Oxygen and carbon dioxide transporting qualities of hemocyanin in the hemolymph of a Natant Decapod Palaemon adspersus

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Oxygen and Carbon Dioxide Transporting Qualities of Hemocyanin in the Hemolymph of a Natant Decapod *Palaemon adspersus**

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Summary. 1. The O_2 and CO_2 combining properties of *Palaemon adspersus* hemolymph is studied, aiming to assess respiratory function and the environmental and metabolic adaptations of hemocyanin of natant decapods where, in contrast to the intensively-studied, larger and predominantly less active reptant decapods, virtually no information is available.

2. The hemolymph shows a high O_2 carrying capacity (mean = 2.8 vol%), a low O_2 affinity (at 15 °C half-saturation tension, $P_{50} = 16$ and 37 mm Hg at pH 7.85 and 7.65, respectively), pronounced cooperativity in O_2 binding (Hill's coefficient, $n \simeq 2.8$) and a large, pH dependent Bohr factor ($\phi = \Delta \log P_{50}/\Delta pH = -2.0$ and -0.9 at pH 7.85 and 7.4, respectively) (Figs. 1 and 2). These qualities are distinct from those typifying reptant hemocyanins and appear ill-suited for O_2 transport at low ambient tensions, but well-adapted for O_2 delivery in tissues at high P_{O_2}, supporting high levels of metabolism and activity.

3. CO_2 has a specific, augmenting effect on O_2 affinity at high pH (Fig. 3), indicating carbamate formation with an opposite oxygenation-linkage as in vertebrate hemoglobins. Astrup titrations indicate the presence of a small but distinct Haldane effect at physiological pH, and buffering capacity varies greatly ($\Delta HCO_3/\Delta pH \simeq -4.4$ to -9.3 mmol · l^{-1} · (pH unit)$^{-1}$ depending on hemocyanin concentration) (Fig. 4). Equilibrium curves of total, non-protein-bound CO_2 show large capacitance for transport at low, in vivo CO_2 tensions (Fig. 4).

4. The data are discussed comparatively, particularly as regards hemocyanin function in reptant decapods, and the O_2, CO_2 and proton exchanges involved.

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Introduction

Recent years have witnessed a tremendous increase in the understanding of the respiratory function of crustacean hemocyanin (Mangum 1980). The published data, however, deal almost entirely with one of the two major groups of the decapodan crustaceans, the Reptantia or crawling decapods, which includes the larger, predominantly benthic crabs and crayfishes, and no data on physiological function appear to be available on the Natantia or swimming decapods. The latter includes several families of shrimps that typically are small and exhibit significantly greater capacities and adaptations for neritic life and associated swimming activity. Thus whereas the dorsoventrally flattened reptants have well-developed legs but reduced swimming pleopods the opposite applies to the laterally flattened natants.

The refinement of micromethods over the last years provides opportunity for studying the oxygen and carbon dioxide transporting properties of whole, hemocyanin-containing hemolymph of small natant crustaceans, and thus for discerning its in vivo respiratory functions and the adaptations to environmental conditions and to activity (which may be a major factor determining the adaptive qualities of crustacean hemocyanin; Redmond 1968). This paper represents such a study of the natant shrimp *Palaemon adspersus*, which frequents oligohaline *Zostera*-beds in the Baltic Sea that may become hypoxic at night. An earlier study (Hagerman and Weber 1981) reports hemocyanin concentrations and the influence of water P_{O_2} on respiratory rate and hemolymph P_{O_2} values in individual shrimps.

Materials and Methods

Specimens of *Palaemon adspersus* Rathke weighing about 1.5 to 2.0 g and originating from Aunø Fjord, South Sealand, Denmark (see Hagerman and Weber 1981) were used. The shrimps were

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kept in aquaria containing well-aerated sea water of 18‰ salinity, without temperature control (near 10 °C) and fed on crab meat. Hemolymph was collected from the heart, by dorsal piercing of the arthropodial membrane at the posterior end of the carapace with a 25 gauge hypodermic needle and syringe. Hemolymph from 5–10 specimens was pooled, rapidly shaken using a vortex mixer and centrifuged in 3 ml capped Eppendorf tubes for 5 to 10 min at approximately 14,000 *g* and stored on ice. Gelled hemolymph that formed at the surface showed the same intensity oxy-hemocyanin blue as the remaining fluid and was removed with a needle.

