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THE REGULATION OF HAEMOCYANIN OXYGEN AFFINITY DURING EMERSION OF THE CRAYFISH *AUSTROPOTAMOBIUS PALLIPES*

I. AN *IN VITRO* INVESTIGATION OF THE INTERACTIVE EFFECTS OF CALCIUM AND L-LACTATE ON OXYGEN AFFINITY

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SUMMARY

The haemolymph of the crayfish *Austropotamobius pallipes* exhibits a high affinity for oxygen ($P_{50} = 2{\cdot}7$ Torr at pH 7·9 and 15°C) and a modest Bohr effect ($\varphi = -0{\cdot}455$). The affinity of haemolymph dialysed against a crayfish Ringer was lower with a P_{50} value of 6·4 Torr at the same temperature and pH. The oxygen affinity of the dialysed haemolymph can be increased markedly by increased concentrations of L-lactate and to a greater extent by elevated concentrations of calcium ions.

In the dialysed preparation, the potentiating effects of L-lactate and Ca^{2+} on haemocyanin oxygen affinity were found to be interdependent. Elevating the concentration of one of these two ions reduced the effect of the other.

The increase in the oxygen affinity of the haemocyanin brought about by elevated Ca^{2+} and L-lactate was insufficient to account for the difference in affinity between dialysed and nondialysed haemolymph. The mutually agonistic effects of Ca^{2+} and L-lactate are described both empirically and graphically.

INTRODUCTION

The effects of aerial exposure on respiration in decapod crustaceans have been investigated for a number of species and many of the respiratory responses to emersion are now well understood (e.g. Wheatly & Taylor, 1979; Taylor, Butler & Sherlock, 1973; McMahon & Wilkes, 1983).

The crayfish *Austropotamobius pallipes* has been observed to leave the water when the oxygen tension falls below 40 Torr and to breathe air (Taylor & Wheatly, 1980, 1981). Complete emergence of crustaceans into air often causes, initially, a haemolymph acidosis (Truchot, 1975*a*; Taylor & Wheatly, 1981; deFur & McMahon, 1984*b*), which is normally accompanied by a decrease in post-branchial haemolymph

Key words: crayfish, L-lactate, calcium, haemocyanin, oxygen affinity.

oxygenation (Taylor & Wheatly, 1980, 1981; deFur & McMahon, 1984*a*; deFur, McMahon & Booth, 1983). An increased anaerobiosis leads to an accumulation of both lactate and H^+ in the haemolymph. Taylor & Wheatly (1981) have shown that during extended aerial exposure the initial acidosis that occurs in the haemolymph of *A. pallipes* can be almost completely compensated, over 24 h, by elevated levels of haemolymph HCO_3^-. These authors also demonstrated that the accumulated lactate in the haemolymph of *A. pallipes* was either substantially excreted, sequestered in the tissues, or metabolized.

The role of pH in modulating the oxygen affinity of crustacean haemocyanins is now appreciated, as is the potentiating effect of Ca^{2+} (Larimer & Riggs, 1964; Truchot, 1975*b*; Weiland & Mangum, 1975; Arisaka & van Holde, 1979). The other major haemolymph variable during aerial exposure, L-lactate, is now known to increase haemocyanin oxygen affinity (Truchot, 1980). Although both of these factors are known to fluctuate in the haemolymph of *A. pallipes*, the effect of these ions on the oxygen affinity of the haemocyanin has until now received little attention. The study reported here considers the effects of Ca^{2+} and L-lactate separately and together on the *in vitro* oxygen affinity of haemocyanin from *A. pallipes*.

MATERIALS AND METHODS

Animal collection and haemolymph sampling

Specimens of the common British crayfish, *Austropotamobius pallipes* (Lereboullet) were collected and maintained as described by Taylor & Wheatly (1980, 1981). Haemolymph was withdrawn from intermoult animals *via* the pericardial space. The haemolymph was then frozen at −70 °C and transported under solid CO_2 to Düsseldorf where the investigation was carried out. The frozen material was allowed to thaw on ice and was then pooled. Clotted material was broken up by pressing the haemolymph through a fine nylon mesh and subsequent centrifugation at 10 000 ***g*** for 10 min. The resulting haemolymph was divided into 500 μl aliquots which were frozen until required. The possibility that freezing may affect the oxygenation properties of *A. pallipes* haemocyanin has been discussed (Morris, Bridges & Grieshaber, 1986) and, although some changes in cooperativity may occur, n_{50} is not markedly reduced and changes in P_{50} were considered unlikely.

