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AN ANALYSIS OF THE EFFECTS OF pH ON OXYGEN BINDING BY SQUID (*ILLEX ILLECEBROSUS, LOLIGO PEALEI*) HAEMOCYANIN

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Summary

The *in vitro* oxygen-binding characteristics of haemocyanin were investigated in whole blood of two species of pelagic squid, *Illex illecebrosus* and *Loligo pealei*. pH-independent Haldane coefficients ($\Delta HCO_3^-/\Delta HcyO_2$) (where $HcyO_2$ is haemocyanin-bound oxygen) slightly smaller than −1 were found in both species. Oxygen-linked CO_2 binding was not present. Buffer values ranged between 5 and 5.8 mmol l^{-1} pH unit^{-1}. For further analyses a pH/saturation diagram was selected to show the effect of pH on oxygen binding at constant P_{O_2} in a continuous plot. The slopes of the resulting oxygen isobars ($\Delta HcyO_2/\Delta pH$ or $\Delta S/\Delta pH$) (where S is oxygen saturation) depend on pH. The diagram allows evaluation of both the Bohr coefficients ($\Delta \log P_{50}/\Delta pH$) and the Hill coefficients (n_{50}) at specific pH values. It provides an integrated illustration of the importance of the Bohr effect and cooperativity for oxygen binding.

In accordance with Wyman's linkage equation, Bohr and Haldane coefficients are found to be identical. Both are pH-independent between pH 7 and 8. The changing slopes of the oxygen isobars are likely to reflect changes in cooperativity with pH. Maximum values of n_{50} coincide with maximum steepness of the oxygen isobars in the physiological range of pH and P_{O_2}. Assuming that the haemocyanin acts as a buffer for venous P_{O_2}, this maximum in pH sensitivity and its decrease in the higher and lower pH ranges are discussed in the light of the maintenance of pigment function *in vivo*.

Introduction

Among cephalopod haemocyanins, highly pH-dependent molecules with Bohr coefficients ($\Delta \log P_{50}/\Delta pH$) below −1 predominate (Brix *et al.* 1981, 1989; Houlihan *et al.* 1982). This observation has cast doubt on whether Bohr and Haldane effects in these and other animals (e.g. some crustaceans, Morris *et al.*

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1985) can support oxygen loading and CO_2 unloading of the blood at the gills and oxygen release and CO_2 loading in the tissues, in the classically accepted manner. Since Bohr and Haldane coefficients are expected to be identical (Wyman, 1964), the Haldane effect would cause high proton consumption during deoxygenation. The support of oxygen release in the tissues would require more protons than would be available from the formation and accumulation of CO_2 (linked to the consumption of transported oxygen and to realistic RQ values). Deoxygenation would cause an alkalosis, oxygen affinity would increase, and further oxygen release would require a large drop in venous P_{O_2}. Consequently, instead of supporting venous unloading, large Bohr and Haldane effects would appear to obstruct the oxygen supply to the tissues.

Brix *et al.* (1981) proposed that the large Bohr effect acts predominantly to support oxygen uptake at the gills. Venous alkalization, it is suggested, is minimized by oxygen-linked CO_2 binding. According to this mechanism, both O_2 and CO_2 are bound to the haemocyanin at the gills and are released in the tissues (Lykkeboe *et al.* 1980). Finally, Brix *et al.* (1989) concluded that Bohr and Haldane shifts in cephalopod blood allow the haemocyanin to contribute to pH stabilization and prevent 'any interference with the loading of O_2 at the gills'.

Is oxygen-linked CO_2 binding widely distributed among cephalopods? This study is designed to investigate oxygen and CO_2 binding in squid (*Loligo pealei* and *Illex illecebrosus*) haemocyanin. The potential role of pH in the oxygenation/deoxygenation cycle is also addressed. For this purpose, a graphical depiction is chosen (Figs 1A and 2A) which presents changes in oxygen saturation with pH at various P_{O_2} values. This presentation illustrates the dependence of haemocyanin oxygen-binding on pH and allows the conditions required for optimum function of the pigment in oxygen transport and delivery to be defined.

Materials and methods

Animals

Squid (*Illex illecebrosus*, 300–500 g, *Loligo pealei*, 200–400 g) were caught by commercial fishermen in St Margarets Bay or close to Herring Cove, Nova Scotia, from October to December 1986. The animals were placed in plastic bags filled with oxygenated sea water at 2–6°C and transported to Halifax. There they were held in running sea water at ambient temperatures of 8–15°C. At high ambient temperatures they were used as soon as they recovered from transport and handling (after 2–4 h). When ambient temperatures fell below 12°C, the animals were brought close to the experimental temperature for 12–24 h before being used.

Anaesthesia and sampling procedure

Squid, some of them cannulated in the vena cava (H. O. Pörtner, D. M. Webber, R. G. Boutilier and R. K. O'Dor, in preparation), were placed in a Beamish-type respirometer filled with approximately 100 l of sea water at

15±0.5°C. Water flowed through the system continuously, some of it being circulated through the animal chamber at approximately 0.1 m s^{-1}. After about 1.5 h of recovery from handling, a blood sample was taken, the respirometer was closed and the animal anaesthetized by adding 2 l of pure ethanol to the water circulation downstream of the animal. Full anaesthesia (indicated by the cessation of ventilation) was reached after 3–5 min. The animal was removed from the respirometer and quickly decapitated. Additional blood samples were collected from the gill hearts and the ventricle. Blood samples from all experimental animals were pooled, frozen and stored at close to −20°C until utilized for *in vitro* studies of oxygen binding.

Analysis of oxygen equilibria

Oxygen-binding characteristics of squid haemocyanin were studied using a tonometer (Instrumentation Laboratory, model 273, Padorno Dugano, Italy) on whole blood at 15°C. Samples of pooled blood were equilibrated with humidified gas mixtures of varying P_{O_2} (between 0 and 98.6 kPa, 1 mmHg=0.1333 kPa, 1 kPa=7.502 mmHg) provided by gas-mixing pumps (type M 303/a-F; Wösthoff, Bochum, FRG). Blood pH was varied by changing the P_{CO_2} (between 0.09 and 12.0 kPa) or by replacing small volumes (<10 μl per 2 ml of blood) of supernatant plasma after ultracentrifugation (1 h at 120 000 *g*; see Morris *et al.* 1985) with fixed acid (1 mol l^{-1} HCl) or base (2 mol l^{-1} NaOH), thereby causing a pH shift of approximately 0.3 units in the acid or alkaline direction, respectively. Total CO_2 was analysed in 50 μl blood samples using the gas chromatography method of Lenfant and Aucutt (1966) modified after Boutilier *et al.* (1985). The pH was measured for each data point by using a microcapillary pH electrode (G299, Radiometer, Copenhagen, Denmark) thermostatted at 15±0.1°C and calibrated with precision phosphate buffers (Radiometer, Copenhagen). 75 μl blood samples were analysed for oxygen content by measuring the increase in P_{O_2} after release of bound oxygen in a solution containing 6 g l^{-1} KCN and 3 g l^{-1} saponin (Bridges *et al.* 1979) using a 'Tucker' chamber (Tucker, 1967).

