L is the equilibrium constant between the tense (T) and relaxed (R) states in the fully deoxygenated form, K_T and K_R are the association equilibrium constants for the low-affinity (T, tense) and high-affinity (R, relaxed) forms, respectively, P is the partial pressure of O_2 and q is the number of interacting binding sites (Monod et al., 1965). Curve-fitting to obtain K_T, K_R and the allosteric constant L, estimation of the standard errors and calculation of the derived parameters P_{50}, P_m (the median O_2 tension), n_{50}, n_{max} (the maximum cooperativity along the equilibrium curve) and ΔG (the free energy of cooperativity) were carried out as detailed previously (Weber et al., 1995).

Statistical analyses

Statistical differences were assessed using Student's *t*-tests, assigning statistical significance at $P<0.05$.

Results

Effects of emersion

Tissue D-lactate and tauropine concentrations in the foot and adductor muscles of abalones settled in water were low (mean values 0.4–0.8 mmol kg^{-1} fresh mass) and were below the detection limit in approximately one-third of cases (Table 1) (Fig. 1). Significant haemolymph concentrations of D-lactate were present only in the adductor haemolymph samples, but tauropine levels generally similar to tissue values were recorded in haemolymph from all three sites. After 24 h of air exposure, D-lactate and tauropine concentrations were elevated in all haemolymph and tissue samples. D-Lactate was present at approximately similar concentrations in haemolymph and tissue samples (mean values approximately 1.7 mmol l^{-1}/mmol^{-1} kg^{-1}, ranging up to 2.6–2.7 mmol l^{-1}/mmol^{-1} kg^{-1}, respectively). Tauropine concentrations, although highly variable, were higher than the D-lactate values in both foot and adductor tissues (mean values approximately 5.5 mmol kg^{-1}, ranging up to 11.4 mmol kg^{-1}) but lower in the haemolymph

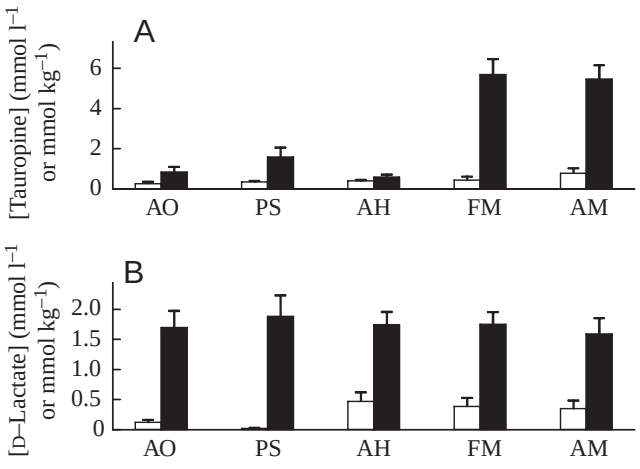

Fig. 1. The concentrations of tauropine (A) and D-lactate (B) in the haemolymph (AO, aorta; PS, pedal sinus; AH, adductor haemocoel; mmol l^{-1}) and the tissues (FM, foot muscle; AM, adductor muscle; mmol kg^{-1} fresh mass) in *Haliotis iris* settled in water (open columns) or emersed in air for 24 h (filled columns) at 15 °C. Values are means + S.E.M.; see Table 1 for *N* values.

Table 2. *Effects of handling disturbance on the concentrations of Ca^{2+}, Mg^{2+} and haemocyanin and on pH and P_{O_2} in the haemolymph of* Haliotis iris

	[Ca^{2+}] (mmol l^{-1})	[Mg^{2+}] (mmol l^{-1})	pH	P_{O_2} (mmHg)	[Haemocyanin]* (functional units)	[D- or L-lactate] (mmol l^{-1})
Control	12.96±0.40	48.78±2.85	7.02±0.03	20.3±6.4	0.21±0.03	ND
Experimental	12.87±0.57	49.34±2.12	6.83±0.28	16.1±4.9	0.23±0.02	ND

Values are means ± S.E.M. (N=7), except for P_{O_2}, where N=5.
*Functional units, based on 2Cu per unit.
ND, not detected.
1 mmHg=0.133 kPa.

samples (0.6–1.6 mmol l^{-1}, highest value 2.8 mmol l^{-1} in the pedal sinus). L-Lactate was absent from the haemolymph and tissues of both resting and air-exposed animals (Table 1).

A simple index of the different distributions of D-lactate and tauropine was obtained from the ratio of their haemolymph concentration (either the mean of the aortic and pedal samples or the adductor haemolymph value, mmol l^{-1}) to the tissue concentration (mean value for foot and adductor muscle) for the 11 animals for which matched data were available. These ratios were 1.28±0.23 for D-lactate and 0.20±0.06 for tauropine. The difference is highly significant (P=0.0004, paired t-test, two-tailed).

Effects of handling disturbance

Haemolymph Ca^{2+} and Mg^{2+} concentrations were almost identical in the control (undisturbed) and disturbed (3 h-handled) groups (Table 2). The pH was slightly, but not significantly, higher in the control group, both groups exhibiting near-neutral values (7.02 and 6.83, respectively). P_{O_2} values in the cephalic arterial sinus were variable and did not differ in the control and disturbed groups (20.3 and 16.1 mmHg, respectively; 1 mmHg=0.133 kPa), as also was the case with Hc concentrations (0.21 mmol l^{-1} and 0.23 mmol l^{-1}, respectively) (see Table 2). Neither D- nor L-lactate was detected in the blood of either group.

Native haemolymph from both control and disturbed animals showed a high O_2 affinity (P_{50}=3.9 and 4.0 mmHg, respectively) and slight cooperativity (n_{50}=1.1 and 1.2, respectively) at pH 6.9 (Table 3). Increasing the pH to 7.7 significantly decreased O_2 affinity, with P_{50} increasing to 11.6 and 12.2 mmHg for the control and disturbed groups, respectively. Cooperativity was also pH-sensitive, increasing with rising pH to 1.6 and 1.4, respectively. These data show a large reverse Bohr effect ($\varphi=\Delta\log P_{50}/\Delta pH$=+0.65 for the control group, and +0.68 for the disturbed group; Table 3). On the basis of the similar O_2-binding parameters (and identical haemolymph ion concentrations), the overall mean values were used for comparison with dialysed samples.

