EFFECT OF MAINTAINED HYPOXIC EXPOSURE ON THE CRAYFISH *ORCONECTES RUSTICUS:*

II. MODULATION OF HAEMOCYANIN OXYGEN AFFINITY

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SUMMARY

Haemolymph Na^+, Cl^-, K^+, Mg^{2+}, Ca^{2+}, Cu^{2+} and protein levels, *in vivo* postbranchial acid-base status (total CO_2, pH and P_{CO_2}), *in vitro* haemolymph buffer value, Bohr value and oxygen affinity were measured before and after a $3\frac{1}{2}$-week period in which control crayfish were maintained at normoxia and experimental crayfish were maintained at an ambient oxygen tension of 50–55 torr. Analysis of haemolymph Cu^{2+} and protein levels in control and experimental crayfish indicated no increase in haemocyanin and therefore oxygen carrying capacity of the haemolymph. Although the Bohr value was not significantly different between control and experimental crayfish, the haemocyanin oxygen affinity was elevated in the hypoxic crayfish by two mechanisms. The first was dependent upon the haemolymph H^+ concentration, i.e. a Bohr shift resulting from a respiratory alkalosis. The second mechanism was independent of haemolymph H^+ concentration in that at a given pH haemolymph from experimental crayfish had a significantly higher oxygen affinity. The decrease in P_{50} probably cannot be attributed to a specific cation effect.

INTRODUCTION

Changes in haemocyanin oxygen affinity often play an important role during respiratory compensation for environmental change (Mangum & Towle, 1977). Modulation of oxygen affinity resulting from change in H^+ concentration (i.e. Bohr effect) are well known (Butler, Taylor & McMahon, 1978; McMahon, Butler & Taylor, 1978b; Burnett, 1979; Wilkes & McMahon, 1982), but other modulating mechanisms are available. Of these, the most completely documented is control of the ionic environment of the haemocyanin molecule. Oxygen affinity varies directly both with the total ionic strength of the haemolymph, as in *Callinectes sapidus* (Mangum & Weiland, 1975) and *Carcinus maenas* (Truchot, 1975), and with divalent cation concentration, as in *Procambarus simulans* (Larimer & Riggs, 1964), *Carcinus mediterraneus, Potoman edulis* (Chantler, Harris & Bannister, 1975) and *Carcinus maenas* (Truchot, 1975). Additionally, there is evidence of an unidentified, dialysable component of haemolymph in *C. maenas* (Truchot, 1975) and *Pacifasticus leniusculus*

(M. G. Wheatly & B. R. McMahon, in prep.) which can also affect oxygen affinity. Finally, crustacean haemocyanin is a complex protein which can exist in discrete associations of a number of subunits, changing combinations of which may allow control of oxygen affinity (van Holde & van Bruggen, 1971; Sullivan, Bonaventura & Bonaventura, 1974; C. P. Mangum, in prep.).

Although the increased affinity of oxygen binding observed during hypoxic exposure in crustaceans has previously been associated with increase in haemolymph pH (Butler *et al.* 1978; McMahon *et al.* 1978b; Burnett, 1979; Wilkes & McMahon, 1982), the roles of other modulating factors are not known. The present study on the crayfish *Orconectes rusticus* demonstrates the presence of additional factors which supplement the Bohr effect in maintaining elevated oxygen affinity during chronic hypoxic exposure.

METHODS AND MATERIALS

Orconectes rusticus (10–15 g body weight) were obtained from the Kawartha Lakes district around Peterborough, Ontario, and shipped by air to the University of Calgary where this work was performed. All animals were maintained in normoxic non-recirculated water at 15 °C for a minimum of 2 weeks prior to experimentation. After this period an experimental group of 73 animals was maintained in a 10 × 61 × 225 cm opaque aquarium while 33 control animals were maintained in a similar 10 × 76 × 122 cm aquarium. Both tanks were supplied with recirculated water at 15 °C (*ca.* 4 l/animal). All animals were allowed complete freedom of the tanks, but normally occupied crevices between rocks placed throughout the aquaria. Visual disturbance was reduced by covering the tanks with dark plastic. Animals were fed a diet of frozen smelt and Tetramin 2–3 times weekly.