Calculations and graphical analysis

Changes in haemocyanin oxygenation (measured as total oxygen minus physically dissolved oxygen) and pH were plotted in a pH/saturation diagram (Figs 1A, 2A). The resulting oxygen-binding curves appear as isobars delineating the change in oxygenation with pH at constant P_{O_2}. The points of intersection of the oxygen isobars with the line of half saturation show at which pH $\log P_{O_2}$ of the isobar is equivalent to $\log P_{50}$ of the pigment. The values of $\log P_{50}$ are shown on the line in the upper part of the diagram. Since, from these intersections, the change in $\log P_{50}$ with pH was found to be constant over the whole range of pH, the Bohr coefficient, $\Delta \log P_{50}/\Delta pH$ could be evaluated by linear regression analysis.

Proton release or consumption during oxygenation/deoxygenation was calculated in accordance with the linkage equation of Wyman (1964), as used by Siggaard-Andersen (1974) and Brix *et al.* (1981). The Haldane coefficient

($\Delta HCO_3^-/\Delta HcyO_2$) was evaluated from the vertical distance between buffer lines (see Figs 5 and 6). The linkage equation is:

$$\frac{\Delta \log P_{50}}{\Delta pH} = \left(\frac{\Delta HcyH}{\Delta HcyO_2}\right)_{pH} = \left(\frac{\Delta HCO_3^-}{\Delta HcyO_2}\right)_{pH}, \quad (1)$$

where HcyH and $HcyO_2$ are the concentrations of protonated and oxygenated binding sites, respectively, in haemocyanin. By definition (as indicated by the subscript), this linkage is valid for one specific pH.

pH changes in the blood, which result from deoxygenation and the corresponding CO_2 production in the tissues, can be determined from model calculations similar to those outlined by Pörtner (1987). The increase in C_{CO_2} in venous blood can be calculated taking into account the amount of transported oxygen and assuming a respiratory quotient (RQ) of 0.8–0.85 for the animals (based on the assumption that amino acid and protein metabolism are predominant, see Hoeger *et al.* 1987). When calculating the increase in venous apparent bicarbonate levels, P_{CO_2} was assumed, as a first approximation, to be constant:

$$\Delta[HCO_3^-] = \Delta C_{CO_2} - \alpha P_{CO_2}, \quad (2)$$

where α is the solubility of CO_2. The pH change caused by deoxygenation results from the corresponding shift in the buffer line at constant P_{CO_2}. This shift is only a fraction of the calculated increase in bicarbonate levels (Figs 5 and 6). Adding the missing amount gives an approximate value for the venous pH along the buffer line of deoxygenated blood. At this pH, P_{CO_2} can then be calculated from C_{CO_2} by using the Henderson–Hasselbalch equation and adequate values of pK''' and CO_2 solubility (calculated according to Heisler, 1986).

$$P_{CO_2} = C_{CO_2}/(\alpha \times 10^{pH-pK'''} + \alpha). \quad (3)$$

This value of P_{CO_2} is then used to recalculate the increase in venous bicarbonate levels (equation 2). Following on from this, the values of venous pH, P_{CO_2} and bicarbonate levels are readjusted in an iteration procedure.

pH changes required to bring about deoxygenation can be estimated both from a consideration of Bohr coefficients and from the slopes of the oxygen isobars (in Figs 1A, 2A, 4A). The steepness of these slopes can be quantified from $\Delta HcyO_2/\Delta pH$ or $\Delta S/\Delta pH$ (where S is oxygen saturation) at constant P_{O_2}.

Along each isobar, values of S depend on pH values and the P_{O_2} of the isobar. The pH/saturation diagram allows comparison of S, pH and P_{O_2} with P_{50} at $S=0.5$ and the same pH ($=pH_{50}$, Fig. 3A). This provides values of S, $\log P_{O_2}$ and $\log P_{50}$ which are needed for the analysis of cooperativity at a specific pH. If this is done in the range of saturation S between 0.4 and 0.6, the analysis allows a close estimate of Hill coefficients (n_{50}) to be made according to (see Fig. 3B):

$$\log \frac{S}{1-S} = n(\log P_{O_2} - \log P_{50}), \quad (4)$$

where P_{O_2} is the P_{O_2} of the isobars shown in Figs 1A, 2A, 3A, and 4A; S results

from the P_{O_2} at a specific pH (pH_{50}, Fig. 3A) and P_{50} is the P_{O_2} for S=0.5 at the same pH.

Results and discussion

Illustration of pH effects

The pH-dependence of oxygen binding can be described by plotting the change in the P_{O_2} of half-saturation (P_{50}) against H^+ activity on a double logarithmic scale. pH effects on cooperativity can be evaluated in the region of P_{50} as the change of slope of the oxygen-binding curve in the Hill plot. The Hill coefficient n_{50} characterizes this change in cooperativity. pH effects for other ranges of oxygen saturation may be analysed in the same fashion. It rapidly becomes clear, however, that these analyses of pH effects focus on only one value of saturation at a time and do not provide a comprehensive illustration of pH effects within the whole range of oxygenation.

Such an illustration is also lacking in the classical presentation of oxygen-binding curves using a P_{O_2}/saturation diagram. For pH-sensitive pigments, the oxygen-binding curve shifts with pH; this can be taken into account by presenting several curves, each valid for one pH value. No pH scale is included in the presentation, with the result that the course of transitions in oxygen binding with changing pH cannot easily be quantified. In highly pH-sensitive pigments such as cephalopod haemocyanin this presentation is also problematical for practical reasons. During the analysis of oxygen binding with changing P_{O_2}, the changes in oxygenation will change pH according to the Haldane coefficient. According to the Bohr coefficient, saturation values are affected by these pH changes and, consequently, the oxygen-binding curve will not be valid for a specific pH. For this reason, buffers have been used in pigment solutions to minimize pH fluctuations (e.g. Miller and Mangum, 1988). The use of buffers, however, is unphysiological and may not always be advisable (as discussed by Brix *et al.* 1989), for example, if oxygen-binding data *in vitro* and *in vivo* are to be compared.