Table 3. *Effects of handling disturbance on the O_2-binding characteristics of the native haemolymph of* Haliotis iris *at 15 °C*

	pH	P_{50} (mmHg)	n_{50}	Bohr factor φ
Control	6.94±0.01	3.85±0.19	1.12±0.11	
	7.69±0.01	11.55±1.62	1.63±0.26	0.65±0.05
Experimental	6.94±0.00	4.02±0.63	1.18±0.05	
	7.70±0.01	12.19±1.30	1.44±0.22	0.68±0.04

Values are means of four individuals ± S.E.M.
1 mmHg=0.133 kPa.
P_{50}, half-saturation oxygen tension; n_{50}, Hill's cooperativity coefficient.

Effects of Ca^{2+}, Mg^{2+}, D-lactate and L-lactate

The effects of divalent cations and lactate are illustrated in Figs 2 and 3 and Table 4. Dialysis of the Hc increased O_2 affinity (decreased P_{50} values from 11.9 to 4.5 mmHg at pH 7.7 and from 3.9 to 3.2 mmHg at pH 7.0). The greater effects at high pH resulted in a marked reduction of the reverse Bohr effect (φ falling from +0.66 to +0.21). Dialysis also reduced n_{50} (from 1.5 to 1.0 and from 1.3 to 1.1 at pH 7.7 and 7.0, respectively).

This pronounced effect of dialysis on O_2-binding properties prompted an examination of the effects of 10 mmol l^{-1} Ca^{2+} and 50 mmol l^{-1} Mg^{2+} (which approximate the *in vivo* values)

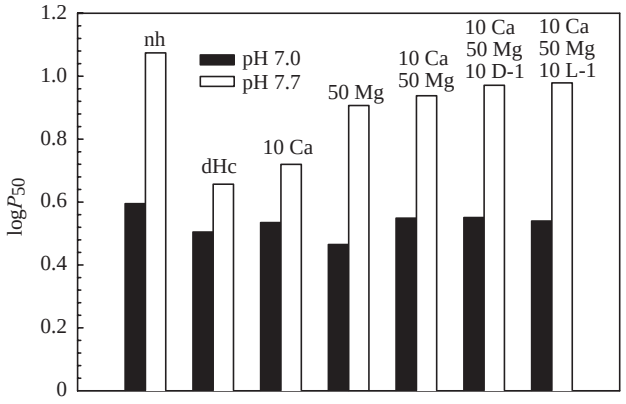

Fig. 2. The effects of dialysis against various ions on the O_2-affinity (logP_{50}) of *Haliotis iris* haemocyanin at 15 °C, pH 7.0 (filled columns) and pH 7.7 (open columns). Labels above the columns refer to the concentrations (mmol l^{-1}) of Ca^{2+} (Ca), Mg^{2+} (Mg), D-lactate (D-l), and L-lactate (L-l). nh, native haemolymph; dHc, dialysed haemocyanin without added cofactor.

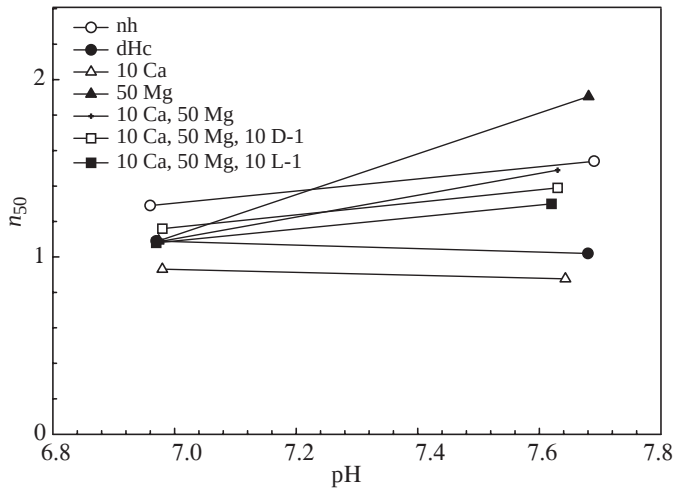

Fig. 3. The effects of various ions on the cooperativity (n_{50}) of *Haliotis iris* haemocyanin O_2-binding at 15 °C. Labels in the legend refer to the concentrations (mmol l^{-1}) of Ca^{2+} (Ca), Mg^{2+} (Mg), D-lactate (D-l), and L-lactate (L-l). nh, native haemolymph; dHc, dialysed Hc without added cofactor.

and lactate. The addition of 10 mmol l^{-1} Ca^{2+} decreased the affinity at both high and low pH, and also affected the reverse Bohr factor, which increased to +0.28. P_{50} changed more at pH 7.6, increasing from 4.5 mmHg (intrinsic affinity) to 5.3 mmHg, than at pH 7.0, and cooperativity became slightly negative (n_{50}=0.9 at both pH values). The presence of Mg^{2+} (50 mmol l^{-1}) markedly decreased O_2 affinity at pH 7.7 (P_{50}=8.1 mmHg), such that the pH-sensitivity of O_2 binding and cooperativity were regained (φ=+0.62 and n_{50}=1.9; see Table 4). A small increase in O_2-binding affinity was seen at pH 7.0 (P_{50}=2.9 mmHg).

The effects of both D- and L-lactate on O_2 binding of dialysed Hc were investigated at low pH (assumed to favour binding of the negatively charged ion to positively charged sites on the Hc molecule). Neither D- nor L-lactate (5 mmol l^{-1}) changed the affinity (at pH 7.0, P_{50}=3.0 and 3.3 mmHg, respectively), although cooperativity was slightly decreased in both cases (n_{50}=0.9 and 1.0, respectively).

To determine the possible contributions of Ca^{2+} and Mg^{2+} to Hc-O_2 affinity *in vivo*, the combined effects of 10 mmol l^{-1} Ca^{2+} and 50 mmol l^{-1} Mg^{2+} were measured and were found to lower O_2 affinity more than Mg^{2+} alone, P_{50} increasing to 3.5 and 8.7 mmHg at pH 7.0 and 7.7, respectively. The Bohr factor was high (+0.60) and virtually unchanged compared with that with 50 mmol l^{-1} Mg^{2+} (+0.62). Curiously, Ca^{2+} exerted the opposite effect compared with Mg^{2+} on cooperativity at pH 7.6, decreasing n_{50} to 1.5 (compared with 1.9 with 50 mmol l^{-1} Mg^{2+}). The effects of 10 mmol l^{-1} D- or L-lactate in the presence of 10 mmol l^{-1} Ca^{2+} plus 50 mmol l^{-1} Mg^{2+} were also investigated. The additive effect of D-lactate was pH-sensitive; P_{50} increased only at pH 7.6 (to 9.4 mmHg), resulting in a small augmentation of the Bohr factor (to +0.65). A similar pattern was found for L-lactate: P_{50} increased to 9.5 mmHg at pH 7.6 and was largely unaffected at pH 7.0 (3.5 mmHg), such that the reverse Bohr effect remained high (+0.68) and n_{50} decreased slightly (to 1.30) at high pH.