In vivo hemolymph pH was measured in individual shrimps using a Blood Micro System (BMS II mk 2) and a PHM 72 millivoltmeter (Radiometer, Copenhagen) avoiding contact with air. This was done by fitting the sharp end of a 25 gauge hypodermic needle onto the polycarbonate capillary tube of the BMS pH microelectrode, then using this to pierce the pericardial cavity (as above) sucking the hemolymph directly into the electrode capillary, within 10–15 s after removing the shrimps from the water.

O_2 carrying capacity was measured in air-equilibrated hemolymph samples, using a technique where pigment-bound O_2 is chemically liberated and recorded as increases in O_2 tension (Tucker 1967, as modified by Bridges et al. 1979). Hemocyanin concentrations were estimated from peak absorbances near 335 nm, using an $A_1^{1\,\%}_{cm}$ value of 2.33 as applies to *Carcinus* hemocyanin (Nickerson and Van Holde 1971). With a functional subunit mass of 75,000 as characterizes arthropod hemocyanin (Lontie and Witters 1973) this reflects an ε_{mmol} value of 17.25 for calculation of the theoretical O_2 capacity. Replicate estimations of O_2 capactiy by both methods showed good agreement.

Blood pH at different CO_2 tensions were measured at 15 °C after twenty-minute equilibration periods in Radiometer BMS II tonometers using appropiate mixtures of O_2 or N_2 and CO_2 (supplied by Wösthoff gas mixing pumps). Bicarbonate and carbonate concentrations were calculated from the Henderson Hasselbach equation

$$pH = pK_1' + \log \frac{[HCO_3^-]}{\alpha_{CO_2} \cdot P_{CO_2}} = pK_2' + \log \frac{[CO_3^=]}{[HCO_3^-]}$$

using pK_1', pK_2' and α_{CO_2} values of 6.07, 9.45 and 0.0525 mmol·l^{-1} ·mm Hg^{-1}, respectively, corresponding to the values in *Carcinus* haemolymph at 15 °C and 18‰ salinity (Truchot 1976a). Total CO_2 was calculated as

$$C_{CO_2}(\text{mmol} \cdot l^{-1}) = [HCO_3^-] + (CO_3^=) + \alpha_{CO_2} \cdot P_{CO_2},$$

neglecting a small amount of CO_2 that may be bound to hemocyanin as carbamate (Truchot 1976b).

Oxygen equilibria of 10 µl samples of the hemocyanin were measured at 365 nm in a gas diffusion chamber (with modifications of Sick and Gersonde 1969, and Weber et al. 1974), using serially connected gas mixing pumps for delivery of calibration gases of increasing O_2 tension, while keeping CO_2 tensions constant. The solutions applied (path length <0.1 nm) were allowed to equilibrate with a gas mixture with the required CO_2 tension for 20 min or more before the recordings. Full O_2 saturation was estimated after equilibration with O_2. pH values in the haemolymph samples were measured by equilibrating parallel 70 µl samples to the same CO_2 tensions in BMS tonometers for at least 20 min.

Results and Discussion

1. Hemolymph pH and O_2 Carrying Capacity

pH values in the hemolymph of *Palaemon adspersus*, measured at 15 and 20 °C, were 7.85 (s.d. = 0.02, N = 5) and 7.65 (s.d. = 0.12, N = 7) respectively. The latter value agrees closely with those measured at 20–22 °C in larger, reptant marine decapods (Mangum and Shick 1972) and in smaller isopods from littoral habitats (Sevilla and Lagarrique 1979).

O_2 carrying capacity measured in 8 individual shrimps that had been kept in the laboratory for 2 weeks in November was 2.82 (s.d. = 0.60) vol%. Subtraction of dissolved O_2 leaves 2.20 ± 0.60 vol% hemocyanin bound O_2. This compares with a mean value of 3.0 vol% (range 1.7 to 4.2 vol%) earlier calculated for 16 individual shrimps on the basis of hemolymph copper content (Hagerman and Weber 1981). The pigment levels also varied greatly in different batches of shrimps; five pooled samples of hemolymph used in the gas-binding experiments (collected from shrimps maintained in the laboratory for 2–4 weeks) showed O_2 capacities of 2.19 ± 1.45 vol%.