Measurements in whole haemolymph

The concentrations of key ions in the haemolymph were determined using the methods described by Bridges, Morris & Grieshaber (1984), Morris, Bridges & Grieshaber (1985*a*) and Morris, Taylor, Bridges & Grieshaber (1985*b*), in which Ca^{2+} and Mg^{2+} were measured spectrophotometrically (test kits: 1028, Roche, Basel, Switzerland; Merkotest 3338, Darmstadt, FRG) and Cl^- was measured with a chloride titrator (CMT 10, Radiometer, Copenhagen, Denmark). The concentration of L-lactate in the haemolymph was measured according to the method of Gutmann & Wahlefeld (1974), modified by the addition of EDTA to the assay

(Engel & Jones, 1978; Graham, Mangum, Terwilliger & Terwilliger, 1983). Each determination was made at least twice.

The concentration of haemocyanin was determined by spectrophotometric scanning of 10 μl of haemolymph in 1 ml Ringer solution between 200 and 450 nm (Uvikon 810, Kontron, Munich, FRG). The haemocyanin concentration was then calculated from the absorbance maximum near 335 nm, assuming an extinction coefficient of 2·69 $E^{1\%}_{1\,cm}$ (Nickerson & van Holde, 1971).

Preparation of dialysed haemolymph

Aliquots of *A. pallipes* haemolymph (0·5 ml) were dialysed against a series of Ringer solutions. In each case the dialysis was carried out at 4°C for 24 h with the Ringer solution (2 l) being replaced after approximately 12 h. The stock Ringer was formulated on the basis of measured and literature values and had the following composition in mmol l^{-1}: NaCl, 181; KCl, 4·7; $CaCl_2$, 17; $MgCl_2$, 1·0 and $NaHCO_3$, 3·0 (pH = 8·0). Ringer solutions were also prepared with $CaCl_2$ concentrations of 49 mmol l^{-1} and 9 mmol l^{-1} to encompass the *in vivo* variation in $[Ca^{2+}]$ that occurs during aerial exposure of the crayfish (E. W. Taylor, unpublished results). The concentration of NaCl was changed correspondingly to ensure that the concentration of Cl^- remained constant. In addition to this each of the three Ringer solutions was prepared with either low lactate (1·0 mmol l^{-1} L-(+)-lactate, Sigma Chemie GmbH, Taufkirchen, F.R.G.) or high lactate (approx. 8·0 mmol l^{-1}) to give a total of six different Ringer solutions against which native *A. pallipes* haemolymph was dialysed. The exact concentrations of Ca^{2+} and L-lactate in each dialysed haemolymph preparation were measured subsequent to the construction of oxygen equilibrium curves.

The possible existence of a specific effect of CO_2 that might increase the oxygen affinity of *A. pallipes* haemocyanin was investigated using the methods of Morris *et al.* (1985*b*) and of Morris & Bridges (1985). Dialysed haemolymph samples (100 μl) were centrifuged in an 'air-fuge' (Beckman, California, USA) for 45 min at 140 000 ***g*** in a precooled rotor. After centrifugation, 40 μl of the supernatant was removed and a measured amount of this replaced by either 0·1 mol l^{-1} HCl or 0·1 mol l^{-1} NaOH (5–30 μl) in *A. pallipes* Ringer. The resulting mixture was carefully remixed with the haemocyanin pellet. Control measurements were made using solutions in which a 30 μl replacement was made with Ringer alone. Oxygen equilibrium curves were then constructed at constant P_{CO_2} tensions of 0·7 and 10·5 Torr for haemolymph in which the pH had been fixed by the addition of acid or base. By comparing this 'fixed acid' Bohr shift to the Bohr shift induced by CO_2, any specific effect of CO_2 on oxygen affinity could then be distinguished from the CO_2 Bohr shift.

