J. exp. mar. Biol. Ecol., 1980, Vol. 43, pp. 11–27

THE EFFECTS OF SALTS ON HAEMOCYANIN-OXYGEN BINDING IN THE MARINE PULMONATE SNAIL *AMPHIBOLA CRENATA* (Martyn)

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Abstract: Dilution of the haemolymph from the estuarine pulmonate *Amphibola crenata* (Martyn) increased the oxygen affinity of its haemocyanin down to 25% sea water although the sigmoidal shape of the oxygen equilibrium curve was unchanged.

When the haemocyanin was 'stripped' of its natural ionic environment it exhibited a hyperbolic oxygen equilibrium curve between pH 6.5 and 8.0. Under these conditions the haemocyanin molecules were dissociated into subunits when viewed with an electron microscope. These effects were reversed when the divalent cations Ca^{2+} and Mg^{2+} were added to the 'stripped' haemocyanin.

Increasing the concentrations of various ions (Cl^-, SO_4^{2-}, Na^+, Ca^{2+}, and Mg^{2+}) decreased the haemocyanin's oxygen affinity and augmented a reverse Bohr effect. In the estuarine environment where *Amphibola* lives, the reverse Bohr and salt effects might act in opposite directions to stabilize oxygen delivery under conditions of fluctuating salinity.

INTRODUCTION

A number of molluscs and arthropods possess the copper protein haemocyanin which imparts a bluish colour to their blood in the presence of oxygen. The oxygen-transporting function of haemocyanin has been investigated by many authors who have substantiated the notion that the oxygen-binding properties of this protein balance an organism's demand for oxygen with its access to air (see Jones, 1972; Ghiretti & Ghiretti-Magaldi, 1975). In the Malacostraca, recent attention has been focused on the relationship between haemocyanin and whole animal physiology by taking into account the influences of the extracellular and external environments on haemocyanin function (Truchot, 1973, 1975; Sullivan *et al.*, 1974; Weiland & Mangum, 1975; Mangum, 1976a; Mangum *et al.*, 1976). In crustacean haemocyanins and some annelid haemoglobins, a number of salts appear to induce an increase in oxygen affinity which is opposed by a normal Bohr effect, decreasing the oxygen affinity in the presence of hydrogen ions (Larimer & Riggs, 1964; Pickett *et al.*, 1966; Everaarts & Weber, 1974; Weiland & Mangum, 1975; Mangum, 1976b; Ellerton *et al.*, 1977; Brouwer *et al.*, 1978). In a remarkable study for its day, Redfield & Ingalls (1932) described the effects of salts and hydrogen-ion concentration on the oxygen equilibrium of the haemocyanin of the prosobranch *Busycon canaliculatum*. Moreover, these authors attributed the integrity of native

haemocyanin molecules and the sigmoidal nature of the equilibrium curve to the presence of calcium ions. Their essential findings have been re-affirmed 47 years later by Mangum & Lykkeboe (1979). By contrast, in gastropod and xiphosuran haemocyanins and in vertebrate haemoglobins, salts decrease the oxygen affinity which in the case of haemocyanin, is accompanied by a reverse Bohr effect (Redfield & Ingalls, 1932; Spoek *et al.*, 1964; Er-el *et al.*, 1972; Vannoppen-Ver Eecke & Lontie, 1973; Sullivan *et al.*, 1974; Hall *et al.*, 1975; Mangum, 1976a).

The pulmonates are a particularly interesting group of molluscs in that they include terrestrial, freshwater, and marine forms, all having pulmonary respiration. The influence of salts on oxygen-binding by pulmonate haemocyanin has been examined in the terrestrial snail *Helix pomatia* (Spoek *et al.*, 1964; Er-el *et al.*, 1972; Vannoppen-Ver Eecke & Lontie, 1973) and in the freshwater snail *Lymnaea stagnalis* (Hall *et al.*, 1975). Although the presence of a reverse Bohr effect has also been demonstrated in the marine snail *Siphonaria zelandica* (Wells & Wong, 1978), the effects of salts on the oxygen-binding properties of haemocyanin from a marine pulmonate are entirely unknown.

Amphibola crenata (Martyn) is a primitive basommatophoran pulmonate endemic to New Zealand where it is common on estuarine mud flats, occupying a transitional habitat between marine and terrestrial conditions. These areas are subject to considerable variations in salinity from freshwater run-off and evaporation. We have re-affirmed Farnie's (1919) observation that these snails survive for considerable periods of time in tap or rain water and, therefore, we have addressed ourselves to the problem of whether *Amphibola* haemocyanin, like its terrestrial and freshwater relatives, is sensitive to changes in ionic concentration. Furthermore, we have speculated on these findings in relation to the animal's natural habitat.

Materials and Methods

Preparation of haemocyanin

A. crenata were collected from an estuarine mud flat exposed at low tide at Whangateau, North Auckland (36°20'S: 174°46'E). At the time of collection, the temperature of the water overlying the mud was 24.1 °C while 5 cm below the surface a temperature of 24.2 °C was recorded. Oxygen-binding experiments were conducted at 25 °C.