As with the natant shrimp *Crangon crangon* this variation likely relates to season, moulting cycle and starvation, and the internal compartmentalization of copper and apohemocyanin (Djangmah 1970a, b; Busselen 1970). A recent study on *Carcinus* (Boone and Schoffeniels 1979) moreover documents hyposmotic stress as a factor inducing rapid hemocyanin synthesis. Uglow (1969) earlier demonstrated a rapid decrease in the level of an electrophoretically-'slow' hemocyanin component in *Carcinus* during starvation suggesting that this component may be an organic reserve while the 'fast' component serves more specifically for O_2 transport. Schoffeniels (1976) analogously hypothesized that hemocyanin may serve as a store of amino-acids for increasing cellular osmotic pressure during hyperosmotic stress. In *Crangon crangon* increased cellular osmolalities during hyperosmotic stress is attributable almost entirely to raised levels of proline, alanine and glycine (Weber and Van Marrewijk 1972).

The hemocyanin-O_2 carrying capacity of *Palaemon* hemolymph is higher than in most other aquatic crustaceans. It varies from 0.58 to 1.53 vol% in five species of marine crabs (Redmond 1968), and averages 0.29, 2.08, 1.6 and 1.7 vol%, respectively, in the spider crab *Libinia emarginata*, the ghost crab *Ocypode quadrata*, the blue crab *Callinectes sapidus* and the burrowing shrimp *Callianassa californiensis* (Burnett 1979; Mangum and Weiland 1975; Miller et al. 1976). For *Cancer magister* Johansen et al. (1970), however, report an O_2 capacity of 3.44 vol%. The methods used for estimating the values in *Callianassa, Cancer magister* and *Callinectes sapidus*, however, use potassium ferricyanide for liberating hemocyanin-bound oxygen, which unlike potassium cyanide used in the present study may yield artefactual values (Cook 1927; Miller et al. 1976). Warburg manometric measurement of hemocyanin O_2 capacity of hemo-

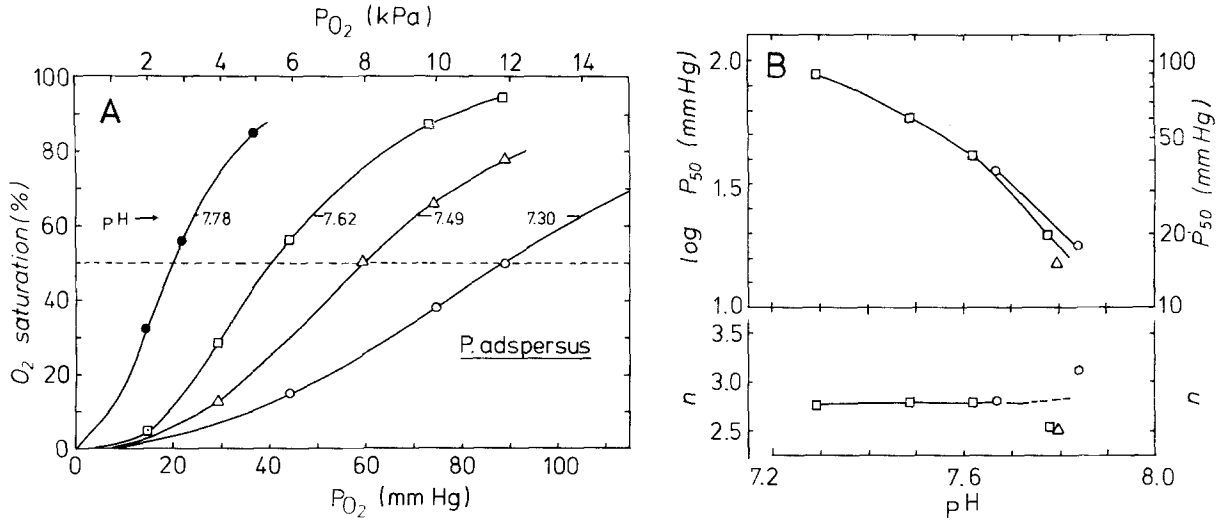

Fig. 1. **A** O_2 equilibrium curves of *P. adspersus* haemolymph at 15 °C in the presence of increasing CO_2 tensions (from left to right, 0.7, 3.7, 7.3 and 15.0 mm). **B** Variation of O_2 halfsaturation tension, P_{50}, and cooperativity coefficient, n_{50}, with pH at 15 °C. □ data from **A**; ○ and △ two separated samples of pooled hemolymph (P_{CO_2}=0.27 and 3.6 mm)