Extended Hill plots

Precise O_2 equilibrium measurements in the absence and presence of Mg^{2+} were made at pH 7.7, where this modulator exerted a pronounced effect on the cooperativity of dialysed samples. As no cooperativity was observed in the dialysed Hc (Fig. 4) it was impossible to fit the MWC model to the data. Instead, P_{50} and n_{50} were determined from the linear regression

Table 4. P_{50}, n_{50} *and* φ *values for* Haliotis iris *haemolymph and dialysed haemocyanin in the absence and presence of* Ca^{2+}, Mg^{2+} *and* D- *or* L-*lactate at the indicated concentrations and 15 °C*

	pH	[Ca^{2+}] (mmol l^{-1})	[Mg^{2+}] (mmol l^{-1})	[D-lactate] (mmol l^{-1})	[L-lactate] (mmol l^{-1})	P_{50} (mmHg)	n_{50}	Bohr factor, φ
Pooled native	6.96	0	0	0	0	3.94	1.29	
Pooled native	7.69	0	0	0	0	11.87	1.54	0.657
Dialysed	6.97	0	0	0	0	3.20	1.09	
Dialysed	7.68	0	0	0	0	4.54	1.02	0.212
Dialysed	6.98	10	0	0	0	3.43	0.93	
Dialysed	7.64	10	0	0	0	5.25	0.88	0.277
Dialysed	6.97	0	50	0	0	2.92	1.09	
Dialysed	7.68	0	50	0	0	8.07	1.90	0.624
Dialysed	6.95	0	0	5	0	2.98	0.87	
Dialysed	6.95	0	0	0	5	3.30	0.97	
Dialysed	6.98	10	50	0	0	3.54	1.09	
Dialysed	7.63	10	50	0	0	8.68	1.49	0.600
Dialysed	6.98	10	50	10	0	3.56	1.16	
Dialysed	7.63	10	50	10	0	9.35	1.39	0.645
Dialysed	6.97	10	50	0	10	3.47	1.08	
Dialysed	7.62	10	50	0	10	9.53	1.30	0.675

P_{50}, Half-saturation oxygen tension; n_{50}, Hill's cooperativity coefficient.

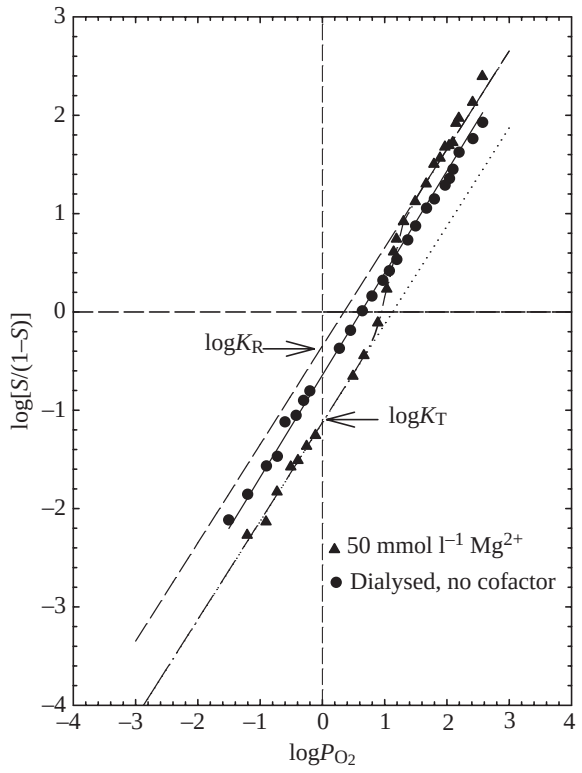

Fig. 4. Hill plot for dialysed haemocyanin of *Haliotis iris* measured in the absence (circles) and in the presence (triangles) of 50 mmol l^{-1} Mg^{2+}, in 0.1 mol l^{-1} Bis-Tris buffer at pH 7.7 and 15 °C. K_T and K_R are estimates of O_2 association equilibrium constants (mmHg^{-1}) for the low-affinity (T, tense) and high-affinity (R, relaxed) forms, respectively. *S*, fractional oxygen saturation.

to be 4.3 mmHg and 1.0, respectively. With K_T=K_R=1/P_{50}, the corresponding association constant is 0.23 mmHg^{-1} (Table 5). The addition of 50 mmol l^{-1} Mg^{2+} altered the affinities of both the tense and relaxed forms of the Hc molecules, the greatest effect being seen on K_T, which decreased to 0.075 mmHg^{-1}, while K_R increased to 0.449 mmHg^{-1}. The number of interacting O_2-binding sites (*q*) providing the best possible fit was 15.48±2.17; mean ± S.E.M.) (Table 5). The high *q* value together with a very high value of *L* reflect the narrow P_{O_2} range in which the molecule shifts from the tense to the relaxed state (Fig. 4). The Mg^{2+}-induced variations in K_T and K_R were also obtained from analyses with *q* fixed at multiples of 8 (8, 16, 24 and 32, respectively; data not shown), and similar results were obtained. The P_m value was lower than the P_{50} (7.2 mmHg compared with 8.9 mmHg), and n_{max} was higher than n_{50} (3.3 compared with 2.7), reflecting asymmetry of the O_2-binding curves. The free energy of cooperativity Δ*G* was 4.291 kJ mol^{-1} at pH 7.7.

Discussion

Haemolymph and muscle lactate and tauropine levels

The accumulation of D-lactate and tauropine in the muscle tissue of abalone during environmental and functional hypoxia has been reported previously and has been discussed in terms of the differing strategies available for anaerobic energy production among molluscs (Wieser, 1981; Fields, 1983; Sato et al., 1985; Wijsman, 1985; Gäde, 1988). The elevation of D-lactate levels has also been observed in the blood of pulmonate gastropods during periods of anoxia (Wieser, 1981; Wijsman, 1985), but we believe that this is the first demonstration of the presence of D-lactate in the blood of an archaeogastropod and the first published report of the presence of tauropine in molluscan blood. Similar observations have been made previously for the haemolymph of *Haliotis rubra* (J. P. Elias, unpublished data). The blood:tissue ratios (0.20 for tauropine, 1.28 for D-lactate) in emersed animals are uncorrected for tissue dry matter and haemolymph content. Nevertheless, they imply a rather rapid equilibration of D-lactate between the intracellular and extracellular pools and a greater retention of tauropine intracellularly. Some caution must be exercised: although the foot and adductor muscles account for a large proportion of the body mass, the release (and uptake) of these products at other sites is likely, and further studies along these lines should aim to quantify this.