Immediately on return to the laboratory, the snails were bled by foot puncture and the haemolymph was pooled and centrifuged for 5 min at 5400 *g* to remove particulate matter. The dark blue supernatant was then eluted with deionized water on a 12 × 100 mm Sephadex G-25 column to strip the haemocyanin of its salt content. The resulting solution was stored at 4 °C until used. Aliquots of the haemocyanin solution to be used for oxygen affinity studies were subsequently dialysed in Visking tubes exhaustively against appropriate buffers and brought to

known ionic composition by the addition of various salts. All experiments were carried out within 3 days after preparation.

LIGHT ABSORPTION SPECTRA

Solutions of haemocyanin in pH 9.15 Tris buffer were scanned in the U.V.-visible range using 1-cm silica cells in a Unicam SP 1750 recording spectrophotometer. Oxyhaemocyanin was prepared by flushing the sample in a tonometer with pure oxygen and deoxyhaemocyanin by repeated evacuation.

MEASUREMENT OF HAEMOCYANIN CONCENTRATION

Haemocyanin concentration was measured spectrophotometrically from a 1 : 100 dilution of blood in oxygen-saturated 0.05 M Hepes buffer pH 8.6. The absorbance of this solution was measured at 345 nm and the concentration of haemocyanin was calculated using the extinction coefficient $E^{1\%}_{1cm} = 3.28$ at 345 nm, given for gastropod haemocyanin by Nickerson & Van Holde (1971), assuming the same coefficient.

ELECTRON MICROSCOPY

Samples of haemocyanin, at the dilutions used in the oxygen-binding experiments, were negatively stained with 4% uranyl acetate on carbon grids and examined at 10 000 times magnification using a Philips 301 electron microscope. In this way, the degree of association of the subunits comprising the haemocyanin molecules could be clearly seen.

HAEMOCYANIN-OXYGEN-BINDING

Oxygenated and deoxygenated solutions of haemocyanin show considerable differences in light absorption between 300 and 700 nm (see Fig. 1). These differences in the visible spectrum are well known (blue oxygenated haemocyanin becomes colourless upon deoxygenation) and this observation had been the basis for spectrophotometric analyses of haemocyanin–oxygen-binding in a number of earlier publications. More recently, Nickerson & Van Holde (1971) have shown that the absorption bands of molluscan haemocyanin in the visible spectrum are complex and require large light-scattering corrections. Accordingly, extinction coefficients for these bands are not available for these haemocyanins. In contrast, the absorption band in the near-U.V. at 345 nm is unaffected by anomalous dispersion and we have found that the spectrum near this wavelength is insensitive to changes in hydrogen and divalent cations over the range of concentrations used in our experiments. We have, therefore, measured the percentage saturation of haemocyanin with oxygen by measuring the light absorbed at 345 nm according to the following procedure.

The reaction of haemocyanin with oxygen is carried out inside a tonometer which may be constructed as shown in Fig. 2. A 1-cm silica cuvette is fused to a

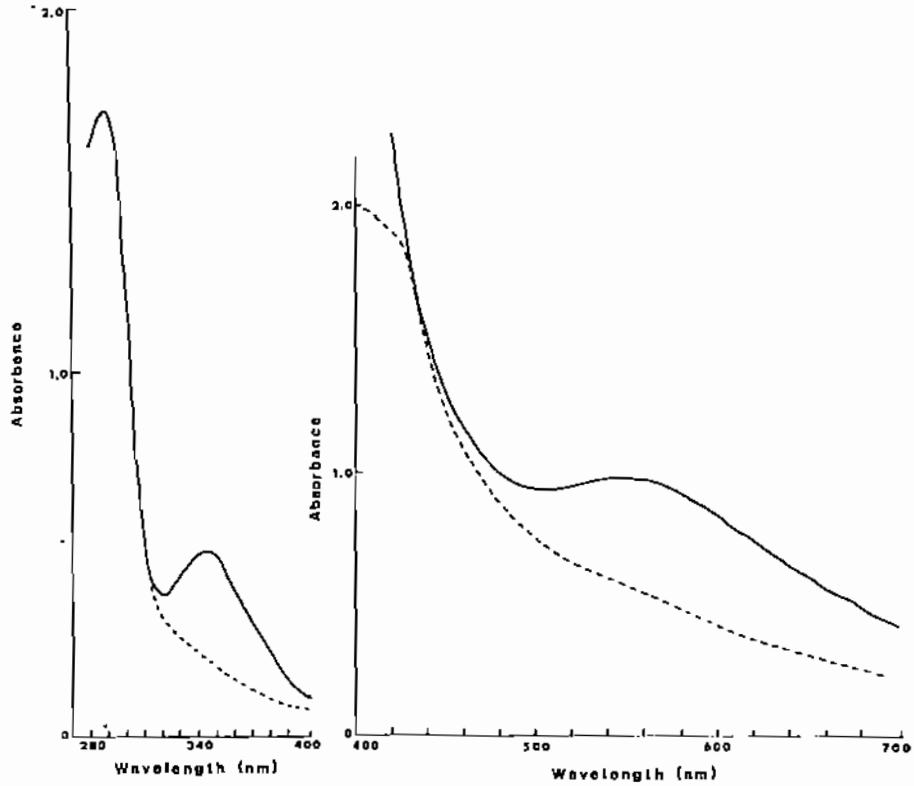

Fig. 1. Absorption spectra of *A. crenata* oxyhaemocyanin (solid line) and deoxyhaemocyanin (broken line) in 0.05 M Tris buffer, pH 9.15 and 24°C: in the U.V. spectrum (a), concentration of protein is 1 mg/ml and in the visible spectrum (b), 2 mg/ml.