cyanin of the gastropod mollusc *Haliotis* treated with cyanide similarly yield values greatly in excess of those calculable from protein concentrations (Ainslie 1980). Evidently, control measurements of O_2 capacity and concentrations of hemocyanin by various techniques are sorely needed.

The relatively high O_2 capacity of *Palaemon* hemolymph will potentially reduce the blood convection requirement ($\dot{Q}/\dot{V}_{O_2}$, expressing the ratio of blood flow to O_2 uptake and thus the volume of blood that must perfuse the tissues per unit O_2 delivered). This is well illustrated by comparison of the spider- and ghost crabs (Burnett 1979), where a low total O_2 capacity in the former (0.76 compared to 2.55 vol% in the ghost crab) correlates with a four fold higher $\dot{Q}/\dot{V}_{O_2}$. The low capacity thus needs compensation by an energetically expensive increase in cardiac output. At 35–95 beats/min the heart rate in *Palaemon* (Hagerman and Uglow 1979) is significantly lower than the 127/min in the hemocyanin-poor spider crab. Although the convection strategy has been postulated as an alternative to high hemocyanin levels that provides greater sensitivity to short-term environmental perturbations (Burnett 1979), it should be borne in mind that the advantages of variable blood convection may also be exploited in hemocyanin-rich species like *P. adspersus*.

2. *Hemolymph-O_2 Equilibrium Properties*

The whole hemolymph shows a low in vitro O_2 affinity; at pH 7.85 and 7.65 (which approximate in vivo values at 15 and 20 °C – above) P_{50} values were ~16 and 37 mm, respectively, whereby O_2 tensions of at least 30 and 70 mm are required for 90% O_2 saturation of the hemocyanin (Fig. 1 A). These affinities are much lower than those previously reported in reptant decapods; at 11 to 22 °C and pH 7.5 to 7.7, seven species of crabs and crayfish show P_{50} values of 12 to 22 mm (see Mangum and Weiland 1975; Angersbach and Decker 1978). The *Palaemon* values, however, resemble those in the whole hemolymph of another natant shrimp, *Crangon crangon* (P_{50}=20.5 and 55.5 at 15 °C and P_{CO_2} values of 1.9 and 7.5 mm; Weber, unpublished). Considered in conjunction with the steep decrease in O_2 tension across the respiratory surfaces (ΔP_{O_2}=60 mm in aerated water; Hagerman and Weber 1981) the low affinity suggests that the *P. adspersus* hemocyanin will only serve in O_2 transport at high ambient tension. This property correlates with observations that in nature *P. adspersus* avoids hypoxic *Zostera* meadows at night, returning during daytime when O_2 levels are high through photosynthetic activity (Muus 1967).

The Hill coefficient n_H averages 2.7 in the whole hemolymph, showing pH invariance below 7.7 (Fig. 1 B). A Hill plot of the hemolymph-O_2 equilibrium (Fig. 2) shows two distinct phases, with non-cooperativity below 5% O_2 saturation and pronounced, monophasic cooperativity at higher saturations, extending to at least 97.5% saturation. Whilst extrapolation of the non-cooperative phase to the half-saturation point (cf. Weber 1981) reflects a low O_2 association equilibrium constant for the deoxygenated hemolymph (K_{deoxy}=0.012 mm Hg^{-1}), the absence of a reduction in cooperativity at extreme high O_2 saturation (~97.5% – see Fig. 2) reflects a phenomenal increase in O_2 affinity upon oxygenation (K_{oxy}/K_{deoxy}>60) and