Regulation of the O_2-binding properties of Haliotis iris *haemocyanin*

The respiratory adaptations of gastropod Hc are poorly understood compared with those in arthropods. Adaptations in Hc function to different conditions may occur *via* changes in the pigment concentration, changes in its intrinsic structural

Table 5. *Effect of 50 mmol l^{-1} Mg^{2+} on MWC and derived parameters for dialysed* Haliotis iris *haemocyanin at 15 °C*

	pH	P_{50} (mmHg)	P_m (mmHg)	n_{50}	n_{max}	K_T (mmHg^{-1})	K_R (mmHg^{-1})	log*L*	Δ*G* (kJ mol^{-1})	*q*
No cofactor	7.69	4.34	–	0.98	–	0.231	0.231	–	–	–
50 mmol l^{-1} Mg^{2+}	7.58	8.89	7.17	2.73	3.31	0.075±0.004	0.449±0.023	7.86	4.291	15.48

MWC, Monod–Changeux–Wyman model; n_{max}, maximum cooperativity along the equilibrium curve; P_m, median O_2 tension; K_T, O_2 association equilibrium constant of the low-affinity state of haemocyanin; K_R, O_2 association equilibrium constant of the high-affinity state of haemocyanin; *L*, equilibrium constant between the tense and relaxed states in fully deoxygenated haemocyanin; Δ*G*, free energy of cooperativity; *q*, number of interacting binding sites; n_{50}, Hill's cooperativity coefficient; P_{50}, half-saturation O_2 tension.

1 mmHg=0.133 kPa.

Values for K_T, K_R and Δ*G* are means ± S.E.M.

and functional properties or changes in the type and concentration of 'cofactor' molecules that modulate its O_2-binding properties. Unlike vertebrates, in which organic phosphates are potent regulators of Hb-O_2 affinity, the affinity of invertebrate pigments is mainly dependent on inorganic cations (Truchot, 1975; Mangum and Lykkeboe, 1979). In contrast to crustacean Hc, in which Mg^{2+} and Ca^{2+} increase O_2 affinity (e.g. in the shore crab *Carcinus maenas*) (Truchot, 1975), these cations decrease O_2 affinity in *Haliotis* Hc. The O_2 affinity of some crustacean Hcs is, moreover, increased by L-lactate and urate (Truchot, 1980; Morris and Bridges, 1986).

The present data for *H. iris* Hc illustrate new modes of effector modulation of O_2 affinity. Removal of divalent cations through dialysis increased affinity and obliterated cooperativity (Figs 2, 3). In addition, the pH-sensitivity of O_2 binding (Bohr effect) was markedly reduced. The addition of 50 mmol l^{-1} Mg^{2+}, which approximates the *in vivo* value, at pH 7.7 (which falls within the physiological range in marine gastropods) (Mangum and Shick, 1972; Brix et al., 1979; Mangum and Lykkeboe, 1979) almost fully restored blood P_{50} and even increased cooperativity beyond the level seen in native blood. The small additional effect of Ca^{2+} (at approximately *in vivo* concentrations) further decreased affinity and, curiously, opposed the positive effect exerted by Mg^{2+} on cooperativity, resulting in the O_2-binding properties seen in the native blood (Figs 2, 3).

Evidently, the *in vivo* P_{50} is strongly dependent on the Mg^{2+} concentration. That the difference in the effects of Ca^{2+} and Mg^{2+} does not appear to be due to ion-specific binding by the Hc molecule, but simply to result from their *in vivo* concentration differences, is suggested by the observation (data not shown) that the two cations exerted similar effects when tested at equivalent concentrations. However, unlike Mg^{2+}, Ca^{2+} did not induce cooperativity even when tested at concentrations far exceeding physiological values (data not shown), indicating a specific cation effect on n_{50} (Fig. 3). The reverse Bohr effect favours O_2 binding at low pH (Brix et al., 1979; Mangum and Lykkeboe, 1979). Protons neutralise the negatively charged binding sites of the Hc molecule, decreasing cation binding and the effect of cations on P_{50} and decreasing n_{50} in the case of Mg^{2+}. The cumulative effects of Mg^{2+} and Ca^{2+} in dialysed blood at low pH resulted in O_2-binding properties comparable with those in native blood.

Crustacean Hcs show highly specific but variable sensitivities to L-lactate (Truchot, 1980; Bridges et al., 1984; Morris and Bridges, 1986; Lallier and Truchot, 1989), which interacts with all four positions of the chiral carbon of the protein, thus explaining the difference in binding compared with D-lactate and other structural analogues (Johnson et al., 1984; Graham, 1985). The Hc of the whelk *Busycon contrarium* was reported to be insensitive to both stereoisomers of lactate (Mangum, 1983, 1992). However, the effect of lactate on the O_2 binding of *Haliotis iris* Hc has not previously been investigated. The almost identical, minor depressant effects of the two isoforms on affinity and n_{50} indicate that the binding sites on *Haliotis iris* Hc cannot distinguish between these organic ions or that the effects reflect a more general anion sensitivity. In any case, it appears that neither form is an important modulator *in vivo*. However, before abandoning the concept of organic modulators of molluscan Hc, the potential role of tauropine should be examined. Unfortunately, tauropine is unavailable commercially, but methods have been published for its synthesis and purification (Sato et al., 1985, 1991).

Allosteric control mechanisms of O_2 affinity

Analyses of the precise O_2 equilibria in *H. iris*, represented in the extended Hill plots (Fig. 4), indicate a new allosteric control mechanism in which Mg^{2+} decreases K_T and also increases K_R, resulting in a sigmoid O_2 equilibrium curve. Hence, it increases the O_2 affinity of the oxygenated (relaxed) state but also (and proportionally more) decreases the affinity of the tense state. This differs from vertebrate Hbs, in which erythrocytic organic phosphates (such as diphosphoglycerate and ATP) and increased proton concentrations decrease O_2 affinity by decreasing K_T (Tyuma et al., 1971, 1973; Weber et al., 1987), annelid extracellular Hbs, in which divalent cations and increased pH increase O_2 affinity by increasing K_R (Weber, 1981; Fushitani et al., 1986), and pulmonate snail (*Biomaphalaria glabrata*) extracellular Hb, in which cations modulate K_T and protons modulate K_R (Bugge and Weber, 1999). While little is known about the control mechanisms in Hcs, the available data indicate that in the Hc of the shrimp *Callianassa californiensis* Mg^{2+} raises both K_T and K_R (Miller and van Holde, 1974), whereas in the Hc of the opisthobranch gastropod *Aplysia limacina* Ca^{2+} decreases K_T and increases K_R (Ghiretti-Magaldi et al., 1979). However, in the latter case, the Hill plots in the presence and absence of Ca^{2+} were measured at different pH values (pH 8.5 and 8.0, respectively), and the effects of cations cannot be separated from those of protons. The effect of Mg^{2+} on *Haliotis iris* Hc is analogous to that of Ca^{2+} on *Limulus polyphemus* Hc, in which this cation appears to enhance the stability of the deoxy conformation (Topham et al., 1998).