round glass bulb which presents a large surface area for gas exchange during equilibration. At the top of the tonometer is a Quickfit fitting with a small hole for letting in air. A small scratch extends the hole sideways and is used for allowing small amounts of air to enter the tonometer steadily. The volume of the tonometer is about 70 ml and is carefully determined before use. The tonometer with its attached cell fits into the sample position of most single or double beam narrow-bandwidth spectrophotometers. It is, however, necessary to construct a light-proof box to accommodate the tonometer in the cell compartment – a black top-hat works admirably.

About 100 μl 'stripped' haemocyanin solution were added to 3 ml buffer until its absorbance read about 0.3 when finshed with oxygen, indicating a protein

concentration of ≈2 mg/ml. Three ml of buffered haemocyanin solution were put into the tonometer which was then evacuated using an Edwards high vacuum pump. The haemocyanin was then equilibrated by rapidly rotating the tonometer in a water bath at 25 ± 0.2 °C for 3 min. For routine measurements, it is convenient to mount the tonometer in a cradle and use a mechanical means of rotation. Since the speed of rotation determines the equilibration time, we found that the samples were equilibrated within 3 min provided that rotation was not < 50 r.p.m. The evacuation procedure was repeated twice and the absorbance of the deoxyhaemocyanin then recorded.

A known volume of air was now allowed to enter the tonometer from a 3-ml graduated pipette connected to the side arm of the tonometer through a rubber tube using a mercury bubble to indicate the amount of air that had been added. On cautiously opening the tap, the atmospheric air contained in the pipette was sucked into the tonometer. The volume of air introduced was measured by the displacement of the mercury bubble. The tonometer was then equilibrated for 3 min in the waterbath, transferred to the spectrophotometer, and the absorbance recorded. This procedure was repeated until all the haemocyanin was in the oxygenated form, that is, the further addition of air fails to increase the absorbance. Full saturation was confirmed using pure oxygen for a number of the low oxygen-affinity curves. An inherent difficulty in the method is that the assumption of 100% saturation of the oxygenated sample is required. The error introduced by this assumption, however, is negligible in the analysis of the middle part of the oxygen equilibrium curve (Winslow *et al.*, 1977).

PREPARATION OF SOLUTIONS

Buffer solutions were made up from 0.05 M solutions of the free acid and sodium salt of Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid). Hepes is an ideal buffer for this kind of study because its effective buffering range is pH 6.5–8.6 at 20 °C and its effect on ions other than H^+ (especially Ca^{2+}, Mg^{2+}) is negligible (Gueffroy, 1975). When it was essential that no divalent cations remained, EDTA was added to a final concentration of 10 mM. The chloride salts of Ca^{2+}, Mg^{2+}, and Na^+ were used unless otherwise stated. pH measurements were checked using a Radiometer PHM64 meter with a type B electrode which has a low salt error.

CALCULATIONS

The pressure of oxygen in the tonometer corresponding to each introduction of air was calculated by the following formula:

$$pO_2 = \frac{(pB - (RH \times SVP)) \times 0.2093}{Vt - Vs} \times \frac{Te}{Ta} \times Vx,$$

where, pB = barometric pressure in mm Hg; RH = relative humidity; SVP = water vapour pressure at Ta; 0.2093 = fraction of oxygen in air; Te = temperature of equilibration in °K; Ta = ambient temperature iu °K; Vt = volume of tonometer iu ml; Vs = volume of buffered haemocyanin solution in ml; and Vx = total volume of air added in ml.

The percentage saturation of haemocyanin was calculated from the following formula:

$$\%S_x = \frac{A_{345}\text{ partially oxygenated Hcy} - A_{345}\text{ deoxy Hcy}}{A_{345}\text{ fully oxygenated Hcy} - A_{345}\text{ deoxy Hcy}} \times 100,$$

where $\%S_x$ is the saturation at a pO_2 of X mm Hg, and A_{345} is the absorbance.

The $\%S$ is then plotted against pO_2 for a dissociation curve. Knowledge of the absolute absorbance is not required as only the difference in absorbance is used in calculation.

Results

In addition to the usual protein band at 280 nm, 'stripped' oxyhaemocyanin had absorption maxima in the near-U.V. and in the visible region (Fig. 1a,b). These maxima, at 345 nm and 570 nm disappear in deoxygenated solution as is characteristic of other gastropod haemocyanins (Nickerson & Van Holde, 1971; Ghiretti & Ghiretti-Magaldi, 1972; Hall *et al.*, 1975; Wells & Wong, 1978). Identical U.V. spectra were obtained at pH 6.5 and when whole blood was diluted with sea water, indicating the stability of the 345-nm peak in the presence or absence of various ions. Accordingly, the oxygen-binding studies were carried out using absorbance measurements at 345 nm.