Seventy-two hours prior to the initial sampling in normoxia animals were equipped with numbered plastic tags glued to the carapace and with haemolymph sampling ports as described by Wilkes & McMahon (1982). A further 72 h period to allow recovery from sampling preceded hypoxic exposure. To minimize blood loss in these small animals, both control and experimental crayfish were subdivided into four subgroups from which samples were taken for specific measurements as follows. Post-branchial haemolymph samples (obtained as described by Wilkes & McMahon, 1982) from the three subgroups were used to assess *in vivo* levels of:

Group (i): Na^+, Cl^-, K^+, Ca^{2+}, Mg^{2+} and total protein concentration (100 μl).
Group (ii): Cu^{2+} and total protein concentration (200 μl).
Group (iii): pH, total carbon dioxide (100 μl).

Samples from the 4th subgroup were limited to 250 μl (6% haemolymph volume, Kerley & Pritchard, 1967) and were pooled and declotted to provide serum of which aliquots were used to determine both buffer curves ($\Delta C_{CO_2}/\Delta pH$) and oxygen equilibrium curves. All four sets of measurements were repeated on both control and hypoxic exposed crayfish after the $3\frac{1}{2}$-week experimental period. Additionally the haemolymph acid-base status of both control and experimental crayfish was re-examined 6 days following return to normoxic conditions.

Hypoxic water was produced by passing re-circulated water through an oxygen

stripping column as described by Wilkes & McMahon (1982). The oxygen tension of the water was continuously monitored by an E51443/0 oxygen electrode connected to a TOX 40 oxygen transmitter and displayed on a CME 40 on/off controller (Radiometer) (McMahon *et al.* 1978*b*). Controller limits were set at 50 and 55 torr and ambient P_{O_2} was thus maintained at $53 + 2$ torr for the $3\frac{1}{2}$-week experimental period. The large surface area and re-circulation maintained ambient oxygen tension in the control tank near saturation (110-120 torr, elevation 1048 m at Calgary).

Analysis of haemolymph cations were made using an atomic absorption spectrophotometer (Jarrell Ash 850) calibrated with certified atomic absorption stock solutions (Fisher) diluted with double-distilled water. Single-element hollow cathode lamps (Corning) were used.

Haemolymph, 100 μl, samples were diluted in double-distilled water to appropriate concentrations for each ion. Samples used for Ca^{2+} and Mg^{2+} measurements were made in $LaCl_3$ to suppress chemical interference. Cl^- concentration was determined using an Orion 94–17 chloride specific electrode and a 90–01 double junction reference electrode. Potentials were displayed on a Beckman Model 4500 digital pH meter. A calibration curve was constructed using appropriate dilutions of Orion 100 ppm chloride standard in double-distilled water. Total haemolymph protein concentration was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, technical bulletin 1051, 1977).

Oxygen equilibrium curves were constructed on 1·0 ml of pooled serum using a mixing method (Haab, Piiper & Rahn, 1960) that has recently been verified for rabbit and human blood by Schied & Meyer (1978). Briefly, serum was equilibrated with either humidified air or nitrogen containing equal P_{CO_2} levels obtained using Wöstoff gas-mixing pumps. Accurate volumes of the samples were mixed in a 100 μl Hamilton syringe and P_{O_2} measured with a Radiometer E5046 O_2 electrode thermostatted to 15 °C and displayed on an Acid-Base Analyzer (Radiometer PHM 71, 72, or 73). The volume of oxygen carried by the haemolymph was determined from the product of percent saturation and the oxygen content of the 100 % saturated serum, as measured using a Lex-O_2-Con Oxygen Analyzer (Lexington Inst.) with modifications as per McMahon *et al.* (1978*a*). The difference between the oxygen content of the haemolymph and the volume of oxygen carried in physical solution is the oxygen capacity of the haemocyanin molecule itself. The fraction of dissolved oxygen was calculated from the oxygen tension and the oxygen solubility coefficient corrected for temperature and osmotic strength of the haemolymph. The osmotic strength of the haemolymph was measured by freezing-point depression (Advanced Osmometers, Ltd.) on ten individual haemolymph samples.

The Bohr shift ($\phi = \Delta \log P_{50}/\Delta pH$) was quantified by constructing oxygen equilibrium curves at 3–4 different pH levels produced by altering P_{CO_2} of the equilibrating gases between 1·5–7·0 torr with Wöstoff gas-mixing pumps.