Gastropod Hc didecamers each possess 160 O_2-binding sites. Since this very large number of binding sites is not reflected by correspondingly high values of the Hill coefficient, n_H (cf. van Holde and Miller, 1995; van Holde et al., 2000), the value for the number of interacting sites (q) may correspond to the number of O_2-binding sites within the subunits that behave like allosteric entities (van Holde et al., 2000). Accordingly, it may be more appropriate to fit the standard MWC model, fixing q as the number of binding sites per subunit or multiples of these. In *Octopus dofleini*, the Hc dissociates into subunits upon removal of divalent cations at pH 8.0 (Miller, 1985; Connelly et al., 1989). This is also the case for *Sepioteuthis lessioniana* Hc (van Holde et al., 2000), in which the cooperativity expressed in the native decamer is obliterated in the subunits upon removal of divalent cations. Non-cooperativity in isolated subunits seems to be general in molluscan Hcs. In the Hc of the crustacean *Callianassa californiensis*, both Ca^{2+} and Mg^{2+} promote the association of subunits (Roxby et al., 1974; Miller and van Holde, 1974). Is

it then the presence of divalent cations that induces distinct T and R states, or is the observed cooperativity in the presence of cations simply due to preservation of the native aggregated state? Although results with *Sepioteuthis lessoniana* Hc (van Holde et al., 2000) indicate that cooperativity is correlated with the presence of decameric quaternary structure rather than with Mg^{2+} concentration *per se*, this view is not supported by the present results. Thus, if the native aggregated state were the only prerequisite for cooperativity, then addition of either Ca^{2+} or Mg^{2+} would have exerted similar effects in establishing cooperativity. The unique correlation between the presence of Mg^{2+} and cooperativity in *Haliotis iris* Hc (Fig. 3) indicates either that the molecule can distinguish between these two divalent cations or that Ca^{2+} does not promote subunit aggregation.

The K_T/K_R ratio in the presence of Mg^{2+} indicates a sixfold higher affinity for the last compared with the first O_2 molecule bound to the macromolecules. This is a low factor compared with those of 313 (pH 7.4) and 68 (pH 7.4) observed in vertebrate and annelid extracellular Hbs, respectively (Tyuma et al., 1973; Weber, 1981). Similarly, the free energy (per binding site) of interaction between O_2-binding sites (ΔG=4.3 kJ mol^{-1} in the presence of Mg^{2+}) is low compared with that of stripped Hbs (ΔG=8.7 kJ mol^{-1} for human Hb and ΔG=8.4 kJ mol^{-1} for extracellular lugworm Hb) (Tyuma et al., 1973; Weber, 1981). However, given the large number of interacting O_2-binding sites (q=15.48 compared with q=4 in tetramers), the total free energy of interaction between O_2-binding sites may be large. The Hill plot (Fig. 4) indicates that the transition from the tense to the relaxed conformation occurs late in the oxygenation process, at log[S/(1–S)]=–0.2, i.e. approximately 17 % saturation, compared with approximately 8 % in human Hbs (Tyuma et al., 1973), reflecting the high degree of stabilisation of the tense conformation by internal bonds.

Recently, it has been shown that the Hc of *Haliotis tuberculata* exists in two isoforms (HtH1 and HtH2) (Keller et al., 1999; Lieb et al., 1999, 2000; Harris et al., 2000; Meissner et al., 2000). The two isoforms, and their component functional units (*a* to *h*), are all immunologically distinct but correspond respectively to the much-studied KLH1 and KLH2 isoforms of another archaeogastropod, the keyhole limpet *Megathuria crenulata* (Gebauer et al., 1994; Söhngen et al., 1997). The haemolymph concentrations of the different isoforms may vary independently (e.g. during starvation) and also differ in their tendencies to associate into didecamers and multidecamers in the presence of divalent cations. Clearly, future studies must establish the extent to which the properties of *H. iris* Hc reported here relate to one or both isoforms.

Physiological implications of a reverse Bohr and Root shift

Repeated handling and air exposure over a period of 3 h appeared not to affect the haemolymph composition or pH (Table 1). Although the mean pH values are markedly lower than those reported previously for marine gastropods (Mangum and Shick, 1972; Brix et al., 1979; Mangum and Lykkeboe, 1979), they agree well with those of Wells et al. (1998), who found the *in vivo* pH of pedal sinus blood of *H. iris* at 20 °C to be 7.39±0.2 at rest and 6.51±0.3 following 10 min of intensive exercise that caused metabolic acidosis. The fact that the reverse Bohr shift is operative in this pH range implies that O_2 unloading in the relatively acid tissues may be impaired.

Interestingly, the Hc of the gastropod *Buccinum undatum* acclimated to normoxic, high-salinity (35 ‰) water shows a normal Root effect (reduction in O_2 carrying capacity with decreasing pH) compared with a reverse Root effect following exposure to hypoxic conditions or lowered salinity (Brix et al., 1979; Brix, 1982). The shift was attributed to a hyporegulation of the blood ion levels, leading to a lowered Cl^- concentration and a concomitant increase in P_{CO_2} that lowered pH. The associated decrease in Cl^- blockage of the Hc-O_2 binding sites increases the O_2 saturation of the Hc when pH decreases and augments the effective levels of the O_2 carrier in the blood (Brix and Torensma, 1981). The combined effect of the reverse Bohr and Root shift was shown to increase the venous O_2 content significantly under hypoxic conditions, thus compensating for the reduced amount of physically dissolved O_2 in the blood (Brix, 1982).