Construction of oxygen equilibrium curves

The curves were constructed using a spectrophotometric method on 8 μl samples in a diffusion chamber (Sick & Gersonde, 1969) as described by Bridges, Bicudo & Lykkeboe (1979). Briefly, oxygen tension was varied using gas mixing pumps

(Wösthoff, Bochum, FRG), and the pH of the haemolymph was varied by adjusting the CO_2 content of the gas mix between 0·1 and 2·0%. The same gas mixtures were also supplied to the tonometers of a BMS II (Radiometer) to enable the haemolymph pH to be measured near the P_{50} using the microelectrode (G299, Radiometer) of the BMS II. All determinations were made at 15°C. The values of P_{50} and cooperativity (n_{50}) were calculated by regression analysis of the saturation values between 25% and 75% using the Hill equation. Where appropriate, values throughout this paper are given as means ±1 S.D. unless otherwise stated.

RESULTS

The haemocyanin concentration of the native *A. pallipes* haemocyanin (Hcy) used in the investigation was 53·5 mg Hcy ml^{-1} and the dialysed haemolymph preparations had a mean concentration of 50·1 ± 6·8 mg Hcy ml^{-1}. Consequently, the concentration of haemocyanin was assumed to remain effectively constant throughout the experiment.

In the pooled haemolymph, L-lactate averaged 1·01 ± 0·02 mmol l^{-1}. The concentrations of L-lactate measured in the dialysed haemolymph preparations are given in Figs 1, 2, 3. The measured ion values of untreated *A. pallipes* haemolymph were 9·7 ± 2·1 mmol l^{-1} for Ca^{2+} and 198 ± 1·0 mmol l^{-1} for Cl^-, the concentration of Mg^{2+} was low at 1·9 ± 0·2 mmol l^{-1}. In the dialysed haemolymph preparations, these ions, with the exception of Ca^{2+}, were maintained constant. The concentration of Ca^{2+} is given, in each case, together with the appropriate figure.

The P_{50} values obtained from fixed acid preparations were subjected to least-squares regression analysis in order to determine whether a specific effect of CO_2 was present in this species. The calculated equations for the dependence of $\log P_{50}$ on pH in fixed acid preparations were:

$$0{\cdot}1\%\ CO_2 \quad \log P_{50} = 3{\cdot}875 - 0{\cdot}437\text{pH} \quad (r = -0{\cdot}97)$$
$$2{\cdot}0\%\ CO_2 \quad \log P_{50} = 3{\cdot}299 - 0{\cdot}369\text{pH} \quad (r = -0{\cdot}86).$$

Analysis of covariance indicated no significant difference in the elevation of the slopes described by these equations, nor could they be demonstrated to differ from the data describing the change in $\log P_{50}$ that occurred as a result of a CO_2 Bohr shift. The CO_2 Bohr effect ($\varphi = -0{\cdot}455$) and the fixed acid Bohr effect were not statistically different. No specific effect of CO_2 on the oxygen affinity of the haemocyanin was observed.

The result of dialysing the haemolymph was quite clearly to reduce the apparent affinity of *A. pallipes* haemocyanin for oxygen (Fig. 1A,B). The data show a clear specific effect of L-lactate (Fig. 1A), which increased the oxygen affinity of dialysed haemolymph. In the example given (Fig. 1A), increasing L-lactate from 0·9 mmol l^{-1} to 7·2 mmol l^{-1} reduced the P_{50} from 6·4 Torr to 4 Torr (pH = 7·9).

Similar results were obtained when pH and L-lactate concentration were maintained at whole haemolymph values and the concentration of calcium ions was increased (Fig. 1B). Increasing the concentration of Ca^{2+} from the whole

haemolymph value of 9·7 mmol l^{-1} to 17 mmol l^{-1} produced an increase in oxygen affinity of 2·8 Torr. Increasing the concentration of Ca^{2+} further to 45 mmol l^{-1} elicited a further increase.

Interactive effects of these two modulators were investigated by increasing the concentration of both Ca^{2+} and L-lactate together (Fig. 2), which increased oxygen affinity from a P_{50} value of 6·4 Torr to a P_{50} value of 2·9 Torr. A simple additive effect of the two ions (i.e. adding the lactate effect in Fig. 1A to the Ca^{2+} effect in Fig. 1B) would be expected to increase oxygen affinity beyond this to a P_{50} value near 1·5 Torr (Fig. 2). There was, therefore, clear evidence of agonism in the effects of L-lactate and calcium ions.