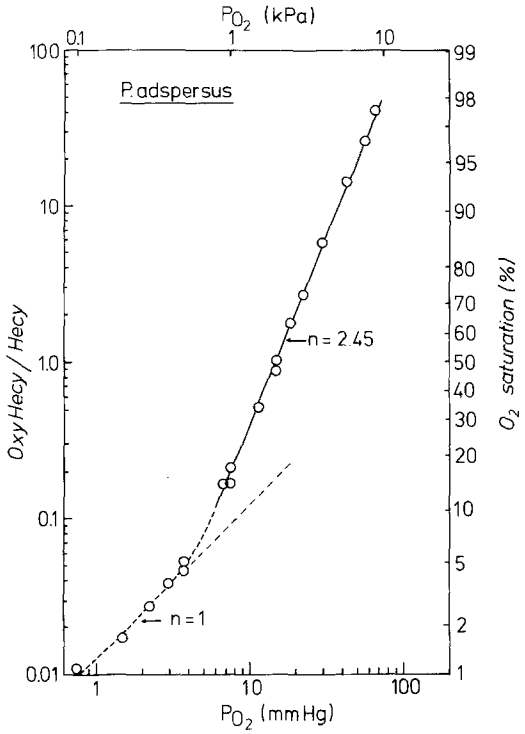

Fig. 2. Hill plot at 1 to 97.5% saturation of O_2 equilibrium of *P. adspersus* hemolymph at 15 °C, pH 7.80 and P_{CO_2} 0.7 mm

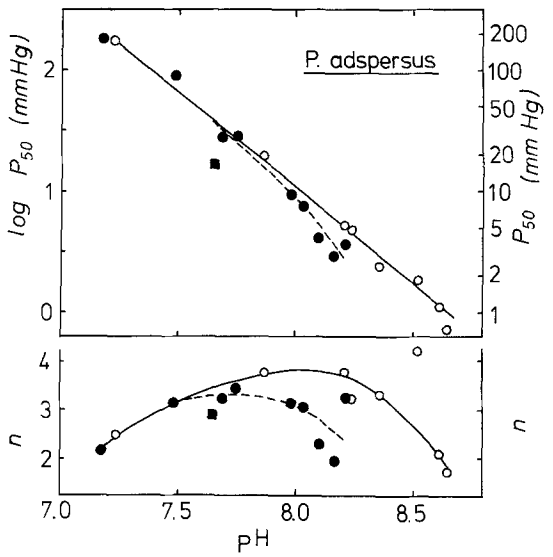

Fig. 3. Variation of P_{50} and n_H of hemolymph buffered to 0.05 molar Tris and bisTris, with pH at 15 °C. ○ P_{CO_2} 0 mm; ● P_{CO_2} 7.2 mm; ■ P_{CO_2} 29.9 mm

a large free energy for interaction between the O_2 binding sites on the hemocyanin molecule.

The O_2 affinity shows very high pH-sensitivity (P_{50} = 16 and 72 mm at pH 7.85 and 7.4 respectively) and the Bohr shift ($\phi = \Delta \log P_{50}/\Delta$pH) increases with pH (−0.9 and −2.0 at pH 7.4 and 7.85, respectively). These values even exceed the 'high' ones characterizing most larger reptant decapods (Mangum 1980). In *Carcinus maenas* ϕ equals −0.67 and −0.47 at pH 7.6 and 7.4 respectively (Truchot 1971a). In *Astacus leptodactylus* and *Cancer magister* the ϕ values are, however, only −0.19 and −0.27 (Angersbach and Decker 1978; Johansen et al. 1970).