Buffer curves ($\Delta C_{CO_2}/\Delta pH$) were constructed on 0·5–1·0 ml of pooled serum sequentially equilibrated in tonometers at 15 °C with gas-mixing pumps (Wöstoff) to P_{CO_2} levels of 2·4, 5·0, 8·3 and 13·5 torr. Full equilibration at 30 min was ensured by the addition of bovine carbonic anhydrase (1 μg.ml^{-1}, Sigma) to the haemolymph. For both *in vitro* determination of buffering capacity and *in vivo* measurements of

Table 1. *A comparison of the number of deaths, moults, and moults resulting in death between the control (normoxic) crayfish and experimental crayfish maintained under hypoxic conditions for $3\frac{1}{2}$ weeks*

	Hypoxia		Normoxia	
	Number	%	Number	%
Start	73	100	33	100
Total moults	14	19	18	54
Total deaths	15	20	4	12
Deaths from moults	8	11	3	9
Total loss of experimental animals	29	40	22	67

haemolymph acid-base status total carbon dioxide (C_{CO_2}) and pH were measured as described by Wilkes & McMahon (1982). *In vivo* haemolymph P_{CO_2} was calculated from an empirically determined relationship between P_{CO_2}, C_{CO_2} and pH (Wilkes, deFur & McMahon, 1980).

Since the crayfish were all from a single population, prehypoxic measurements from both experimental and control groups were combined. During the course of the experiment, daily records were made of moults and deaths in each group. All data are from non-moulting survivors. All values are reported as $\bar{X} \pm$ S.E.M. and are reported significantly different when $P < 0.05$ according to Student's t test. The Bohr curves ($\Delta \log P_{50}/\Delta pH$) for control and experimental crayfish were statistically evaluated by analysis of covariance according to Snedecor (1956).

<div align="center">RESULTS</div>

The data were analysed in three groups: prehypoxic (P), $3\frac{1}{2}$-week controls (C) and experimentals (E). A number of deaths and/or moults occurred during the experimental period (Table 1). Although the number of moults was similar in both groups, the number of deaths and unsuccessful moults which resulted in death were higher in the experimental group. In order to avoid anomalies resulting from either ecdysis or poor health, data from these individuals were deleted from all analysis, including prehypoxic measurements.

Effects of hypoxic exposure of haemolymph acid-base status

Prior to the experimental regime, mean postbranchial pH was 7.716 ± 0.02 at a mean C_{CO_2} level of 6.4 ± 0.4 mmol.l^{-1}. After $3\frac{1}{2}$ weeks, hypoxic exposed animals had become significantly alkalotic (pH $= 7.915 \pm 0.042$, $C_{CO_2} = 4.9 \pm 0.1$ mmol.l^{-1}) when compared with controls (7.795 ± 0.034; 6.6 ± 0.8 mmol.l^{-1}) (Fig. 1). Following 6 days recovery in normoxic water the significant difference in pH had been abolished. For reasons unknown, C_{CO_2} levels rose significantly during the recovery in both normoxic and previously hypoxic groups (Fig. 1).

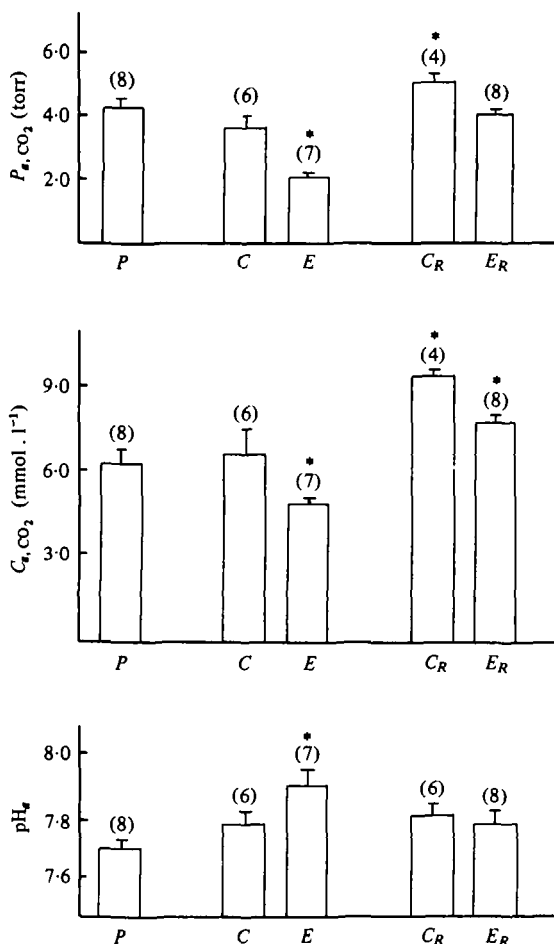Due to the limited haemolymph sample volumes available from these small crayfish