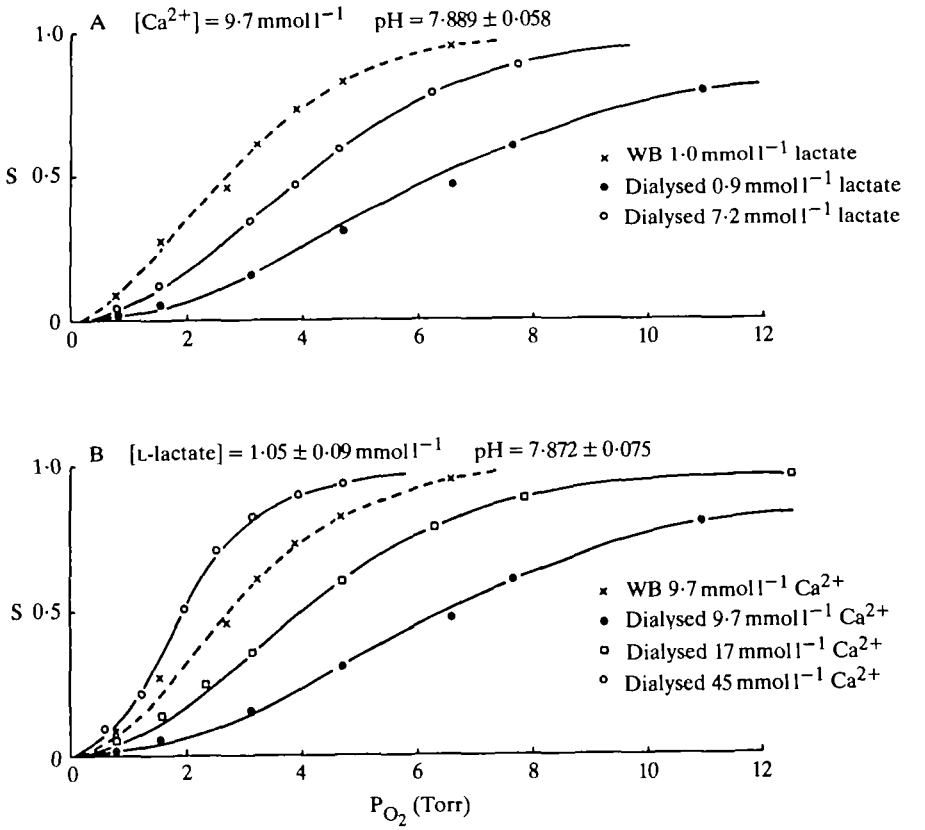

Fig. 1. (A) Oxygen equilibrium curves showing the decrease in the oxygen affinity at 15 °C of *Austropotamobius pallipes* haemolymph when dialysed against crayfish Ringer in which the concentrations of L-lactate and calcium ions are maintained at the concentration in whole haemolymph. Also shown is the effect of increasing the L-lactate concentration in dialysed haemolymph above the concentration in whole haemolymph. (B) The effect of calcium ions on the oxygen affinity of dialysed blood at a constant L-lactate concentration. The oxygen affinity of dialysed haemolymph containing calcium ions at a similar concentration to that in whole haemolymph is compared to the affinity of undialysed *A. pallipes* haemolymph. WB, whole blood.