Haemocyanin concentration in haemolymph

The mean haemocyanin concentration of the haemolymph from 16 animals was 16.98 (±0.89) mg/ml. The volume of blood obtained from single animals was too small to measure directly the oxygen combining capacity of the blood. Nevertheless, assuming that 50 000 g haemocyanin binds 1 mol oxygen (Klarman *et al.*, 1972) and including the 0.47 vols % oxygen carried in physical solution at 35‰ salinity and 25 °C, the capacity of *Amphibola* blood is 1.23 vols %.

Electron micrography

Haemocyanin dialysed against 50 mM Ca^{2+} and negatively stained, showed rod-like molecules with dimensions of ≈ 100 × 32 nm but these structures were not seen after exhaustive dialysis against 50 mM EDTA (see Fig. 10).

OXYGEN EQUILIBRIUM

Oxygen affinity was quantified by the P_{50}, or pO_2 at which half of the haemocyanin is in the oxygenated form, while the shape of the oxygen-equilibrium curve, which reflects the degree of cooperativity between oxygen-binding sites in the pigment, was quantified by Hill's (1910) coefficient, n, from the equation $Y = 100\ (p/P_{50})^n / (1 + (p/P_{50})^n)$, where Y is the percentage of haemocyanin combined with oxygen and p is the pressure of oxygen in mm Hg.

The oxygen-binding properties of gastropod haemocyanin are independent of protein concentration over the range 0.5–4 $mg \cdot ml^{-1}$ (Vannoppen-Ver Eecke & Lontie, 1973). At the 2 $mg \cdot ml^{-1}$ used throughout this study, there was no evidence of concentration-dependent subunit dissociation of the haemocyanin (see Fig. 10a).

The effect of diluting whole haemolymph with 100, 50, and 25% sea water is

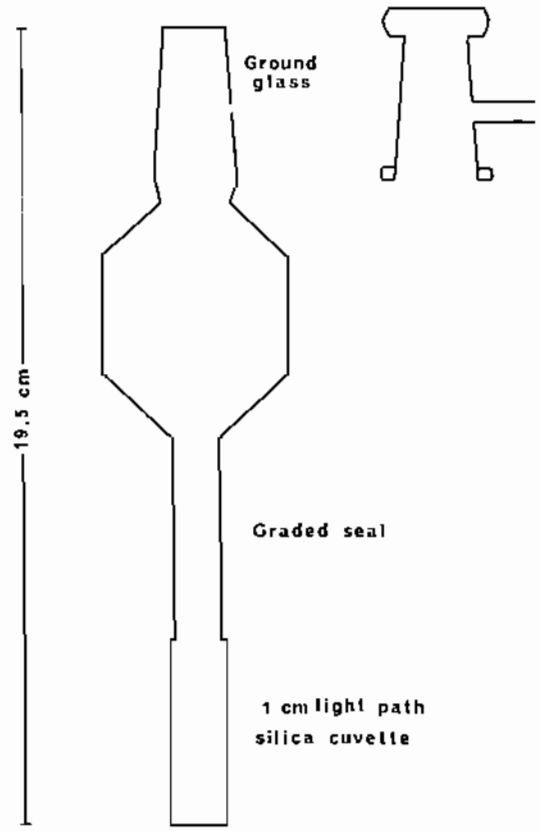

Fig. 2. Diagram of the glass tonometer used to equilibrate buffered solutions of haemocyanin to known pO_2: the Pyrex bowl of the tonometer has an optical cell fused to one end and to the other end the inner part of a 3-way vacuum stopcock; the outer part of the stopcock has the vertical arm sealed and forms a lid with a side arm for admitting or removing air; the ground joint should be liberally coated with silicone grease before vacuum is applied to the side arm.

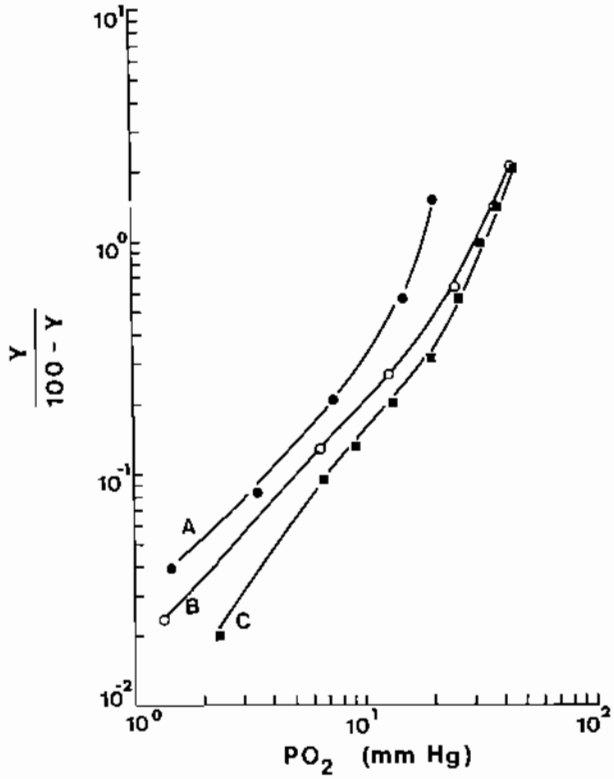

Fig. 3. Hill plots of oxygen equilibrium data for whole blood diluted with 25% (●), 50% (○), and 100% (■) buffered sea water at pH 8.08 and 25 °C.