Fig. 1. Postbranchial values of P_{a,CO_2}, C_{a,CO_2} and pH_a. P, Initial control values prior to experimental period; C, control values after $3\frac{1}{2}$ weeks normoxia; E, experimental values after $3\frac{1}{2}$ weeks hypoxia; R, represents recovery values for E and C respectively 6 days after $3\frac{1}{2}$-week treatment. The number in parentheses represents n; * denotes values significantly different from P.

and to the remarkable clotting properties of crayfish haemolymph, postbranchial carbon dioxide tensions (P_{a,CO_2}) were not measured directly, but rather were calculated from measured C_{CO_2} and pH using the method of Wilkes *et al.* (1980). Prior to hypoxic exposure mean P_{a,CO_2} was calculated to be $4·2 \pm 0·03$ torr; $3\frac{1}{2}$ weeks later the control values did not differ significantly but P_{a,CO_2} of the hypoxic group had declined significantly to $2·1 \pm 0·03$ torr. Six days following return to normoxic water P_{a,CO_2} levels of these crayfish had risen to levels not significantly different from the pre-hypoxic values. P_{a,CO_2} levels, however, also rose significantly in control crayfish at this time.
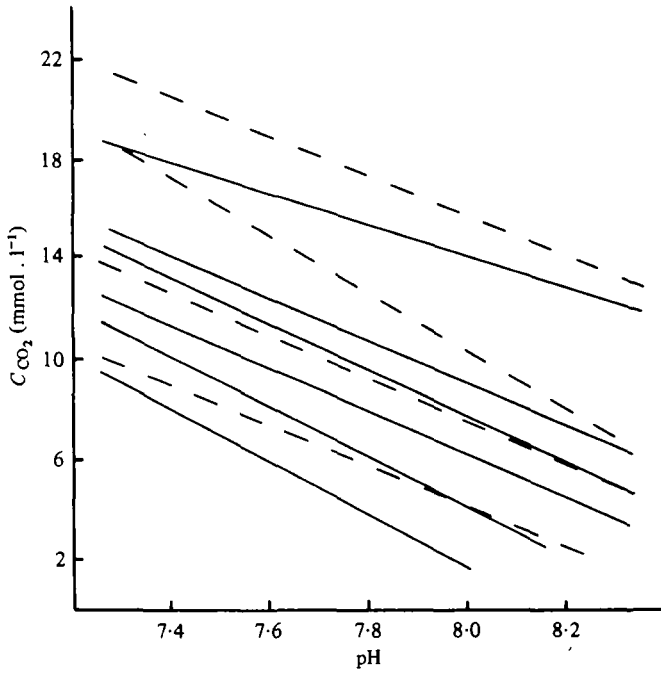
Fig. 2. Haemolymph buffer curves from experimental animals (- - -) after 3½ weeks hypoxia, and from control animals (———) from both before and after 3½ weeks normoxia.
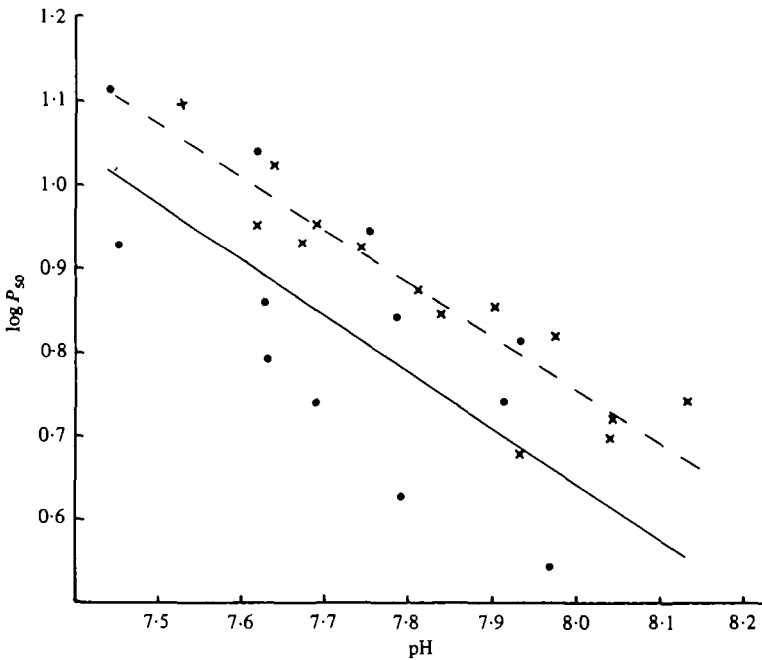
Fig. 3. Log P_{50} v. pH for experimental animals after 3½ weeks hypoxia (●) and controls (×) from both before and after 3½ weeks normoxia.
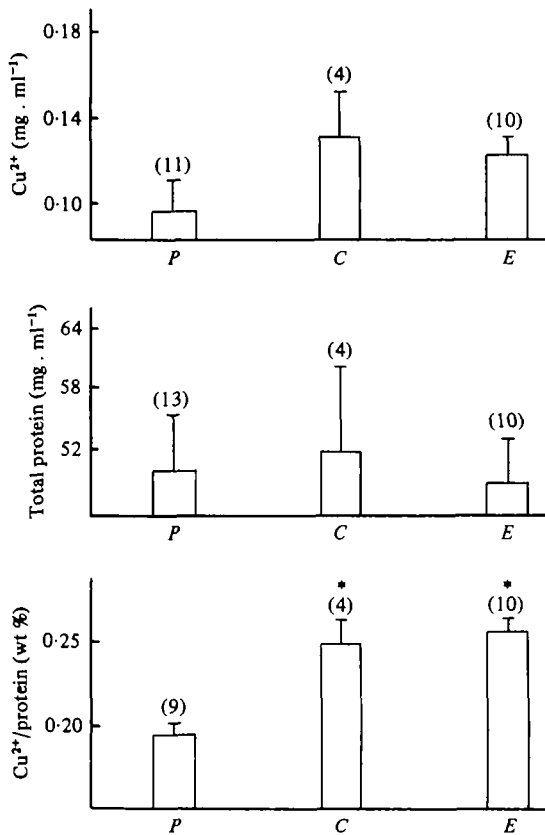
Fig. 4. Haemolymph Cu^{2+} (mg.ml^{-1}), total protein (mg.ml^{-1}), and Cu^{2+}/protein ratio (wt %); *P*, initial control values prior to 3½-week experimental period; *C*, control values after 3½ weeks normoxia; *E*, experimental values after 3½ weeks hypoxia. The number in parentheses represents *n*; * denotes values significantly different from *P*.