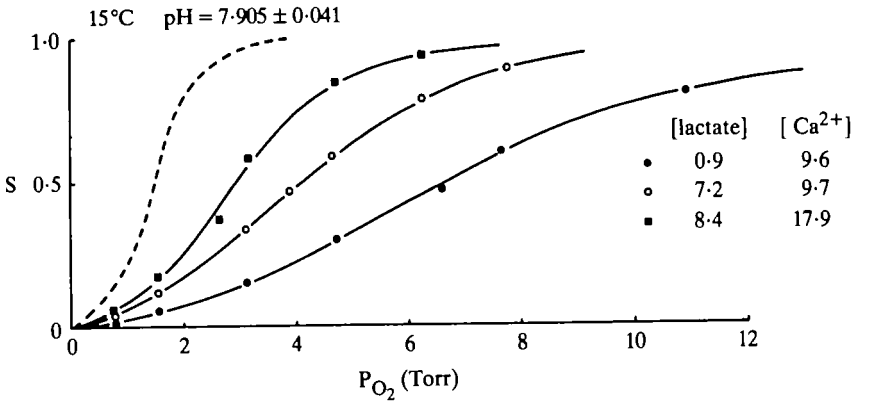

Fig. 2. Oxygen equilibrium curves demonstrating the cumulative effects of L-lactate and Ca^{2+} on the oxygen affinity of dialysed *Austropotamobius pallipes* haemolymph. The broken line represents the hypothetical curve that results from a simple addition of the effects of Ca^{2+} and L-lactate calculated from the data in Fig. 1. The curve actually determined for haemocyanin in the presence of elevated concentrations of Ca^{2+} and L-lactate is shown to the right of the hypothetical curve. Concentrations are given in mmol l^{-1}.

The interdependent nature of the potentiating effects of Ca^{2+} and L-lactate on the oxygen affinity of *A. pallipes* haemocyanin was investigated further and a larger range of experimental conditions were considered (Fig. 3). Examination of these data revealed that: (a) the increase in haemocyanin oxygen affinity brought about by increasing L-lactate concentrations became progressively reduced as the concentration of Ca^{2+} became more elevated; and (b) the effect of Ca^{2+} on oxygen affinity was similarly reduced by increasing the concentration of L-lactate. It was not possible to conclude that either L-lactate or Ca^{2+}, alone or together, were able significantly to affect the cooperativity (n_{50}) or the Bohr value ($-0{\cdot}455 \pm 0{\cdot}033$). The interdependence of these modulators can be more accurately expressed by the equations in Table 1. The specific effect of L-lactate has been shown, when present, to be correlated as log [lactate$^-$] with logP_{50} (see Truchot, 1980; Bridges *et al.* 1984) and identical methods have been employed here. Although Truchot (1975*b*) correlated $\sqrt{[Ca^{2+}]}$ with logP_{50}, a more significant relationship was found in this study when log$[Ca^{2+}]$ was used (see also Mason, Mangum & Godette, 1983). All equations in Table 1 were calculated by least-squares regression analysis of values calculated from the regression equations of the data in Fig. 3. The progressive decrease in the magnitude of the lactate and calcium effect coefficients (Table 1) clearly demonstrates the decreased efficacy of lactate when $[Ca^{2+}]$ was increased, and also the reduced effect of Ca^{2+} when [L-lactate] was increased. Covariance analysis of the P_{50} *vs* pH data obtained in the presence of high and low L-lactate concentrations and at three different concentrations of calcium ions further demonstrated this interdependence. Increasing the calcium concentration from 9 mmol l^{-1} to 17 and then to 47 mmol l^{-1} could be correlated with a decrease in the variance ratio ($F = 228$, 34 and 13·6, respectively) obtained from comparisons of data from the high and low [L-lactate] preparations. The third F value (highest Ca^{2+} concentration) was significant at the 1 % probability level, whereas those for data obtained at the middle and