The present study tries to improve on these drawbacks by analysing haemocyanin oxygen-binding in whole blood and applying a method of analysis that allows continuous and quantitative monitoring of pH effects. This is simply achieved by plotting saturation and pH (instead of P_{O_2}) and including P_{O_2} in the form of oxygen isobars. Figs 1A and 2A show the influence of pH and P_{O_2} on the oxygenation of *Illex illecebrosus* and *Loligo pealei* haemocyanin in a pH/saturation diagram. This diagram is reminiscent of that presented by Redfield and Goodkind (1929) (also a Cartesian diagram), which showed total CO_2 (not pH) and total O_2 (not bound O_2). In the pH/saturation diagram, each oxygen isobar represents an oxygen-binding curve. At constant P_{O_2}, transitions in oxygenation are depicted as they occur with changes in pH. Oxygen isobars show changing slopes which are linear in the range around P_{50}. Each isobar indicates saturation (S, $\bar{x}\pm$S.D.) where it runs parallel to the pH axis (S=1.37±0.07 mmol l^{-1}, N=20 for *Illex illecebrosus*, S=1.38±0.04 mmol l^{-1}, N=14 for *Loligo pealei*).

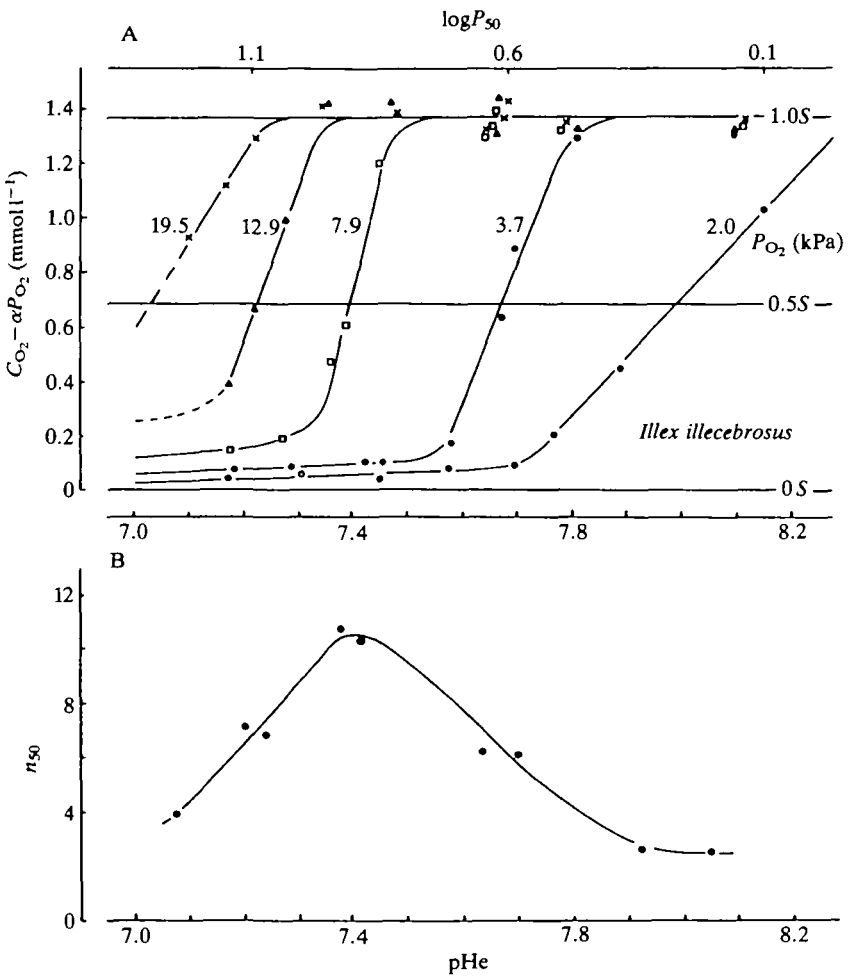

Fig. 1. Graphical presentation of the interrelationship between oxygen binding, pH and P_{O_2} in squid (*Illex illecebrosus*) whole blood (A). Oxygen isobars are linear around 0.5*S* (where *S* is oxygen saturation) but exhibit a changing slope in accordance with a maximum in pH sensitivity. P_{O_2}=○ 2.0 (14.7), ● 3.7 (28), □ 7.9 (59), △ 12.9 (97), × 19.5 (146) kPa (mmHg). Hill coefficients (n_{50}) (B) have been analysed following the procedure described in Fig. 3A. A change in cooperativity with pH is likely to determine the slopes of the oxygen isobars in A. For further explanation see Table 2 and text.

Modulation of the haemocyanin oxygen-affinity by organic and inorganic cofactors and by temperature fluctuations would lead to changes in the location of the oxygen isobars. Modulation of cephalopod haemocyanins by organic cofactors can probably be excluded (C. R. Bridges, personal communication), while modulation by inorganic cofactors would largely depend on changes in the environment, which are unlikely to occur in the case of pelagic squid.