Effects of hypoxic exposure on haemolymph buffer value

Haemolymph buffer values (Fig. 2) demonstrated considerable variability in the elevation of the buffer curves reflecting marked variability in C_{CO_2} in both control and experimental groups. No significant difference in buffer value (i.e. the slope of the relationship $\Delta C_{CO_2}/\Delta pH$) apparently resulted from the hypoxic treatment.

Effects of hypoxic exposure on the Bohr shift and haemolymph oxygen affinity

There was no significant difference in either the variance or the magnitude of the Bohr value, which was 0·629 and 0·683 for sera from hypoxic and control animals respectively. However, under identical *in vitro* conditions the Bohr curve for sera from experimental animals was significantly lower ($P < 0·01$) than in controls (Fig. 3). Thus, at any given pH the haemocyanin of the crayfish exposed to 3½ weeks hypoxic water had a significantly greater oxygen affinity (i.e. a lower P_{50}) than normoxic crayfish.
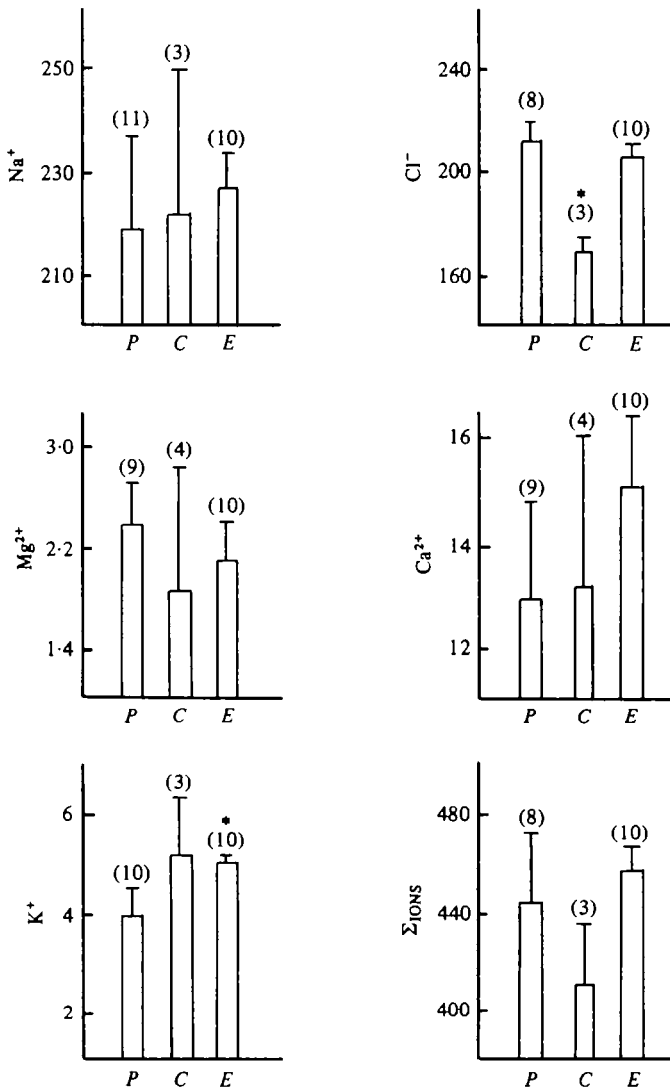
Fig. 5. Haemolymph ion levels in m-equiv. l⁻¹. P, Initial control values prior to experimental period; C, control values after 3½ weeks normoxia; E, experimental values after 3½ weeks hypoxia. The number in parentheses represents n; * denotes values significantly different from P.

Effects of hypoxic exposure on haemocyanin levels

Total haemolymph protein and Cu^{2+} levels were measured before and after 3½ weeks hypoxic exposure to establish whether additional haemocyanin had been produced (Senkbeil & Wriston, 1981). No significant increase resulted (Fig. 4). A small but significant increase in the Cu^{2+}/protein ratio was noted following the experimental period but occurred equally in both control and experimental groups.

Effects of hypoxic exposure on haemolymph ion levels

Haemolymph ion levels were measured because of their importance in controlling haemocyanin oxygen affinity. However, considerable variability was observed in levels of all ions over the experimental period. Perhaps as a result, between group analysis revealed only two significant changes (Fig. 5). Haemolymph Cl^- concentration decreased significantly below prehypoxic values in the control animals, while K^+ was elevated above prehypoxic values in the experimental group. There was, however, no significant difference in K^+ concentration between control and experimental crayfish.

DISCUSSION

In *Orconectes rusticus*, as well as in some brachyuran crustaceans, *H. vulgaris* (Mc-Mahon *et al.* 1978*b*), and *C. maenas* (Truchot, 1975; Burnett, 1979), oxygen uptake from hypoxic water is promoted by an increase in oxygen affinity of haemocyanin. In *H. vulgaris* (McMahon *et al.* 1978*b*) this increase has been attributed to a Bohr effect resulting, at least initially, from hyperventilation and the ensuing respiratory alkalosis. A similar mechanism has also been reported to contribute to an increased oxygen affinity of haemocyanin in *O. rusticus* (Wilkes & McMahon, 1982) during 6 days hypoxia. In the present study the elevated pH and reduced P_{CO_2} and C_{CO_2} levels in the haemolymph would indicate that the scaphognathite rate remains elevated in *O. rusticus* even after 24 days hypoxic exposure. Nevertheless the sustained respiratory alkalosis accounts only for 2·2 torr depression of P_{50} or about 50% of the actual increase in oxygen affinity. Clearly, following 24 days hypoxic exposure additional mechanisms are used to supplement the Bohr effect.