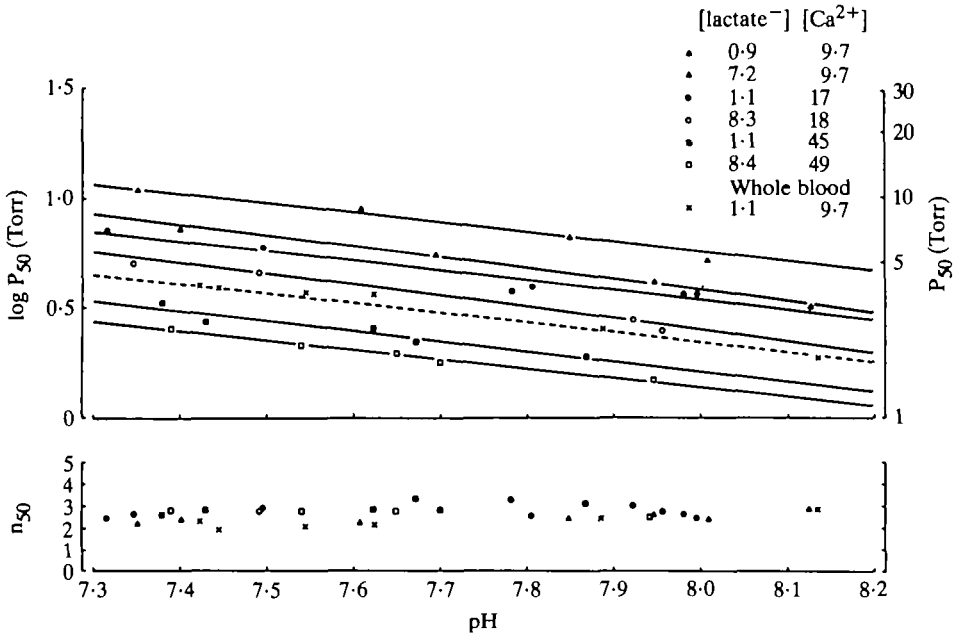

Fig. 3. The relationship between pH and $\log P_{50}$ at 15 °C in dialysed *Austropotamobius pallipes* haemolymph containing different concentrations of L-lactate and Ca^{2+}. The dependence of $\log P_{50}$ on pH in whole haemolymph containing the *in vivo* concentration of these cofactors is also shown. The effect of changing pH on the cooperativity (n_{50}) of the haemocyanin is shown for all of the combinations of Ca^{2+} and L-lactate in the lower panel. Concentrations are given in $mmol\,l^{-1}$.

lower concentrations showed a more significant lactate effect (probabilities of 0·5 and 0·1 %).

DISCUSSION

The potentiation of oxygen affinity by calcium and L-lactate

The strong potentiating effects of Ca^{2+} on the oxygen affinity of *A. pallipes* haemocyanin reported here concur with the early observations of Hogben (1926) working on *Homarus* and of Stedman & Stedman (1926) working on *Cancer* haemocyanin. Investigations of the binding of Ca^{2+} to haemocyanin (e.g. Morimoto & Kegeles, 1971; Brouwer, Bonaventura & Bonaventura, 1978; Arisaka & van Holde, 1979) have usually employed conditions that are outside the physiological range considered here for *A. pallipes*. Nevertheless, these more mechanistic studies demonstrate several features of Ca^{2+}–haemocyanin interaction. For example, Kuiper *et al.* (1979) demonstrated that Ca^{2+}-binding by the haemocyanin caused a pH-dependent H^+ liberation and that the binding of Ca^{2+} was dependent on oxygenation state. Although a dependency of cooperativity (n_{50}) on $[Ca^{2+}]$ has been reported (Miller & van Holde, 1981; Larimer & Riggs, 1964; Chantler, Harris & Bannister, 1973), the present study, like that of Mason *et al.* (1983), found no significant increase in n_{50} when $[Ca^{2+}]$ was increased. These authors (Mason *et al.* 1983) also compared the relatively high value for $\Delta\log P_{50}/\Delta\log[Ca^{2+}]$ of $-0{\cdot}82$ in

Table 1. *The effect* in vitro *of different concentrations of calcium ion and* L*-lactate on the oxygen affinity of* Austropotamobius pallipes *haemocyanin*

A The calcium dependency of the lactate effect

$[Ca^{2+}]$ (mmol l^{-1})	pH 7·4	pH 7·9
9	$\log P_{50} = 1{\cdot}025 - 0{\cdot}175 \log[\text{lactate}^-]$	$\log P_{50} = 0{\cdot}787 - 0{\cdot}194 \log[\text{lactate}^-]$
17	$\log P_{50} = 0{\cdot}816 - 0{\cdot}124 \log[\text{lactate}^-]$	$\log P_{50} = 0{\cdot}589 - 0{\cdot}147 \log[\text{lactate}^-]$
47	$\log P_{50} = 0{\cdot}485 - 0{\cdot}115 \log[\text{lactate}^-]$	$\log P_{50} = 0{\cdot}253 - 0{\cdot}080 \log[\text{lactate}^-]$

B The lactate dependency of the calcium effect

$[\text{lactate}^{-1}]$ (mmol l^{-1})	pH 7·4	pH 7·9
1	$\log P_{50} = 1{\cdot}743 - 0{\cdot}750 \log[Ca^{2+}]$	$\log P_{50} = 1{\cdot}493 - 0{\cdot}735 \log[Ca^{2+}]$
8	$\log P_{50} = 1{\cdot}524 - 0{\cdot}676 \log[Ca^{2+}]$	$\log P_{50} = 1{\cdot}116 - 0{\cdot}528 \log[Ca^{2+}]$

All determinations were made in dialysed haemolymph and ion concentrations were determined as mmol l^{-1}.