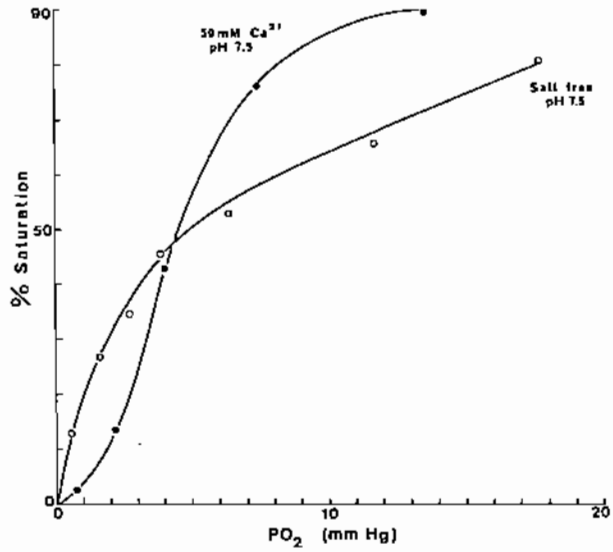

Fig. 4. The effect of 50 mM Ca^{2+} on the shape of the oxygen equilibrium curve of *A. crenata* haemocyanin in 0.05 M Hepes buffer, pH 7.5 and 25 °C.

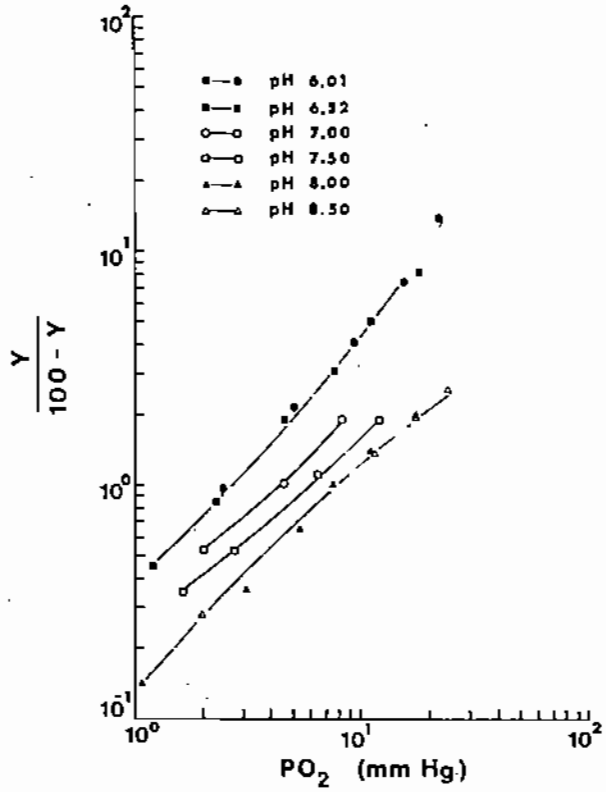

Fig. 5. Hill plots of the oxygen equilibria at different pH for 'stripped' *A. crenata* haemocyanin in 0.05 M Hepes buffers + 10 mM EDTA, 25 °C.

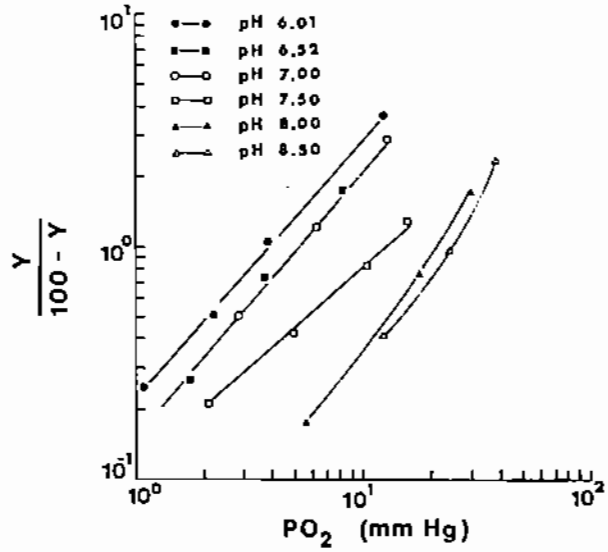

Fig. 6. Hill plots of the oxygen equilibria at different pH for *A. crenata* haemocyanin in the presence of 450 mM NaCl, 0.05 M Hepes buffers, 25 °C.

seen in the Hill plots of Fig. 3, where dilution of the medium increases the haemocyanin–oxygen affinity while the shape of the curve is unaffected over the range presented.

When *Amphibolo* haemocyanin is 'stripped' of its salts at pH 7.5, a hyperbolic curve results, the effect being dramatically reversed in the presence of 50 mM Ca^{2+}, while the P_{50} remains essentially unchanged (Fig. 4). A similar effect was noted when Mg^{2+} was substituted for Ca^{2+}.

Over the pH range 6.5–8.0, the P_{50} of 'stripped' haemocyanin is markedly sensitive to changes in pH, showing a reverse Bohr effect, but at extreme pH values, the Bohr effect is reduced or absent (Fig. 5). Throughout the pH range, the curves are all hyperbolic and n values range from 0.95–1.07.