Active animals need O_2 delivery to the metabolizing tissues at high rate and high tension, which is precisely what may be expected of a system like *Palaemon* hemolymph exhibiting a low O_2 affinity, a large Bohr effect and high values of cooperativity and O_2 carrying capacity. Concrete experimental verification must, however, await measurement of pre- and postbranchial pH and P_{O_2} values, which is technically more difficult in the natant than in the larger reptant crustaceans hitherto studied. Comparative investigations, however, strongly support these considerations. Thus, the hemolymph of the active swimming-crab *Macropipus puber*, shows lower O_2 affinity than five other typically benthic crabs studied by Truchot (1971b). That the low-affinity strategy also becomes exploited by reptant decapods during activity appears from the absence of significant pre-/postbranchial pH differences in specimens of *Carcinus maenas, Callinectes sapidus, Astacus leptodactylus* and *Cancer magister* at rest, and lower pre- than postbranchial values during activity (Truchot 1973a; Mangum and Weiland 1975; Angersbach and Decker 1978; McMahon et al. 1979). This suggests that the Bohr effect becomes operative only during activity when site specific pH differentiation allows increased utilization of the venous reserve (Mangum and Weiland 1975). Low Bohr values may thus correlate with predominant activity. Alternatively stated, the absence of significant tissue-gill pH shifts may obviate the need for a large Bohr effect in inactive species.

Buffered solutions of the hemocyanin show similar O_2 affinities as the whole hemolymph at physiological pH. The 'fixed acid' Bohr shift is, however, pH independent ($\phi = -1.6$) over a wide pH range (Fig. 3). At pH values below 7.8, CO_2 has no specific (pH independent) effect on O_2 affinity indicating that it will not influence O_2 transport under physiological conditions. At high alkalinity, however, CO_2 appears to increase O_2 affinity, in contrast to its depressive effect in vertebrate hemoglobins (Rossi-Bernardi and Roughton 1967; Weber and Johanson 1979), increasing the Bohr effect at high pH. This suggests that the pH dependence of the Bohr shift seen in the whole hemolymph is attributable to oxygenation-linked CO_2 binding rather than to the presence of different types of titrable groups holding Bohr protons or to subunits with different Bohr effects (as postulated for *Upogebia* hemocyanin; Miller et al. 1977).