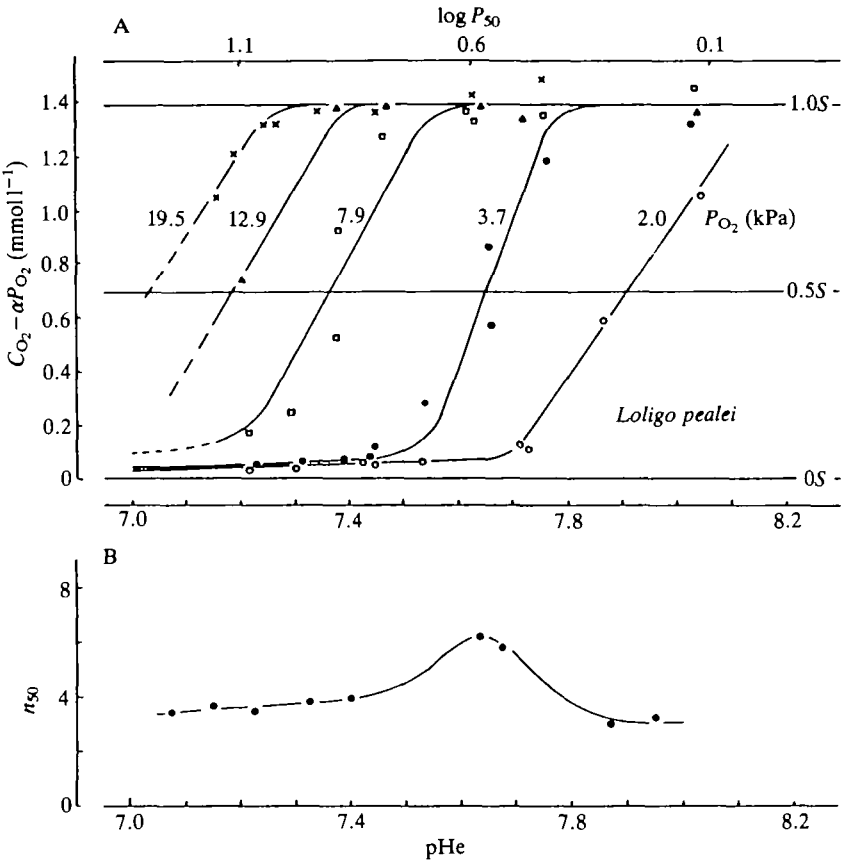

Fig. 2. Graphical presentation of the interrelationship between oxygen binding, pH and P_{O_2} in squid (*Loligo pealei*) whole blood (A). Hill coefficients (n_{50}) (B) have been analysed following the procedure described in Fig. 3A. For further explanation see Fig. 1 and text.

Analysis of Hill coefficients

In whole blood, experimental variation of oxygenation always causes pH changes. This must be considered both when P_{O_2}/saturation curves are constructed (see above) and when Hill coefficients (n_{50}) are analysed (Fig. 3B). For the latter, saturation values slightly smaller or larger than 0.5 are usually selected. Changes in oxygenation are elicited by varying P_{O_2}. In the range of saturation below 0.5, pH is higher than pH_{50} owing to proton consumption during deoxygenation. In the range of saturation above 0.5, however, pH is below pH_{50}. This means that the analysis of n_{50} is affected by the Bohr effect shifting saturation to higher values at higher pH (saturation below 0.5) and to lower values at lower pH (saturation above 0.5). If these saturation values are used for evaluating the Hill coefficient, the real slope of the oxygen-binding curve at pH_{50} is underestimated. The degree to which this error occurs depends on the magnitude of the

Bohr effect (Fig. 3B). The error is independent of oxygen affinity. It is especially large with a high oxygen-binding capacity (that is, with high pigment concentrations in the blood) and at low P_{CO_2}, since, owing to the low CO_2/bicarbonate buffering, the pH change is large under these conditions. The error increases with increasing cooperativity and, therefore, may prevent the detection of a pH-

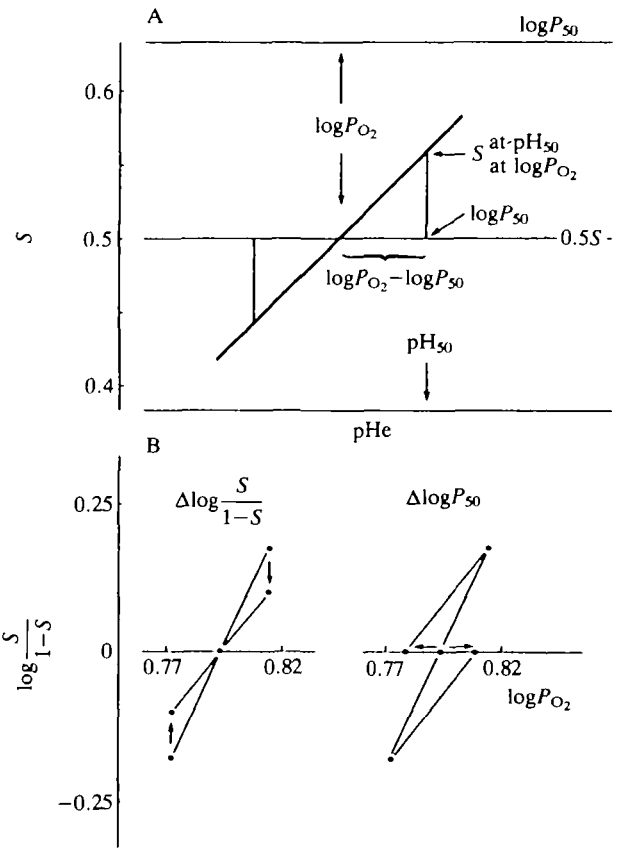

Fig. 3. A comparison of two ways of analysing Hill coefficients (n_{50}). (A) In the analysis based on Figs 1A and 2A, n_{50}, in principle, results from a comparison of saturation values at the same pH, one being depicted on the isobar shown in A, the other one being $0.5S$ at the indicated pH_{50} and the corresponding $\log P_{50}$. In the traditional procedure (B), saturation analysis under varying oxygen tensions, the Bohr effect may play an important role. If, in a model calculation, n_{50} is re-estimated for arbitrary values of pH=7.5, P_{50}=6.23 kPa (46.7 mmHg), n_{50}=8.5, S=0.4 and 0.6 and a Bohr/Haldane coefficient of −1.02, the experimental variation of P_{O_2} between 5.93 kPa (44.5 mmHg) and 6.52 kPa (48.9 mmHg) leads to a low estimate of n_{50}, 4.9. The error in the calculation of n_{50} can be quantified, analysing the shift in $\log[S/(1-S)]$ (±0.073, see left) by readjusting ΔpH (±0.009 based on Fig. 5) and S (0.56, 0.44) in an iteration procedure (Pörtner, 1987). More easily, the shift in pH (±0.015 at $\Delta S = \pm 0.1$) can be analysed based on Fig. 5 and the Haldane coefficient. The indicated shift in $\log P_{50}$ (±0.015) results from the Bohr coefficient. This calculation allows experimental values to be corrected for the influence of the Bohr effect.

sensitivity of cooperativity. Again, buffered pigment solutions may be used (DePhillips *et al.* 1969; Miller, 1985), but the graphical analysis (Fig. 3A) based on Figs 1A, 2A and 4A completely eliminates the introduced error, even for whole blood, since values of S, $\log P_{O_2}$ and $\log P_{50}$ are evaluated for one specific pH.

Figs 1B and 2B show the change in cooperativity with pH for the two squid species. Hill coefficients are found to be largest in the range of pH where oxygen isobars exhibit a maximum steepness (Figs 1A, 2A). The maximum is more prominent in *Illex* and occurs at higher P_{O_2} and lower pH values in *Illex* than in *Loligo* (see Table 2). In both species the largest Hill coefficients are found close to the range of *in vivo* blood P_{O_2} and pH values (based on H. O. Pörtner, D. M. Webber, R. G. Boutilier and R. K. O'Dor, in preparation). The same is likely to be true for *Octopus dofleini* if pH values published by Lenfant and Johansen (1965) are compared with *in vitro* studies of buffered haemocyanin solutions (Miller, 1985; Miller and Mangum, 1988; Fig. 4A,B). The values of n_{50} shown in Fig. 2B are close to the n_{max} values given by DePhillips *et al.* (1969) for buffered *Loligo pealei* haemocyanin solutions at the respective pH.