Oxygen movement from the water, across the gill epithelium to the haemocyanin requires a pressure gradient, i.e. a maintained difference in oxygen partial pressure between water and haemolymph. Wilkes & McMahon (1982) demonstrated that the pressure gradient was reduced in *O. rusticus* during 6 days hypoxic exposure, and cannot be re-established by a reduction in prebranchial P_{O_2}. Nevertheless, the conductance or oxygen transfer factor increased. Thus, despite the reduced pressure gradient across the gill epithelium the ability of the haemocyanin to load oxygen improves and the normoxic rate of oxygen consumption is maintained during 6 days hypoxic exposure (Wilkes & McMahon, 1982). This effect can be attributed to both an increase in cardiac output and an increase in oxygen affinity of the haemocyanin. The former serves to improve gill perfusion so that the oxygen pressure gradient is maintained by the rapid removal of oxygenated haemolymph. The increased oxygen affinity of the haemocyanin results in (i) a greater volume of oxygen being bound to the haemocyanin at a given pressure gradient, and (ii) maintenance of the pressure gradient across the gills by removing oxygen from solution in the haemolymph (Wilkes & McMahon, 1982).

The proton-independent increase in haemocyanin oxygen affinity observed in *O. rusticus* during chronic hypoxic exposure is functionally analogous to the decrease in erythrocytic ATP concentration which occurs during hypoxic exposure in fish

(Wood & Johansen, 1972; 1973; Wood, Johansen & Weber, 1975). Thus, both crustaceans and fish elevate the oxygen affinity of their respiratory pigments to facilitate oxygen loading at the gills. Increasing oxygen affinity during hypoxic exposure, as occurs in water breathers, is opposite to the general mammalian response in which oxygen unloading at the tissues is facilitated by a decrease in haemoglobin oxygen affinity .(Lahiri, 1977). The crustacean response is especially adaptive to *O. rusticus* since there is virtually no venous reserve which could be exploited by a reduction in haemocyanin oxygen affinity (Wilkes & McMahon, 1982).

In fish, in fact in vertebrates generally, hypoxic exposure is also associated with increased haemoglobin production and hence oxygen-carrying capacity (Wood & Johansen, 1972; Lahiri, 1977). However, despite the suggestion that haemocyanin production rate may increase in hypoxic lobsters (Senkbeil & Wriston, 1981), no increase in levels of either protein or Cu^{2+}, and hence no increase in oxygen capacity, accompanied $3\frac{1}{2}$ weeks of hypoxic exposure in the present study. In fact the concentration of haemocyanin in crustacean haemolymph is generally low (C. P. Mangum, in prep.) and Magnum & Johansen (1975) suggest that increased levels might sufficiently raise colloid osmotic pressure so as to generate fluid imbalance between intracellular and extracellular compartments. Increased colloid osmotic pressure may also affect water balance across the gills, especially in freshwater species, and could elevate haemolymph viscosity, increasing the work needed for perfusion of gills and tissues. It is thus possible that the disadvantageous effects of increasing haemocyanin concentration in cardiovascular performance and osmotic balance would outweigh the advantage of an increased oxygen-carrying capacity.

It is evident from these results that during hypoxic exposure the oxygen affinity of the haemocyanin in the crayfish *O. rusticus* increased by at least two mechanisms. The first is dependent upon the pH of the haemolymph, i.e. a Bohr shift resulting from a respiratory alkalosis induced by sustained hyperventilation. The second is independent of haemolymph hydrogen ion concentration. Acting synergistically the two mechanisms effect a 45 % increase in haemocyanin oxygen affinity. As mentioned in the introduction there are several possible ways by which oxygen affinity can be controlled independently of the Bohr shift. The possibility of the H^+-independent decrease in P_{50} resulting from a change in the ionic characteristics of the haemolymph was examined in the present study. Although there was an increase in haemolymph Ca^{2+} concentration, the Ca^{2+}/Cu^{2+}, and the $Ca^{2+}/protein$ ratio in the experimental crayfish, these changes were not significant. Thus the present data suggest that change in the ionic constitution of the haemolymph in *O. rusticus* plays no major role in controlling haemocyanin oxygen affinity during long-term hypoxic exposure.

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