Callinectes sapidus with the lower value of −0·28 in *Carcinus maenas* (Truchot, 1975*b*). Using their criteria, the effect of Ca^{2+} on the haemocyanin O_2 affinity in *A. pallipes* must also be considered to be large when [L-lactate] is at resting values ($\Delta \log P_{50}/\Delta \log[Ca^{2+}] = -0{\cdot}735$).

Data demonstrating the binding of L-lactate to haemocyanin are less plentiful as the discovery of this effect is relatively recent (Truchot, 1980) and is at present limited to the work of Mangum (1983*a*) and of Johnson, Bonaventura & Bonaventura (1984). The effect of L-lactate on the oxygen affinity of haemocyanin from *A. pallipes* is, however, similar to previously quantified lactate effects (Truchot, 1980; Bridges *et al.* 1984; Morris & Bridges, 1985; Morris *et al.* 1985*a*). When compared with the estimates of $\Delta \log P_{50}/\Delta \log[\text{lactate}]$ tabulated by Bridges *et al.* (1984), which ranged from 0 to −0·560 (pH 7·8), the value of −0·194 (pH 7·9) for *A. pallipes* haemocyanin can be seen to represent a marked but unexceptional effect. There is no direct evidence that lactate binding is related to the number of H^+-binding sites on the haemocyanin molecule as is apparently the case with the Ca^{2+} effect (Larimer & Riggs, 1964; Kuiper *et al.* 1979) and the postulated alternatives for the mechanism of the lactate effect (Johnson *et al.* 1984) would seem to indicate that this is not the case.

Comparison of $\Delta \log P_{50}/\Delta \log[Ca^{2+}]$ values with those for $\Delta \log P_{50}/\Delta \log[\text{lactate}]$ indicates that the molecular relationship between haemocyanin and Ca^{2+} is different to that between haemocyanin and L-lactate. In *A. pallipes* Ca^{2+} would appear to be more effective on a molar basis than lactate in enhancing haemocyanin oxygen affinity.

The interaction of the potentiating effects of Ca^{2+} and L*-lactate*

It was concluded that the effect of either Ca^{2+} or lactate on the O_2 affinity of dialysed *A. pallipes* haemocyanin was progressively reduced by the presence of ions

of the other species. The possibility of Ca^{2+} and lactate interacting as free ions was empirically investigated by Ghosh & Nair (1970), who found that the reaction $Ca^{2+} + Lactate^- \rightleftharpoons CaLactate^+$ occurred in aqueous solution but that $CaLactate_2$ was not formed. A similar complexing of lactate with Ca^{2+} was found in turtle blood by Jackson & Heisler (1982). In the anoxic turtle blood, the concentration of Ca^{2+} rose to $68\,mmol\,l^{-1}$ and that of L-lactate to $145\,mmol\,l^{-1}$ which should not be considered comparable to the $8–10\,mmol\,l^{-1}$ that occurs in aerially exposed *A. pallipes* (Taylor & Wheatly, 1981). The formation of $CaLactate^+$ in crustacean haemolymph was shown to be unlikely by the work of Graham *et al.* (1983). These authors showed that the haemocyanin of *Cancer magister* exhibits lactate sensitivity but were unable to demonstrate a change in free Ca^{2+} concentration when lactate concentrations were increased. Interactive effects of Ca^{2+} and lactate may not be a general feature of crustacean haemocyanin.

The effect of these two ions on the oxygen affinity of *A. pallipes* haemocyanin can be summarized graphically. By substituting a given value for $\log P_{50}$ at pH 7·9 into the equations (Table 1) it was possible to solve the relationship for either $[Ca^{2+}]$ or $[lactate^-]$. The resulting points were used to construct Fig. 4. The interdependence of the effects of Ca^{2+} and L-lactate is manifest in the curved isopleths describing given P_{50} values for *A. pallipes* haemocyanin. Interestingly, Mangum (1983*a*) comments on the similar effect of Ca^{2+} on *Callinectes sapidus* haemocyanin O_2 affinity, concluding that a specific effect of lactate added to the large effect of Ca^{2+} would increase affinity so much as to render it non-adaptive. It would appear from the data now available from *A. pallipes* that this additive effect is reduced due to a suppression of one by the other.