Buffer values, Bohr and Haldane coefficients

Analysis of total CO_2 during variations of P_{O_2} and P_{CO_2} in tonometered blood gives the buffer lines shown in the pH/bicarbonate diagrams (Figs 5 and 6). In both species, the position of the buffer lines is different for oxygenated and deoxygenated blood. Non-bicarbonate buffer values (β) are close in the two squid species, that in *Illex* blood ($5.0\,mmol\,l^{-1}\,pH\,unit^{-1}$) being slightly smaller than that in *Loligo* blood ($5.8\,mmol\,l^{-1}\,pH\,unit^{-1}$). The vertical distance between the buffer lines for oxygenated and deoxygenated blood (Figs 5 and 6) yields the Haldane coefficient ($\Delta HCO_3^-/\Delta HcyO_2$). In contrast to reports for other cephalopods (Brix *et al.* 1981; Lykkeboe *et al.* 1980), the Haldane coefficient does not depend on pH.

A comparison of measured and calculated apparent bicarbonate levels allows us to determine whether CO_2 was specifically bound to the haemocyanin. For this purpose values of CO_2 solubility and pK''' calculated according to Heisler (1986) were used. (This calculation procedure had previously been demonstrated to yield valid results in the body fluid of another marine invertebrate, *Sipunculus nudus*, H. O. Pörtner and N. Heisler, unpublished results.) Calculated and measured amounts of apparent bicarbonate agree in both oxygenated and deoxygenated squid blood. Consequently, total CO_2 values include only dissolved CO_2 and apparent bicarbonate levels. Oxygen-linked CO_2 binding, therefore, is not involved in haemocyanin function in *Illex illecebrosus* or *Loligo pealei* (or *Octopus dofleini*, Miller and Mangum, 1988).

The Bohr coefficients obtained by linear regression analysis from Figs 1A and 2A are $\Delta\log P_{50}/\Delta pH=-1.07$, $r^2=0.994$ for *Illex illecebrosus* and $\Delta\log P_{50}/\Delta pH=-1.15$, $r^2=1.0$ for *Loligo pealei*. In the pH range between 7 and 8 the linear relationship between $\log P_{50}$ and pH was not affected by the method used to alter pH [titration with CO_2, fixed acid (HCl) or fixed base (NaOH)]. This

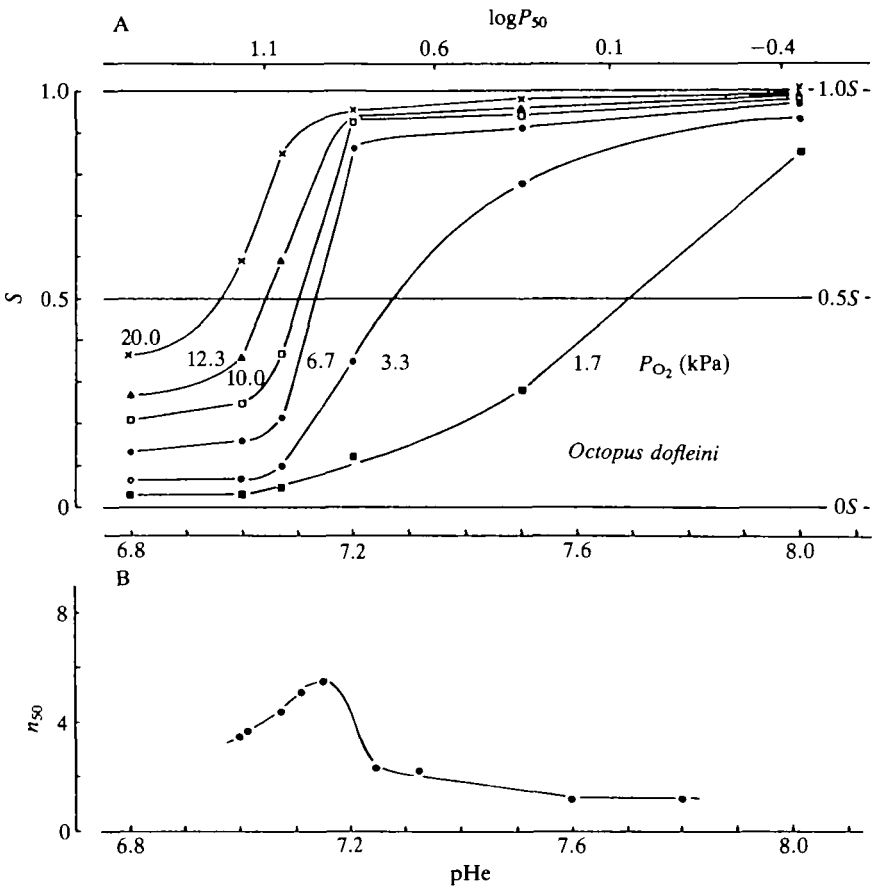

Fig. 4. A similar picture to that obtained for squid results for *Octopus dofleini* blood (at 20°C) if data by Miller and Mangum (1988, their Fig. 2) are transferred into the pH/saturation diagram. Maximum slopes of the oxygen isobars [A, P_{O_2}=■ 1.7 (12.5), ○ 3.3 (25), ● 6.7 (50), □ 10.0 (75), △ 13.3 (100), × 20.0 (150) kPa (mmHg)] are represented by maximum values of cooperativity (B, as analysed in A) in the range of *in vivo* pH (see text). The pH-insensitive reserve of bound oxygen is even larger than in squid (see Figs 1A and 2A). Note that this diagram is based on measurements in Tris-buffered haemocyanin solutions, not in whole blood.

suggests that CO_2 or fixed acid have no specific influence on the change in oxygen affinity with pH; that is, CO_2 and fixed acid Bohr effects are identical. The values for both species are close to the one found by Brix *et al.* (1981) in *Loligo forbesi* and by Brix *et al.* (1989) in *Todarodes sagittatus*. In two other squid species (*Loligo vulgaris* and *Architeuthis monachis*) Brix *et al.* (1989) found Bohr coefficients larger than −1, with the one in the mediterranean species *Loligo vulgaris* being as much as −0.4 at 20°C. The value found for *Loligo pealei* is larger than that (−1.8) formerly cited (Lykkeboe *et al.* 1980; Brix *et al.* 1989) but is close to the value of −1.3 (23°C) which can be recalculated from original data given for *Loligo pealei*

by Redfield and Goodkind (1929, see Table 1). This also agrees with a value of −1.16 recalculated from the data of DePhillips *et al.* (1969) for the pH range between 6.9 and 7.5 (23°C).