A reverse Root shift, independent of hypoxia or low salinity, has been demonstrated in the Hc of *H. iris* (Wells et al., 1998). To date, no convincing hypothesis has been presented for its significance in combination with a reverse Bohr shift and associated loss of cooperativity at the lower end of the physiological pH range. The reverse Root shift may ensure an increased O_2-carrying capacity when pH drops, as inferred by Wells et al. (1998), and has been shown to be of importance in *Buccinum undatum*, in which animals adjusted to hypoxic conditions almost regenerate their normoxic O_2 uptake rate (Brix and Lomholt, 1981). In addition, under hypoxic conditions, the large blood volume of *H. iris* (approximately 52 % of wet mass) (Taylor, 1993; Ragg et al., 2000) constitutes a blood O_2 reserve that offers some protection against internal hypoxia. However, an increased affinity and loss of cooperativity at low pH do not necessarily impair O_2 unloading but only shift it to lower critical levels, where aerobiosis may supplement anaerobic metabolism. The pedal and shell adductor enzyme and metabolite profiles provide evidence that *Haliotis* spp. rely heavily on anaerobic metabolism during exercise or environmentally induced hypoxia (Wells et al., 1998); such results are supported by the accumulation of the pyruvate reductase end-products tauropine and D-lactate (Gäde, 1988; Baldwin et al., 1992; Ryder et al., 1994; Wells and Baldwin, 1995).

Concluding remarks

Taken together, the evidence indicates that *Haliotis iris* has a blood O_2-transport system in which high rates of O_2 delivery to the tissues are not a high priority. Consequently, the reverse Bohr and Root shifts do not constitute a serious impediment. Possibly, *H. iris* evolved under circumstances with no demanding need for O_2 delivery under hypoxia. These cold-adapted animals are found in shallow, sub-littoral, O_2-rich

waters of New Zealand's coasts, achieving their greatest abundance and largest size in the colder southern part of the South Island. The adults are mainly sessile and move only slowly when rasping. Although abalone sometimes glide rapidly to a refuge when disturbed, clamping to the substratum is their primary 'escape' response against predators, and they have little capacity for sustained aerobic locomotion. Intense muscle exercise is thus supported by anaerobic glycolysis. The substantial blood O_2 reserve may provide O_2 for facultatively anaerobic tissues when O_2 availability is restricted, as during clamping.

This study was supported by the Danish Natural Science Research Council (Danish Center for Respiratory Adaptation) and the Marsden Fund of New Zealand (Contract UOC 804). We gratefully acknowledge stimulating discussion and practical assistance from Dr John Baldwin (Monash University), Norman Ragg and David Just (University of Canterbury) and Hans Malte and Anny Bang (University of Aarhus).

References

Ainslie, R. (1980a). Haemocyanin concentrations in field populations of three species of southern Australian abalone. *Aust. J. Mar. Freshwater Res*. **31**, 627–633.

Ainslie, R. C. (1980b). The quantitative role of haemocyanin in the respiration of abalone (genus *Haliotis*). *J. Exp. Zool*. **211**, 87–99.

Albrecht, U., Keller, H., Gebauer, W. and Markl, J. (2001). Rhogocytes (pore cells) as the site of hemocyanin biosynthesis in the marine gastropod *Haliotis tuberculata*. *Cell Tissue Res.* **304**, 455–462.

Andrews, E. B. (1988). Excretory systems of molluscs. In *The Mollusca*, vol. 11, chapter 13 (ed. E. R. Truman and M. R. Clarke), pp. 381–448. New York: Academic Press.

Armstrong, D. A., Armstrong, J. L., Krassner, S. M. and Pauley, G. B. (1971). Experimental wound repair in the black abalone, *Haliotis cracherodii*. *J. Invert. Pathol*. **17**, 216–227.

Baldwin, J., Wells, R. M. G., Low, M. and Ryder, J. M. (1992). Tauropine and D-lactate as metabolic stress indicators during transport and storage of live paua (New Zealand abalone) (*Haliotis iris*). *J. Food Sci.* **57**, 280–282.

Barnhart, M. C. (1986). Hemocyanin function in active and dormant land snails. *Physiol. Zool.* **59**, 725–732.

Bridges, C. R., Morris, S. and Grieshaber, M. K. (1984). Modulation of haemocyanin oxygen affinity in the intertidal prawn *Palaemon elegans* (Rathke). *Respir. Physiol*. **57**, 189–200.

Brix, O. (1982). The adaptive significance of the reversed Bohr and Root shifts in blood from the marine gastropod, *Buccinum undatum*. *J. Exp. Zool*. **221**, 27–36.

Brix, O., Condo, S. G., Colosimo, A. and Giardina, B. (1990). The influence of salinity acclimation on the temperature sensitivity of oxygen binding to the haemocyanin of the prosobranch *Neptunea antiqua*. *J. Exp. Biol*. **149**, 417–424.

Brix, O. and Lomholt, J. P. (1981). The dependence of oxygen consumption of the common whelk (*Buccinum undatum*) on hypoxia, salinity and air exposure. *Comp. Biochem. Physiol.* **71A**, 439–443.

Brix, O., Lykkeboe, G. and Johansen, K. (1979). Reversed Bohr and Root shifts in hemocyanin of the marine prosobranch, *Buccinum undatum*: Adaptions to a periodically hypoxic habitat. *J. Comp. Physiol*. **129**, 97–103.

Brix, O. and Torensma, R. (1981). The molecular basis for the reverse Root effect in the blood of the marine prosobranch, *Buccinum undatum*. *Mol. Physiol*. **1**, 209–212.

Bugge, J. and Weber, R. E. (1999). Oxygen binding and its allosteric control in hemoglobin of the pulmonate snail, *Biomphalaria glabrata*. *Am. J. Physiol.* **276**, R347–R356.

Connelly, P. R., Gill, S. J., Miller, K. I., Zhou, G. and van Holde, K. E. (1989). Identical linkage and cooperativity of oxygen and carbon monoxide binding to *Octopus dofleini* hemocyanin. *Biochemistry* **28**, 1835–1843.

Cuff, M. E., Miller, K. L. van Holde, K. E. and Hendrickson, W. A. (1998). Crystal structure of a functional unit from *Octopus* hemocyanin. *J. Mol. Biol*. **278**, 855–870.

Decker, H. and Terwilliger, N. (2000). Cops and robbers: putative evolution of copper oxygen-binding proteins. *J. Exp. Biol.* **203**, 1777–1782.

Ellerton, H. D., Ellerton, N. F. and Robinson, H. A. (1983). Hemocyanin: a current perspective. *Prog. Biophys. Mol. Biol*. **41**, 143–248.

Ellerton, H. D. and Lankovsky, T. (1983). Structure of the haemocyanin from the paua (abalone) *Haliotis iris*. In *Structure and Function of Invertebrate Respiratory Proteins* (ed. E. J. Wood), pp. 129–132. London: Harwood Academic Publishers.