When brought to 450 mM with NaCl, the curves are displaced to the right and the oxygen affinities are decreased (Fig. 6). Below pH 8.0 the curves are hyperbolic although slight cooperativity ($n = 1.46$) is seen at pH 8.5. To a lesser extent Mg^{2+} increased P_{50} but was more potent in inducing cooperativity at pH > 8.0 where $n = 2.11$ (Fig. 7).

While it was apparent that salt concentration per se contributed to a decrease

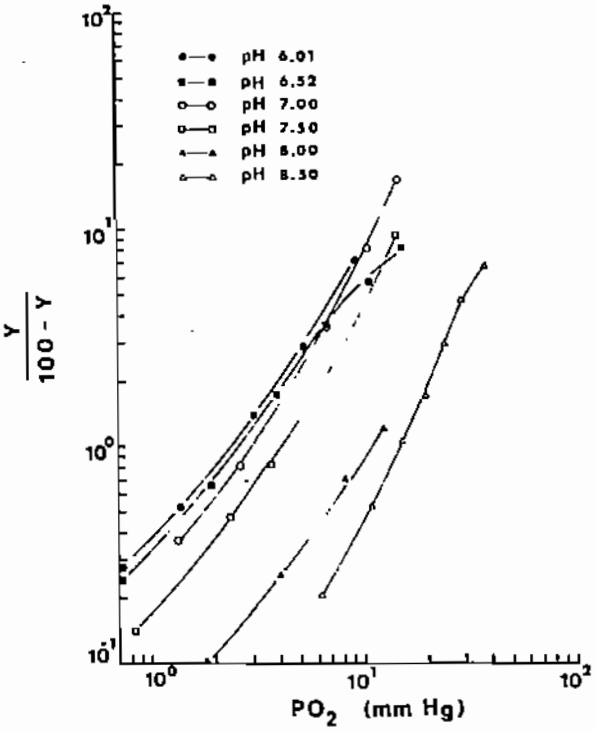

Fig. 7. Hill plots of the oxygen equilibria at different pH for *A. crenata* haemocyanin in the presence of 50 mM Mg^{2+}, 0.05 M Hepes buffers, 25 °C.

in oxygen affinity throughout the pH range (Fig. 8), the influence of different salts augmented the Bohr effect in an unexpected manner (Fig. 9). The maximal value of the Bohr coefficient ($\Delta \log P_{50}/\Delta pH$) varied according to whether monovalent or divalent ions were present. Furthermore, the pH at which the Bohr protons exhibited their greatest influence on P_{50} also varied according to the ions present.

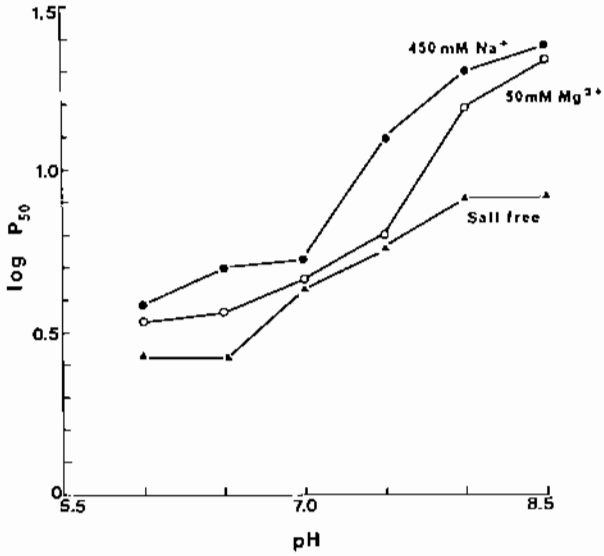

Fig. 8. Logarithmic plot of oxygen half-saturation pressures P_{50} against pH for *A. crenata* haemocyanin, at 25 °C in the absence of salts, and in the presence of 450 mM NaCl, and 50 mM Mg^{2+}.

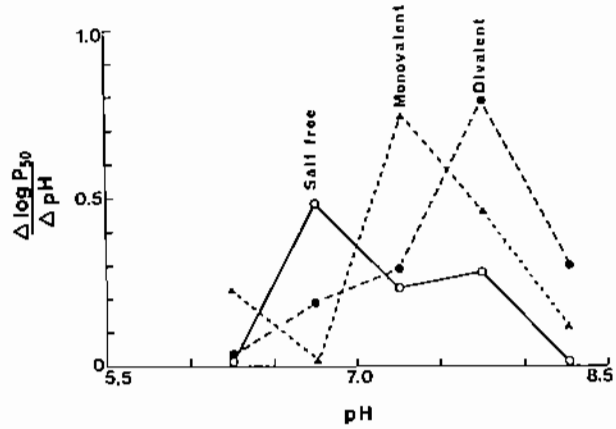

Fig. 9. Magnitude of the Bohr effect as a function of pH in 'stripped' haemocyanin solutions and in the presence of 450 mM NaCl or 50 mM Mg^{2+} at 25 °C.

In the salt-free preparation, the Bohr effect was maximal at pH 6.7, for NaCl, pH 7.2, and Mg^{2+}, pH 7.7.