Bohr and Haldane coefficients are slightly smaller in *Loligo* than in *Illex* blood. For each species, the numerical values of Bohr and Haldane coefficients are virtually identical (cf. Figs 5 and 6). Oxygen-linked CO_2 binding would have caused the (experimental) Haldane coefficient to differ from the numerical value of the Bohr coefficient. The finding of an agreement is in accordance with the

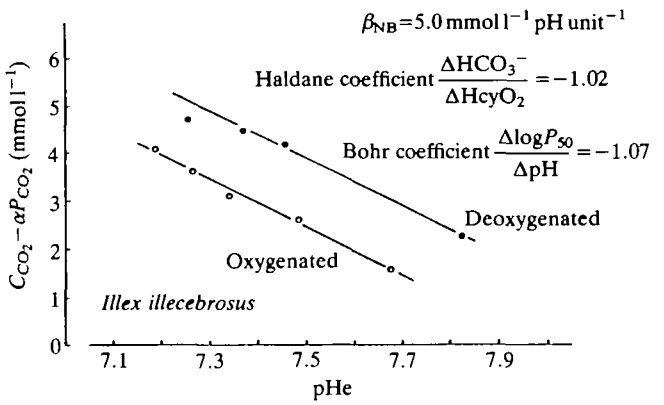

Fig. 5. pH/bicarbonate diagram for squid (*Illex illecebrosus*) whole blood showing the buffer line of oxygenated and deoxygenated blood, the resulting Haldane coefficient, and the Bohr coefficient evaluated from Fig. 1A. Measured and calculated values of apparent bicarbonate concentration yield buffer lines identical in slope and position, indicating that oxygen-linked CO_2 binding is not involved. β_{NB}, non-bicarbonate buffer value.

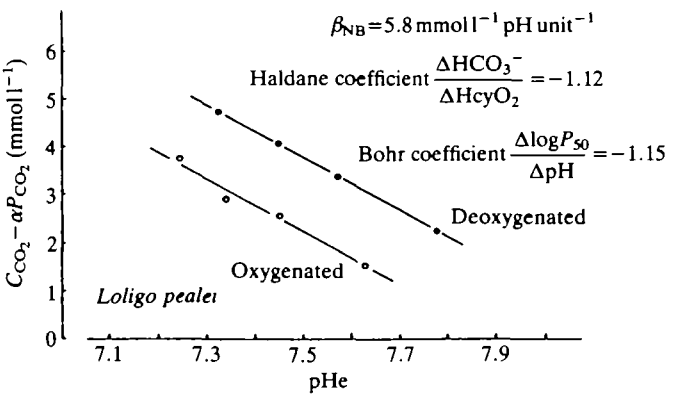

Fig. 6. pH/bicarbonate diagram for squid (*Loligo pealei*) whole blood showing the buffer line of oxygenated and deoxygenated blood, the resulting Haldane coefficient, and the Bohr coefficient as evaluated from Fig. 2A. As for *Illex*, measured and calculated values of apparent bicarbonate yield identical buffer lines. Consequently, oxygen-linked CO_2 binding is not involved. β_{NB}, non-bicarbonate buffer value.

Table 1. *Recalculation of the Bohr coefficient for* Loligo pealei *based on the early work by Redfield* et al. *(1926), Redfield and Goodkind (1929, their Fig. 1 and Table 2, T=23°C) and Redfield and Ingalls (1933)*

	C_{CO_2} (mmol l^{-1})	P_{CO_2} (kPa)	$[HCO_3^-]$ (mmol l^{-1})	pH	logP_{50}
In vitro	5.64	1.73	5.15	7.07	1.26
	2.43	0.40	2.32	7.34	0.90
	1.15	0.13	1.11	7.50	0.71
In vivo					
Arterial blood	1.79	0.29	1.70	7.34	
Venous blood	3.71	0.80	3.49	7.22	

Bohr coefficient: $\Delta\log P_{50}/\Delta pH = -1.28$.

pH and bicarbonate levels have been recalculated from total CO_2 and P_{CO_2} using the Henderson–Hasselbalch equation and values of pK''' and CO_2 solubility (α) calculated according to Heisler (1986). The recalculated parameters can only represent an approximation since Redfield and Goodkind measured the difference in C_{CO_2} between oxygenated and deoxygenated blood at one high value of P_{CO_2} (2 kPa=15 mmHg) and assumed that 'at lower pressures (of CO_2) the difference between the CO_2 content of oxygenated and reduced blood is proportional to the CO_2 content of reduced blood'. This assumption is not confirmed by Fig. 6 of the present study and, thus, their values of C_{CO_2} in oxygenated blood are increasingly overestimated in the range of low P_{CO_2} and high pH. This affects both the arterial value of pH, which may have been overestimated, and the value of the Bohr coefficient, which, because pH_{50} is increasingly overestimated, may have been underestimated to some extent. The final pH values re-evaluated for arterial blood are close to pH values published by Mangum and Shick (1972) and Howell and Gilbert (1976) for squid (handled animals) at 23 °C.

linkage equation (Wyman, 1964) and provides additional evidence that O_2-linked CO_2 binding does not contribute to haemocyanin function in the two squid species.

An integrated view of pH sensitivity

Starting from an initial P_{O_2} in the arterial blood, the pigment releases oxygen on its way through the tissues, thus preserving tissue P_{O_2}. In this way, oxygen gradients are kept high to allow for a high diffusive oxygen flow. By depicting the change in oxygenation at a given P_{O_2}, the pH/saturation diagrams (Figs 1A, 2A) elucidate this buffer function of the pigment. They demonstrate how pH must change to maintain a high venous P_{O_2}. The slope of the resulting isobar indicates its sensitivity to pH. Obviously, this kind of pH sensitivity is different in different ranges of pH and P_{O_2} and cannot be determined by an analysis of the Bohr coefficient. In fact, the magnitude of the Bohr coefficient does not change with pH (see above).