Engel, P. C. and Jones, J. B. (1978). Causes and elimination of erratic blanks in enzymatic metabolite assays involving the use of NAD^+ in alkaline hydrazine buffers: improved conditions for the assay of L-glutamate, L-lactate and other metabolites. *Anal. Biochem.* **88**, 475–484.

Fields, J. H. A. (1983). Alternatives to lactic acid: possible advantages. *J. Exp. Zool*. **228**, 445–457.

Fushitani, K., Imai, K. and Riggs, A. F. (1986). Oxygenation properties of hemoglobin from the earthworm, *Lumbricus terrestris*. Effects of pH, salts and temperature. *J. Biol. Chem.* **261**, 8414–8423.

Gäde, G. (1987). A specific enzymatic method for the determination of taurine. *Biol. Chem. Hoppe-Seyler* **368**, 1519–1523.

Gäde, G. (1988). Energy metabolism during anoxia and recovery in shell adductor and foot muscle of the gastropod mollusc *Haliotis lamellosa*: formation of the novel anaerobic end product tauropine. *Biol. Bull*. **175**, 122–131.

Gebauer, W., Harris, J. R., Heid, H., Süling, M. Hillenbrand, R., Söhngen, S., Wegene-Strake, A. and Markl, J. (1994). Quaternary structure, subunits and domain patterns of two discrete forms of keyhole limpet hemocyanin: KLH1 and KLH2. *Zoology* **98**, 51–68.

Ghiretti-Magaldi, A., Salvato, B., Tallandini, L. and Beltramini, M. (1979). The hemocyanin of *Aplysia limacina*: chemical and functional characterization. *Comp. Biochem. Physiol*. **62A**, 579–584.

Graham, R. A. (1985). A model for L-lactate binding to *Cancer magister* hemocyanin. *Comp. Biochem. Physiol.* **81B**, 885–887.

Gutmann, I. and Wahlfield, A. W. (1974). L-(+)-lactate: determination with lactate dehydrogenase and NAD^+. In *Methods of Enzymatic Analysis*, second edition, vol. 3 (ed. H. U. Bergemeyer), pp. 1464–1491. New York: Academic Press.

Harris, J. R., Scheffler, D., Gebauer, W., Lehnert, R. and Markl, J. (2000). *Haliotis tuberculata* hemocyanin (HtH): analysis of oligomeric stability of HtH1 and HtH2 and comparison with keyhole limpet hemocyanin KLH1 and KLH2. *Micron* **31**, 613–622.

Hazprunar, G. (1996). The molluscan rhogocyte (pore cell, Blasenzelle, cellule nucale) and its significance for ideas on nephridial evolution. *J. Mol. Stud.* **62**, 185–211.

Johnson, B. A., Bonaventura, C. and Bonaventura, J. (1984). Allosteric modulation of *Callinectes sapidus* hemocyanin by binding of L-lactate. *Biochemistry* **23**, 872–878.

Keller, H., Lieb, B., Altenhein, B., Gebauer, D., Richter, S., Stricker, S. and Markl, J. (1999). Abalone (*Haliotis tuberculata*) hemocyanin type 1 (HtH1). Organization of the approximately 400 kda subunit and amino acid sequence of its functional units f, g and h. *Eur. J. Biochem.* **264**, 27–38.

Lallier, F. and Truchot, J. P. (1989). Modulation of haemocyanin oxygen-affinity by L-lactate and urate in the prawn *Penaeus japonicus*. *J. Exp. Biol*. **147**, 133–146.

Lieb, B., Altenhein, B., Lehnert, R., Gebauer, W. and Markl, J. (1999). Subunit organization of the abalone *Haliotis tuberculata* hemocyanin type 2 (HtH2) and the cDNA sequence encoding its functional units d, e, f, g and h. *Eur. J. Biochem.* **265**,134–144.

Lieb, B., Altenhein, B. and Markl, J. (2000). The sequence of a gastropod hemocyanin (HtH1 from *Haliotis tuberculata*). *J. Biol. Chem.* **275**, 5675–5681.

Lieb, B., Altenhein, B., Markl, J., Vincent, A., Van Olden, E., Van Holde, K. E. and Miller, K. I. (2001). Structures of two molluscan hemocyanin genes: significance for gene evolution. *Proc. Natl. Acad. Sci. USA* **98**, 4546–4551.

Mangum, C. P. (1983). Adaptability and inadaptability among HcO_2 transport systems: an apparent paradox. In *Structure and Function of Invertebrate Respiratory Proteins* (ed. E. J. Wood), pp. 333–352. Chur (Switzerland): Harwood Academic Publishers.

Mangum, C. P. (1992). Respiratory function of the molluscan haemocyanins. In *Advances in Comparative and Environmental Physiology*, vol. 13, *Blood and Tissue Oxygen Carriers* (ed. C. P. Mangum), pp. 301–323. Berlin: Springer-Verlag.

Mangum, C. P. and Lykkeboe, G. (1979). The influence of inorganic ions and pH on oxygenation properties of the blood in the gastropod mollusc *Busycon canaliculatum*. *J. Exp. Zool*. **207**, 417–430.

Mangum, C. P. and Shick, J. M. (1972). The pH of body fluids of marine invertebrates. *Comp. Biochem. Physiol*. **42A**, 693–697.

Meissner, U., Dube, P., Harris, J. R., Stark, H. and Markl, J. (2000). Structure of a molluscan hemocyanin didecamer (HtH1 from *Haliotis tuberculata*) at 12 Å resolution by cryoelectron microscopy. *J. Mol. Biol*. **298**, 21–34.

Mikkelsen, F. F. and Weber, R. E. (1992). Oxygen transport and hemocyanin function in the pulmonate land snail, *Helix pomatia*: Physiological and molecular implications of polyphasic oxygen-binding curves. *Physiol. Zool*. **65**, 1057–1073.

Miller, K. I. (1985). Oxygen equilibria in *Octopus dofleini* hemocyanin. *Biochemistry* **24**, 4582–4586.

Miller, K. I. and van Holde, K. E. (1974). Oxygen binding by *Callianassa californiensis* hemocyanin. *Biochemistry* **13**, 1668–1674.

Monod, J., Wyman, J. and Changeux, J. P. (1965). On the nature of allosteric transitions: a plausible model. *J. Mol. Biol*. **12**, 88–118.

Morris, S. and Bridges, C. R. (1986). Oxygen binding by the hemocyanin of the terrestrial hermit crab *Coenobita clypeatus* (Herbst) – the effect of physiological parameters *in vitro*. *Physiol. Zool*. **59**, 606–615.