TABLE I

Parameters of the haemocyanin–oxygen equilibrium from *Amphibola* in the presence or absence of specific ions: data recorded at pH 8.0 and 25 °C.

Ionic species	Hill coefficient n	O_2 affinity P_{50} mm Hg
None	0.93	4.2
NaCl 450 mM	0.86	12.2
Mg_2SO_4 50 mM	1.37	4.6
$MgCl_2$ 50 mM	1.40	6.2
$CaCl_2$ 50 mM	2.01	4.5

When the effects of SO_4^{2-} and Cl^- salts of Mg^{2+} are compared (Table I), it appears that chloride has little influence on the shape of the oxygen-binding curve, but decreases the oxygen affinity. It is also evident that both Ca^{2+} and Mg^{2+} promoted cooperativity at 50 mM; Ca^{2+} generated a higher n value.

DISCUSSION

A. crenata is one of a small number of marine pulmonates and in contrast to freshwater and terrestrial snails, it is subject to considerable fluctuations in salinity. The ecological distribution of this primitive species led Morton (1955) to surmise that *Amphibola* has a wide range of tolerance to salinity. Unpublished observations show that the blood of *Amphibola* remains isosmotic down to an external dilution of 25% sea water and that below this level, the snail is a near-perfect osmoregulator. Thus, although we have observed *Amphibola* to survive for long periods in fresh water, the salt content of the extracellular environment which contains the haemocyanin does not fall below 25% sea water. Our results indicate that if this were not the case and haemolymph was diluted with decreased salinity in the external environment, then the haemocyanin–oxygen equilibrium curve would assume a hyperbolic shape with very high oxygen affinity, thereby being unable to deliver oxygen at pO_2's adequate to sustain a $\dot{V}O_2$ in the normal range. The manner in which salts and H^+ act upon the oxygen equilibrium of *Amphibola* haemocyanin is qualitatively similar to that already observed in freshwater and terrestrial pulmonates (Spoek *et al.*, 1964; Vannoppen-Ver Eecke & Lontie, 1973; Er-el *et al.*, 1972; Hall *et al.*, 1975) and in marine prosobranchs (Mangum, 1976a; Wood *et al.*, 1977). The immediate significance of our observations in the marine environmeut is clear enough: a decrease in salinity increases the haemocyanin's affinity for oxygen. Provided that a period of haemolymph dilution coincided with a period of decreased

oxygen availability, such as might occur when the animal is crawling through fine sediment, then an increase in haemocyanin–oxygen affinity would be advantageous.

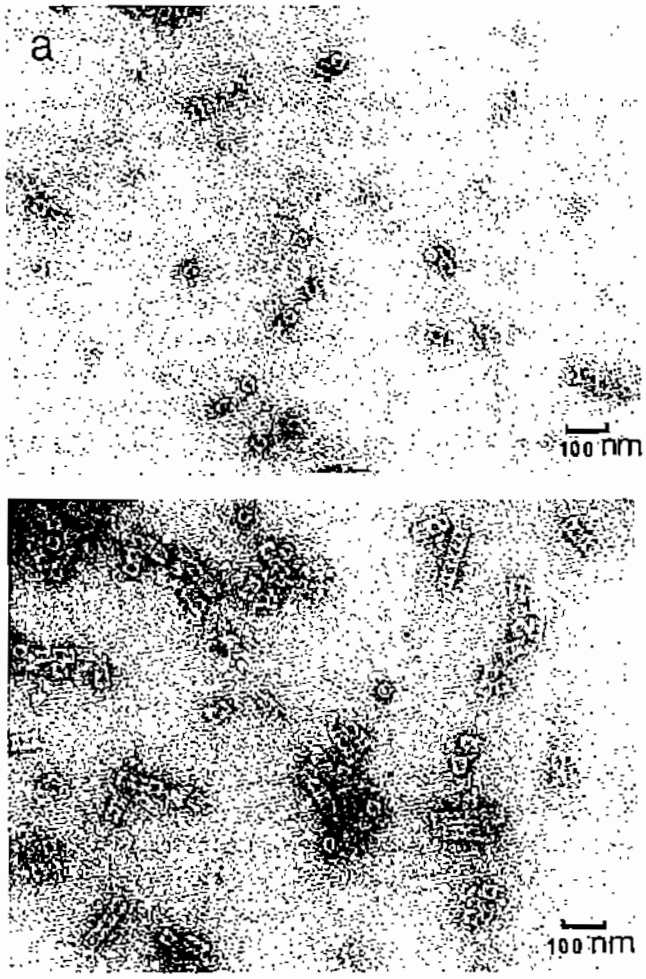

Fig. 10. Electron micrographs of *A. crenata* haemocyanin, negatively stained with 4% uranyl acetate, pH 6.8: a, 'stripped' haemocyanin in 10 mM EDTA; b, in 10 mM Ca^{2+}; whole haemocyanin molecules are seen as cylinders end-on (circles) or side-on (rectangles): some half molecules are also seen.

At low tide, particularly in warm weather, the tendency to haemo-concentration caused by desiccation may be great. It may be significant in this respect that *Amphibola* is the only adult pulmonate known to possess an operculum and thus by closing the shell, water may be preserved at the expense of access to oxygen.