More likely, as suggested by the observation that the maximum cooperativity corresponds to the maximum steepness of the isobars, the change in pH sensitivity may be explained by a change in cooperativity with pH. In this case the oxygen isobar, the steepness of which is given by the ratio $\Delta HcyO_2/\Delta pH$ or $\Delta S/\Delta pH$, would vividly illustrate and quantify the physiological meaning of a sensitivity of

Table 2. *Changes in the slopes of oxygen isobars ($\Delta HcyO_2/\Delta pH$) with pH, based on the graphical analysis in Figs 1A and 2A*

	P_{50} (kPa)	$\Delta HcyO_2/\Delta pH$	pH_{50}	n_{50}
Illex illecebrosus	19.5	3.6	7.04	ND
	12.9	5.6	7.22	7.0
	7.9	8.3	7.40	10.6
	3.7	5.2	7.67	6.2
	4.0	2.1	7.99	3.0
Loligo pealei	19.5	3.2	7.03	ND
	12.9	3.4	7.19	3.6
	7.9	3.7	7.37	3.9
	3.7	5.5	7.65	6.1
	2.0	2.9	7.91	3.1

The slopes are compared with Hill coefficients n_{50} derived from Figs 1B and 2B.

$HcyO_2$, haemocyanin-bound oxygen; pH_{50}, pH at half saturation; ND, not determined; P_{O_2}, P_{O_2} of the isobar.

cooperativity to pH (see below). The linear slope is specific for a combination of P_{50} and pH_{50} values and is valid for up to 80–90 % of the range of saturation (Table 2).

Fig. 7 demonstrates how pH changes may influence oxygen binding in the living animal. Lines *B* and *C* reflect two possible pH changes between arterial and venous blood. Line *C* results from the large Haldane effect, if oxygen is provided only *via* the blood (not through the skin, see below) and if RQ values below 1 prevail (Table 3). The alkalosis causes venous P_{O_2} to fall more than would be observed with a pH-insensitive pigment.

Line *B* is in accordance with a P_{O_2} buffer function of the pigment and follows the isobar for P_{O_2}=7.9 kPa. This isobar exhibits a steeper slope than the isobar of P_{O_2}=12.9 kPa. 12.9 kPa may be close to the arterial oxygen tension (Redfield and Goodkind, 1929). If, in the model, 7.9 kPa is the venous P_{O_2}, this isobar would be crucial for the process of oxygen release. The change from 12.9 to 7.9 kPa would represent an arteriovenous oxygen gradient and would illustrate how the pH sensitivity of the pigment could increase between arterial and venous blood and, thereby, minimize the required arteriovenous pH gradient. The pH change of the blood as it passes through the tissue can be quantitatively predicted as deoxygenation proceeds. Such a pH change has not been confirmed in squid since the early study of Redfield and Goodkind (1929). These authors, however, analysed blood sampled from the branchial hearts of restrained (nailed down) animals (*Loligo pealei*, for the recalculated pH values see Table 1), an experimental situation that may yield different results from those to be observed in unrestrained animals.

This discussion and the observation that pH sensitivity is maximized in the range of *in vivo* pH suggest that deoxygenation *in vivo* may be caused predominantly by pH changes rather than by P_{O_2} fluctuations. If the pigment actually represents a

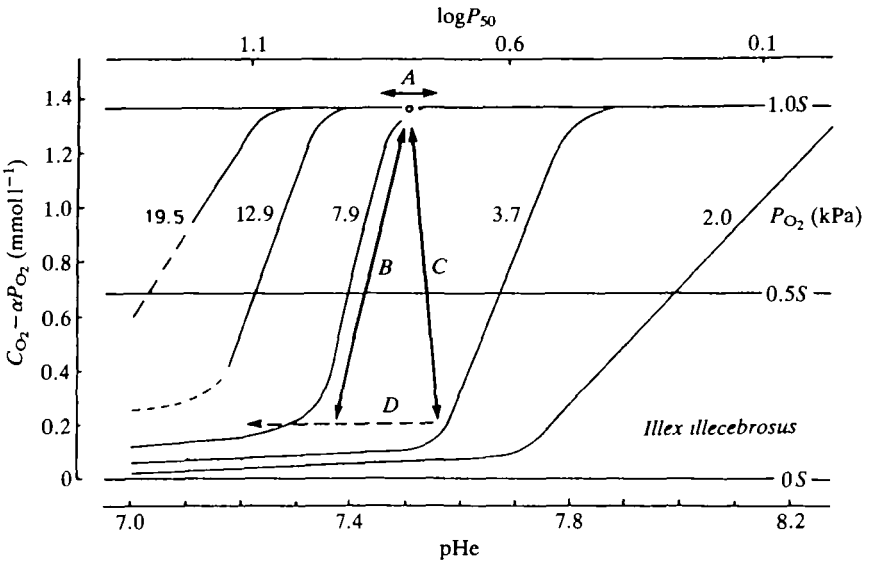

Fig. 7. Modelling of changes in arterial (*A*) and venous (*D*) blood acid–base parameters and their effect on haemocyanin oxygen-binding in the squid *Illex illecebrosus*. *B* and *C* represent two alternative lines of transition between arterial and venous values of oxygenation. Line *C* considers the Bohr effect, the respiratory quotient and the resulting P_{CO_2} changes according to Table 3. Additional venous acidification along line *D* may move line *C* towards line *B*, the latter being in accordance with the P_{O_2} buffer function of the pigment. For further explanation see text.

Table 3. *Model calculation of the venous pH which results from complete deoxygenation of the haemocyanin of* Illex illecebrosus, *assuming CO_2 production in accordance with an RQ of 0.85 (see line* C *in Fig. 7)*

$\frac{\Delta HCO_3^-}{\Delta HcyO_2}$	β	$\Delta HcyO_{2,max}$ (mmol l^{-1})	ΔC_{CO_2} (mmol l^{-1})	$\Delta H^+_{HcyO_2}$ (mmol l^{-1})
−1.02	5.0	−1.37	1.17	−1.40

Blood parameters	pH	$[HCO_3^-]$ (mmol l^{-1})	P_{CO_2} (kPa)
Arterial	7.50	2.5	0.25
Venous	7.57	3.6	0.32

The analysis reveals that the C_{CO_2} increase (ΔC_{CO_2}) expected from complete deoxygenation ($\Delta HcyO_{2,max}$) does not lead to a large enough increase in P_{CO_2} to compensate for the proton uptake by the pigment ($\Delta H^+_{HcyO_2}$). Consequently, a venous alkalosis would result.

buffer system for P_{O_2}, the evolution of pH sensitivity clearly renders deoxygenation without concomitant acidification disadvantageous (Fig. 7, line *C*). Cooperativity and the Bohr effect may, therefore, be coordinated to optimize pH sensitivity with respect to pH fluctuations occurring in the animal. If pH sensitivity by cooperativity is maximized in the range of *in vivo* values, this would suggest that cooperativity compensates for the high proton need during deoxygenation; that is, the high Bohr and Haldane effects. Not much acidification in excess of proton consumption is needed to support venous deoxygenation.