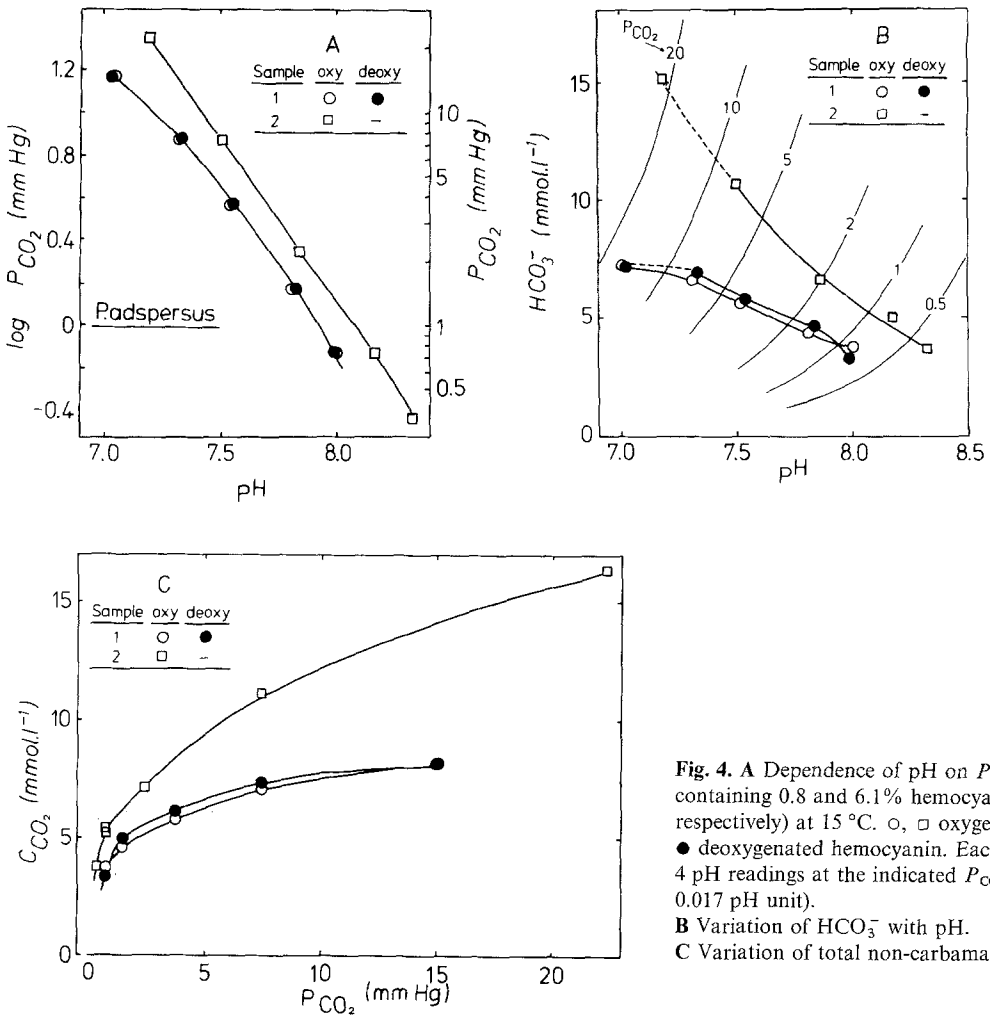

Fig. 4. A Dependence of pH on P_{CO_2} in two hemolymph samples containing 0.8 and 6.1% hemocyanin (circles and squares respectively) at 15 °C. ○, □ oxygenated hemocyanin; ● deoxygenated hemocyanin. Each point gives the average of 4 pH readings at the indicated P_{CO_2} (standard deviations averaged 0.017 pH unit).
B Variation of HCO_3^- with pH.
C Variation of total non-carbamate CO_2 with P_{CO_2}

In buffered solution, the coefficient n_H is high, pH dependent (showing a maximum near pH 8.0) and decreases in the presence of CO_2 (Fig. 3). The restriction of the CO_2 effects on P_{50} and n_H to conditions of high pH suggests that CO_2 binds at uncharged $-NH_2$ groups (Stadie and O'Brien 1936) like those involved in carbamate formation in vertebrate hemoglobin.

Mangum and Lykkeboe (1979) postulate that CO_2 may increase the O_2 affinity in hemocyanin of the mollusc *Busycon* by decreasing the concentration of free divalent cations which depress the affinity. This indirect effect is unlikely to be significant in *Palaemon*, since divalent cations increase O_2 affinity of most crustacean hemocyanins (cf. Miller and van Holde 1974).

Evidence for a 'reverse' CO_2 effect also comes from *Carcinus maenas* hemocyanin (Truchot 1973b) and the extracellular erythrocruorin of the lugworm *Arenicola marina* (Krogh-Rasmussen and Weber 1979). However, unlike *Palaemon* and *Arenicola* where the effect is limited to high pH, CO_2 (at 14.9 mm) reduces P_{50} of *Carcinus* hemocyanin over the entire range (7.2 to 7.8) studied.

Measuring CO_2 wash-out rates and associated pH changes in the hemocyanin of the cephalopod mollusc *Sepia latimanus*, Lykkeboe et al. (1980) observed that part of CO_2 is liberated from the oxygenated pigment without a corresponding uptake of protons. Apart from providing a possible explanation for the stoichiometry of the molar exchanges of O_2, CO_2 and protons when the respiratory ratio is unity and the Bohr factor (protons bound per O_2 liberated) is greater than unity, this reflects greater CO_2 binding to oxygenated pigment as does the specific CO_2 effect in *Palaemon*.

3. *Hemolymph CO_2 and Acid-Base Balance*

Astrup titrations on hemolymph samples with very different hemocyanin concentrations are shown (Fig. 4A). At pH of 7.2 to 7.8, which includes the

physiological condition (above), pH at constant P_{CO_2} was consistently higher in deoxygenated than in oxygenated hemocyanin, in accordance with the presence of a Haldane effect. As in *Carcinus* (Truchot 1976b) the pH difference is small (less than 0.03). The increased pH in the deoxygenated state reflects binding of Bohr protons. That this pH difference is seen at pH values below those where oxygenation-linked CO_2 binding was evident (Fig. 3) suggests that it cannot solely result from carbamate formation which, in contrast to vertebrate hemoglobin, may be favoured by oxygenation in *Palaemon* hemocyanin (as indicated by the 'reversed' CO_2 effect). Transferring the mean in vivo pH value of 7.85 to the log P_{CO_2}/pH diagram (Fig. 4A) reflects in vivo postbranchial CO_2 tensions of 1.5 to 2.5 mm depending on buffering capacity. This compares with a prebranchial value of 2.5 mm in *Carcinus* in 15 °C, full-strength sea water (Truchot 1973a).