While the Amphineura have little or no Bohr effect (Manwell, 1958; Redmond,

1960), and the cephalopod molluscs have a strong normal Bohr effect, gastropod haemocyanins typically show a reverse Bohr effect. The question of the functional significance of a reverse Bohr effect in gastropods has attracted some speculation. When the animal is active at low tide, the carbon dioxide in the near-environment may rise and the pO_2 fall due to the activities of micro-organisms on the mudflats. Under these conditions, an increase in oxygen affinity promoted by the negative Bohr effect might be beneficial. This explanation could also explain the reverse Bohr effect in the marine prosobranch *Busycon* (DePhilips *et al.*, 1970) but is clearly inadequate for fully terrestrial snails. An alternative explanation seems possible. Jones (1972) pointed out that gastropod circulation is rather sloppy and there is scant evidence for rhythmic ventilation of the lung. The presence of a large blood space in the pedal sinus of *Amphibola* may well promote circulation through the movements of the whole animal. Some of the blood enters the pulmonary vein directly from the anterior sinus, but most reaches the heart only after passing through the vessels of the mantle-roof, which constitutes the lung. Because the mantle cavity is filled with water (Morton, 1967), gaseous exchange in the lung is possible when the animal is immersed. Therefore, the release of respiratory carbon dioxide into the lung may be diffusion-limited and consequently the pulmonary pCO_2 may be quite high. Under these hypercapnic conditions, carbon dioxide would impede the uptake of oxygen in molluscs having a normal Bohr effect like the cephalopods (Johansen & Lenfant, 1976). The reverse Bohr effect in snails may actually assist in oxygen uptake by the network of blood vessels in the roof of the mantle. Measurements of in vivo pO_2 and pH under a variety of environmental conditions in the xiphosuran *Limulus*, which also has a reverse Bohr effect, tend to confirm this hypothesis (Johansen & Petersen, 1975). It is certainly clear that the advantages of unloading oxygen are minimal with a reverse Bohr effect.

Mangum *et al.*, (1976) have commented on the interesting observation that H^+ and the ions of inorganic salts have opposite effects on in vitro oxygenation. Salts increase oxygen affinity and there is a normal Bohr effect in crustaceans; in gastropods and in *Limulus*, salts decrease oxygen affinity and there is a reverse Bohr effect. These are not exclusive possibilities, for in spiders, both salts and H^+ decrease oxygen affinity (Loewe & Linzen, 1975).

When the blue crab, *Callinectes sapidus*, is exposed to decreasing salinity, the salt effects on its haemocyanin are opposed by a rise in blood pH and hence restoration of P_{50} by the normal Bohr effect (Mangum & Weiland, 1975; Weiland & Mangum, 1975). On the other hand, the effect of reduced salts on haemocyanin oxygenation in *Limulus* is not opposed by a large increase in blood pH, but by a drop in heart rate and hence blood pO_2 (Mangum *et al.*, 1976). We agree with Mangum that a decrease in blood pO_2 at low salinity is necessary for maximal utilisation of the haemocyanin-oxygen binding properties. The small size of *Amphibola* precluded measurements of blood pH and pO_2 under changing salinity. We would expect, however, a low pO_2 when the animal is covered by water and then burrows into the sedi-

ment. Mangum & Shick (1972) recorded a haemolymph pH from heart puncture of 7.84 in *Busycon*, a value within our range of oxygen-affinity measurements, but considerably higher than the mammalian value of pH 7.4 often assumed for invertebrate bloods. It may be that the in vivo pH is even higher, bearing in mind the sensitivity of pH to sampling stresses in other marine organisms (see Qvist *et al.*, 1977).

The mechanism of respiratory adaptation in estuarine conditions may be different for gastropods and crustaceans, but the end result is similar – a constancy of oxygen supply by the haemocyanin in order to meet respiratory demand. Experiments by one of us (S.E.S.) suggest that this is so in *Amphibola* where oxygen uptake is independent of salinity. Furthermore, the rates are similar in air and water.

We conclude that when the tide is out and *Amphibola* is active on the mudflats, the respiratory rôle of its haemocyanin is very much like that of the terrestrial pulmonates. But when the snails are covered by the tide or by a mixture of seawater and freshwater run-off, and they begin to burrow, then the marked salt effect of the haemocyanin and the reverse Bohr effect act in concert to stabilize the oxygen supply and minimize the environmental perturbations. The oxygen-combining capacity of the haemocyanin (1.2 vols%), which is modest as in all haemocyanin-containing bloods (see Mangum *et al.*, 1975), may contribute at low tide to greater unloading potential as the amount of oxygen physically dissolved in the haemolymph decreases.

The haemocyanins of the Mollusca and Arthropoda are built on entirely different plans of organization (Nickerson & Van Holde, 1971; Ghiretti *et al.*, 1973; Waxman, 1975; Bonaventura *et al.*, 1976, 1979) so perhaps it is not surprising that the physiological properties of haemocyanins possessed by marine species function in different ways.

Acknowledgements

The authors would like to thank the Director and staff of the Leigh Marine Laboratory for the use of their facilities, Dr C.P. Mangum and Professor J.E. Morton for reading the typescript, and the Auckland University Research Committee for funding the project.

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