Maintenance of function at high and low pH

A pH drop in arterial blood would initially follow the direction of line *A* in Fig. 7. High oxygenation ($S=1$) is maintained. S is not maintained, however, when the arterial isobar is deflected in a downward direction. If the 12.9 kPa oxygen isobar reflects the dependence of arterial oxygenation on pH, haemocyanin saturation can no longer be maintained 0.1–0.15 units below arterial pH. Thus, as already outlined by Redfield and Goodkind (1929), an acid shift in arterial pH would be lethal to the animals.

In the range of pH down to below 6.7–6.8 in *Illex* blood (not shown in Figs 1 and 7), the sensitivity to pH leads to a further decrease in saturation. Even at P_{O_2} values of 93.3 kPa, which are far above the physiological range, saturation values remain below those observed at higher pH ($S=1$). Maximum saturation observed at these high P_{O_2} values and at low pH is equivalent to $S=0.8$ at pH 6.7 or $S=0.6$ at pH 6.3. As a corollary, the scope of oxygenation is reduced. It remains to be investigated whether this effect (which was also observed in *Octopus* blood by Miller and Mangum, 1988) is of any physiological significance, since it occurs outside the range of pH and P_{O_2} values measured in cephalopods *in vivo*.

The slopes of the oxygen isobars are less steep at higher and at lower pH. In the range of low venous pH (e.g. during muscular activity, following line *D*, Fig. 7, in the acid direction), there is an increasing amount of bound oxygen which is resistant to further pH-dependent deoxygenation and which can only be depleted by the lowering of P_{O_2} (Figs 1A, 2A, 4A). This conclusion is based on the observation that, at low saturation, the slope of each isobar decreases with falling pH until it runs almost parallel to the pH axis. With increasing P_{O_2}, this occurs at increasingly higher values of saturation. This may indicate a venous reserve that is protected against excessive pH changes. Such a pH-insensitive reserve would diminish any overshoot in P_{O_2} caused by large pH variations and may help to maintain an equal distribution of oxygen throughout a tissue. Excessively high venous P_{O_2} values would, for example, reduce the O_2 gradient from the environment to the tissue and, thus, reduce the amount of oxygen taken up *via* the skin (see below).

Under conditions of low arterial P_{O_2} and high pH (e.g. during hyperventilation under hypoxia, following line *A*, Fig. 7, in the alkaline direction) a reduction in pH sensitivity may also support a more equal distribution of the oxygen reserves to the tissue. This is only valid, however, if venous pH remains largely unaffected by

hyperventilation. Progressive alkalization of arterial blood would cause an increase in haemocyanin oxygen-affinity, thus compensating for a drop in ambient P_{O_2}. However, an increase in venous pH would cause very low venous P_{O_2} values, because cooperativity would be reduced and there would be less compensation for the large Bohr and Haldane effects. Therefore, an increase in the arteriovenous pH gradient would be required for the maintenance of pigment function.

Venous acidification

The conclusion from the foregoing discussion is that a decrease in pH of the blood as it passes from the arterial to the venous side would be consistent with a P_{O_2} buffering function of the haemocyanin (Fig. 7, line *B*). Under aerobic steady-state conditions venous pH can only decrease by the build-up of an arteriovenous P_{CO_2} gradient. However, such a build-up is obstructed by the large Haldane effect. To compensate for this, a mechanism of oxygen-linked, pH-independent CO_2 binding has been proposed (Lykkeboe *et al.* 1980; Brix *et al.* 1981; see Introduction). According to this mechanism, both O_2 and CO_2 are transported to the tissues and both the CO_2 produced by metabolism and the CO_2 released during deoxygenation lead to the build-up of P_{CO_2} in the tissue.

Alternatively, the uptake of significant amounts of oxygen *via* the skin could lead to additional CO_2 formation by metabolism. Such oxygen uptake can occur, especially in regions of the body that are densely vascularized and close to the body surface, such as the outer layers of the mantle musculature (Bone *et al.* 1981). An elevated metabolic rate would cause P_{CO_2} to increase in the tissue and, therefore, in the venous blood. This process could provide the excess protons needed to acidify the blood and to compensate for Bohr coefficients smaller than -1.

An increase in the amount of CO_2 being transported by the blood would be detectable if the respiratory quotient were measured (a) for the blood going to and returning from the tissues, and (b) for the whole animal. High RQ values of 1.1 were obtained by Redfield and Goodkind (1929) from measurements of oxygen and CO_2 transport in *Loligo pealei* blood. Similar RQ values were also obtained from blood gas transport data in unrestrained, cannulated *Octopus dofleini* (Johansen and Lenfant, 1966). For the whole animal, however, RQ values greater than 1 are exceptional under aerobic steady-state conditions and, if they occur, they are thought to be related to high rates of lipid biosynthesis from carbohydrates. This is improbable in squid, where protein and amino acid catabolism are likely to predominate (Hoeger *et al.* 1987). The observation that blood RQ values exceed those expected for the whole animal supports the conclusion that additional CO_2 is being produced and released into the blood. The additional CO_2 cannot originate from oxygen-linked CO_2 binding, since this process does not influence the net transport of CO_2 from the tissues to the gills. It is therefore very likely that oxygen uptake *via* the skin is responsible for the arteriovenous P_{CO_2} gradient and may allow for a decrease of pH in venous blood below that of arterial

blood. Further evidence for oxygen uptake *via* the skin is discussed by O'Dor *et al.* (1990).

In conclusion, the graphical presentation selected for Figs 1A, 2A and 4A allows Bohr and Hill coefficients to be analysed. By integrating the Bohr effect and the changes in cooperativity with pH, the diagram illustrates the physiological implications of pH sensitivity for both oxygen affinity and cooperativity. In squid haemocyanin, cooperativity probably helps to compensate for the negative influence of a high proton uptake during deoxygenation (Haldane effect) on venous P_{O_2}.

The diagram demonstrates the *in vitro* properties of squid haemocyanin and also appears to be suitable for a presentation of arteriovenous changes in pH and P_{O_2} *in vivo* (Fig. 7). As in a pH/bicarbonate/P_{CO_2} diagram, the whole range of combinations of pH, P_{O_2} and oxygen saturation values is covered. The analysis of *in vitro* data indicates how pH changes can play an important role in squid haemocyanin oxygenation/deoxygenation cycles. The question of whether the predictions of Figs 1A, 2B and 7 can actually hold *in vivo* in squid is the subject of further analysis (H. O. Pörtner, D. M. Webber, R. G. Boutilier and R. K. O'Dor, in preparation). The extent to which the graphical presentation is generally applicable to depict the properties and function of other pH-dependent respiratory pigments *in vitro* and *in vivo* remains to be investigated.

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