The hemolymph illustrates corresponding variation in bicarbonate and in buffering power; at pH 7.65 $[HCO_3^-]$ is 5.2 to 8.2 mmol·l^{-1} and $\Delta[HCO_3^-]/\Delta$pH is -4.4 to -9.3 mmol·(pH unit)$^{-1}$ (Fig. 4B). While $[HCO_3^-]$ in crustaceans is strongly dependent both on environmental factors (e.g. temperature, air exposure, gas tensions) and endogenous moulting cycles (Truchot 1976b), buffering power depends mainly on hemocyanin content, which in turn depends upon nutritional state and osmotic stress (Djangmah 1970a; Boone and Schoffeniels 1979) and which varies by a factor of 3 in individual, freshly-collected specimens of *P. adspersus* (Hagerman and Weber 1981).

Neglecting the small amount of CO_2 that may be bound as carbamate at low in vivo P_{CO_2} and high pH (cf. Truchot 1976b), CO_2 equilibrium curves relating CO_2 content to CO_2 tension can be calculated (Fig. 4C). Extrapolation of these curves to zero P_{CO_2} indicates that as in the crustaceans *Homarus cancer* and *Carcinus*, but unlike the mollusc *Helix* (Wolvekamp and Kruyt 1948; Truchot 1976b), *Palaemon* hemolymph contains enough weak acid to liberate all bound CO_2 and to balance associated cation excess when P_{CO_2} falls to zero. This results in steep equilibrium curves and a high capacitance for CO_2 transport ($\beta_{CO_2}=\Delta C_{CO_2}/\Delta P_{CO_2}$) at low, physiological CO_2 tensions.

Since the Haldane effect is the amount of protons liberated during oxygenation it theroretically corresponds to the Bohr effect reflecting the quantity of O_2 liberated by proton binding. The small differences in pH and in total non-hemocyanin CO_2 (less than 0.03 pH unit and 0.03 mmole·l^{-1}, respectively), are thus in accord with a higher buffering power of crustacean hemolymph relative to its O_2 capacity than in vertebrate blood (cf. Truchot 1976b). Molecularly, this rests on the higher minimum molecular weight in hemocyanin than in vertebrate hemoglobin (74,000 compared to 17,000).

The large Bohr shift ($\phi=-2.0$) indicates that 2.0 mole protons are bound per mole O_2 released. At an exchange ratio (CO_2 produced/O_2 consumed) of unity (normally representing the maximum) this suggests that metabolic CO_2 cannot balance the demand for protons stoichiometrically, which will tend to raise the pH in the tissues. This paradoxical situation, however, need not hold in life, as is evident from equal pH values observed in pre- and postbranchial hemolymph of crustaceans at rest, and lower prebranchial values during activity (Angersbach and Decker 1978; Mangum and Weiland 1975). In cephalopod hemocyanin a decrease in pH during unloading is attributable to an O_2-linked CO_2 component which is not associated with proton uptake (Lykkeboe et al. 1980).

Two other phenomena may, however, explain low tissue pH. Firstly, the relationship between O_2 saturation and proton release may be non-linear as indeed has been demonstrated for human hemoglobin (Tyuma and Ueda 1975), where this phenomenon is consistent with the pH dependence of n_H, which is even more pronounced in hemocyanins. Since decapod hemocyanins do not unload completely (Spoek 1962) it follows that the stoichiometrical relationship given by the equation $\Delta H^+ = -\log P_{50}/\Delta$pH (where ΔH^+ is the maximum saturation-dependent charge difference per O_2 binding site) need not apply in the tissues.

Secondly, in view of the low O_2 capacity of hemocyanin-bearing bloods and hemolymph, a considerable fraction of O_2 is transported in physical solution, whereby O_2 unloaded and therefore protons bound to the pigment in the tissues become correspondingly reduced compared to proton production from the CO_2 source, favouring lower pH values. This will apply particularly to low temperature, where O_2 unloading falls as a result of increased affinity (Mauro 1978) and increased O_2 solubility. The effects of temperature on hemolymph pH and O_2 affinity are subjects of continued study.

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