Other factors affecting haemocyanin oxygen affinity

During emersion the arterial haemolymph P_{CO_2} in *A. pallipes* was reported to rise from 3·0 Torr in submerged normoxic animals to 10·6 Torr after 12 h in air (Taylor & Wheatly, 1981). No role could be attributed to CO_2 in the haemolymph of *A. pallipes*. Literature reports of a specific effect of CO_2 on oxygen affinity are limited to Truchot (1973), Weber & Hagermann (1981) and Morris *et al.* (1985*b*). Other studies such as those of Morris & Bridges (1985) and Burnett & Infantino (1984) were also unable to demonstrate specific CO_2 effects and the absence of such effects would now seem to be the normal condition in crustaceans.

This study has demonstrated, however, the presence of at least one unidentified factor that increases the oxygen affinity of the haemocyanin. Although the low specificity of this factor has been recently demonstrated (Morris *et al.* 1985*a*) little is known about its role in *A. pallipes*. In a recent study (Morris *et al.* 1986) the effects of factors other than L-lactate and Ca^{2+} have been considered and may offer an explanation of the absence of a lactate effect in some species (see Mangum, 1983*b*).

The present observations suggest that the reduction in the oxygen affinity of *A. pallipes* haemolymph during aerial exposure, which might be expected as a result of a Bohr shift, may not occur. Instead it is possible that the combined interactions of

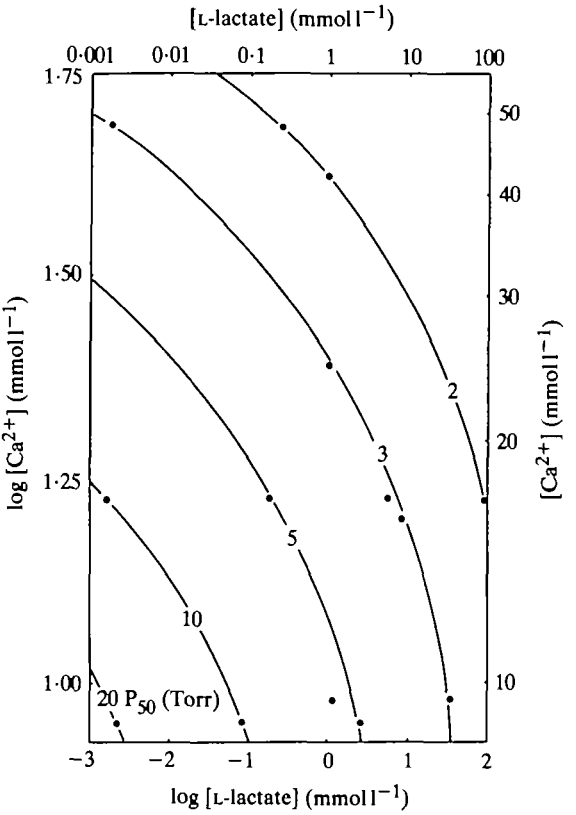

Fig. 4. A graphical representation of the dependency of haemocyanin P_{50} on L-lactate and Ca^{2+} concentration in dialysed *Austropotamobius pallipes* haemolymph at pH 7·9 and 15°C. The data points shown were determined using the data shown in Fig. 3 and the equations in Table 1. The concentrations of Ca^{2+} and L-lactate are shown on both arithmetic and logarithmic scales. The third variable, the P_{50} of the haemocyanin, is shown as a series of isopleths (Torr) which demonstrate how the oxygen affinity of *A. pallipes* haemocyanin responds to changes in the concentration of both Ca^{2+} and L-lactate. For further details see text.

Ca^{2+}, L-lactate and HCO_3^- in the haemolymph compensate for the effects of acidosis on the oxygen affinity of the haemocyanin and may even increase the affinity above that found in resting crayfish under normoxia.

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