Petrovich, D. P., Morris, S. and McMahon, B. R. (1990). Oxygen binding by the hemocyanin of *Busycon canaliculatum*. *Comp. Biochem. Physiol.* **97B**, 745–750.

Pilson, M. E. Q. (1965). Variation of hemocyanin concentration in the blood of four species of *Haliotis*. *Biol. Bull*. **128**, 459–472.

Ragg, N. L. C., Taylor, H. H. and Behrens, J. (2000). Stress and weight loss associated with handling in the blackfoot abalone, *Haliotis iris*. *J. Shell Res.* **19**, 528–529.

Redmond, J. R. (1968). The respiratory function of hemocyanin. In *Physiology and Biochemistry of Haemocyanins* (ed. F. Ghiretti), pp. 5–24. London: Academic Press.

Roxby, R., Miller, K., Blair, D. P. and van Holde, K. E. (1974). Subunits and association equilibria of *Calianassa californiensis* hemocyanin. *Biochemistry* **13**, 1662–1668.

Ryder, J. M., Wells, R. M. G. and Baldwin, J. (1994). Tauropine and D-lactate as indicators of recovery in live paua (New Zealand abalone) (*Haliotis iris*) from handling stress and of postmortem quality. *Food Australia* **46**, 523–526.

Sato, M., Kanno, N. and Sato, Y. (1985). Isolation of D-rhodoic acid from the abalone muscle. *Bull. Jap. Soc. Sci. Fish*. **51**, 1681–1683.

Sato, M., Takeuchi, M., Kanno, N., Nagahisa, E. and Sato, Y. (1991). Characterisation and physiological role of tauropine dehydrogenase and lactate dehydrogenase from muscle of abalone, *Haliotis discus hannai*. *Tohoku J. Agric. Res*. **41**, 83–95.

Sminia, T. and Boer, H. H. (1973). Haemocyanin production in pore cells of the freshwater snail *Lymnaea stagnalis*. *Z. Zellforsch*. **145**, 443–445.

Sminia, T. and Vlugt van Daalen, J. E. (1977). Haemocyanin synthesis in pore cells of the terrestrial snail *Helix aspersa*. *Cell Tissue Res*. **183**, 299–301.

Söhngen, S. M., Stahlmann, A., Harris, J. R., Müller, S. S., Engel. A. and Markl, J. (1997). Mass determination, subunit organization and control of oligomerisation states of keyhole limpet hemocyanin (KLH). *Eur. J. Biochem*. **248**, 602–614.

Spoek, G. L., Bakker, H. and Wolvekamp, H. P. (1964). Experiments on the haemoglobin–oxygen equilibrium of the blood of the edible snail (*Helix pomatia* L.). *Comp. Biochem. Physiol*. **12**, 209–221.

Taylor, H. H. and Anstiss, J. M. (1999). Copper and haemocyanin dynamics in aquatic invertebrates. *Mar. Freshwater Res.* **50**, 907–931.

Taylor, J. E. (1993). Responses to wounding resulting in haemorrhage in the black-footed paua *Haliotis iris* Gmelin (Mollusca: Gastropoda). MSc thesis, University of Canterbury, Christchurch, New Zealand.

Topham, R., Tesh, S., Cole, G., Mercatante, D., Westcott, A. and Bonaventura, C. (1998). Active site disruption in native *Limulus* hemocyanin and its subunit by disulfide-bond reductants: a chemical probe for the study of structure–function relationships in the hemocyanins. *Arch. Biochem. Biophys*. **352**, 103–113.

Truchot, J. P. (1975). Factors controlling the *in vitro* and *in vivo* oxygen affinity of the hemocyanin in the crab *Carcinus maenas* (L.). *Respir. Physiol*. **24**, 173–189.

Truchot, J. P. (1980). Lactate increases the oxygen affinity of crab hemocyanin. *J. Exp. Zool*. **214**, 205–208.

Tyuma, I., Imai, K. and Shimizu, K. (1973). Analysis of oxygen equilibrium of hemoglobin and control mechanism of organic phosphates. *Biochemistry* **12**, 1491–1498.

Tyuma, I., Shimizu, K. and Imai, K. (1971). Effect of 2,3-diphosphoglycerate on the cooperativity in oxygen binding of human adult hemoglobin. *Biochem. Biophys. Res. Commun.* **43**, 423–428.

van Holde, K. E. and Miller, K. I. (1995). Hemocyanins. *Adv. Protein Chem.* **47**, 1–81.

van Holde, K. E., Miller, K. I. and Decker, H. (2001). Hemocyanins and invertebrate evolution. *J. Biol. Chem*. **276**, 15563–15566.

van Holde, K. E., Miller, K. I. and van Olden, E. (2000). Allostery in very large molecular assemblies. *Biophys. Chem.* **86**, 165–172.

Weber, R. E. (1981). Cationic control of O_2 affinity in lugworm erythrocruorin. *Nature* **292**, 386–387.

Weber, R. E., Jensen, F. B. and Cox, R. P. (1987). Analysis of teleost hemoglobin by Adair and Monod–Wyman–Changeux models. *J. Comp. Physiol. B* **157**, 145–152.

Weber, R. E., Malte, H., Braswell, E. H., Oliver, R. W. A., Green, B. N., Sharma, P. K., Kuchumov, A. and Vinogradov, S. N. (1995). Mass spectrometric composition, molecular mass and oxygen binding of *Macrobdella decora* hemoglobin and its tetramer and monomer subunits. *J. Mol. Biol*. **251**, 703–720.

Wells, R. M. G. and Baldwin, J. (1995). A comparison of metabolic stress during air exposure in two species of New Zealand abalone, *Haliotis iris* and *Haliotis australis*: implications for the handling and shipping of live animals. *Aquaculture* **134**, 361–370.

Wells, R. M. G., Baldwin, J., Speed, S. R. and Weber, R. E. (1998). Haemocyanin function in the New Zealand abalones *Haliotis iris* and *H. australis*: relationship between oxygen-binding properties, muscle metabolism and habitat. *Mar. Freshwater Res*. **49**, 143–149.

Wieser, W. (1981). Responses of *Helix pomatia* to anoxia: changes of solute activity and other properties of the haemolymph. *J. Comp. Physiol*. **141**, 503–509.

Wijsman, T. C. M., van der Lugt, H. C. and Hoogland, H. P. (1985). Anaerobic metabolism in the freshwater snail *Lymnaea stagnalis*: haemolymph as a reservoir of D-lactate and succinate. *Comp. Biochem. Physiol.* **81B**, 889–895.