Oxygen binding and its allosteric control in hemoglobin of the pulmonate snail, Biomphalaria glabrata

JON BUGGE AND ROY E. WEBER
Danish Centre for Respiratory Adaptation, Department of Zoophyiology, Institute of Biological Sciences, University of Aarhus, DK 8000 Aarhus C, Denmark

Oxygen binding and its allosteric control in hemoglobin of the pulmonate snail, Biomphalaria glabrata. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R347–R356, 1999.—Pulmonate snails that experience extreme variations in gas tensions and temperatures possess extracellular, high-molecular mass (~1.7 × 10^6 Da) hemoglobins (Hbs) that are little known as regards oxygenation and allosteric characteristics. Biomphalaria glabrata hemolymph exhibits a high O_2 affinity (half-saturation O_2 tension = 6.1 mmHg; pH 7.7, 25°C), pronounced Bohr effect (Bohr factor = −0.5), and pH-dependent cooperativity (Hill’s cooperativity coefficient at half-saturation = 1.1–2.0). Divalent cations increase O_2 affinity, Ca^{2+} exerting greater effect than Mg^{2+}. Analyses in terms of the Monod-Wyman-Changeux model indicate novel O_2 affinity control mechanisms. In contrast to vertebrate Hb, where organic phosphates and protons lower affinity via decreased O_2 association equilibrium constant of Hb in low-affinity state (K_T), and to extracellular annelid Hbs, where protons and cations primarily modulate O_2 association equilibrium constant of Hb in high-affinity state (K_A), in B. glabrata Hb, the Bohr effect is mediated predominantly via K_R and the cation effect via K_T, reflecting preferential, oxygenation-linked proton binding to oxygenated Hb and cation binding to deoxygenated Hb. CO_2 has no specific (pH independent) effect. Nonlinear van't Hoff plots show temperature dependence of the overall heats of oxygenation, indicating oxy-deoxy heat capacity differences. The findings are related to possible physiological significance in pond habitats.

Bohr effect; cation effects; cooperativity; oxygen transport; mollusks

THE OCCURRENCE OF HEMOGLOBIN (Hb) in invertebrates is widespread among invertebrate phyla, where an extensive range of adaptive roles has been postulated (16, 31). Among mollusks that commonly use hemocyanin for gas transport, extracellular Hbs occur in aquatic pulmonate gastropods and two families of bivalves (13, 38).

In contrast to the intracellular vertebrate Hbs, which weigh ~68 × 10^3 Da, extracellular planorbid Hbs are large, multidomain and multisubunit proteins (28) with molecular mass values of 1.60–1.75 × 10^6 Da [1.70 × 10^6 Da for Biomphalaria glabrata (2)]. The molecules contain 3% sugars (1) and 1 heme per 17,700 Da protein (23). Only scant information is available on the quaternary structure of B. glabrata Hb. However, on the basis of electron micrographs, the Hb of the related Planorbis corneus has been interpreted as consisting of hexagonal ring structures (37) and that of the pulmonate Helisoma trivolvis as, variously, a 10-membered ring structure (22), 12 single polypeptide chain subunits that each carry 10 hemes and are grouped in pairs held together by disulfide bonds (10), and a compact two-layer pentameric ring structure of decamers stabilized by disulfide linkages (9). In contrast, the Hbs of the heterodont bivalve families Astartidae and Carditidae exhibit higher molecular masses (8–12 × 10^6 Da), greater size heterogeneity of the native molecules, and subunits of 240,000–390,000 Da that contain 1 mol heme per 17,000–20,000 g protein (20).

As with vertebrate Hbs, planorbid Hbs exhibit inhibitory, heterotropic interactions between O_2 and proton binding sites (Bohr effects) as well as homotropic heme-heme interactions (cooperativity) that are responsible for the sigmoid shape of O_2 binding curves (23, 26, 38). Invertebrate heme-carrying pigments are insensitive to anionic organic phosphates like glycerate-2,3-bisphosphate and ATP (16, 30, 31) that decrease O_2 affinity of Hb in vertebrate red blood cells by lowering the affinity constant of the tense, deoxygenated form of the Hb molecule (K_T). O_2 affinity of the high-molecular weight, extracellular Hbs and chlorocruorins from annelids are increased by inorganic cations (5, 11, 32), and data of van Aardt and Naude (26) provide evidence for similar effects in Biomphalaria. In the annelid pigments, inorganic cations and decreasing proton concentrations increase O_2 affinity primarily by raising the affinity constant of the relaxed, oxygenated form of the Hb molecule (K_A) (8, 11, 25, 32).

The Hbs of pulmonate snails appear to play an important role in O_2 transport. In P. corneus the presence of Hb correlates with a 20-min delay in the onset of anaerobiosis compared with that in the Hb-free pulmonate Lymnaea stagnalis and increased diving potential, lower postdive pulmonary O_2 tensions, and a greater exploitation of the pulmonary O_2 store than in L. stagnalis (12).

Biomphalaria typically inhabits swamps that present drastic variations in the physicochemical factors that affect Hb-O_2 binding. Extreme hypoxia (P_{O_2} commonly falling to 0–7 mmHg) is frequently accompanied by marked hypercapnia [P_{CO_2} values rising to 35 mmHg (13)] and large temperature variations. As with many invertebrates, planorbid snails do not maintain constancy in the hemolymph ionic concentrations in response to changing osmolality of the medium (14).

We have studied the oxygenation properties of B. glabrata hemolymph and Hb and the effects of CO_2, inorganic ions, pH, and temperature, seeking to iden-
ify adaptations to natural environmental conditions and mode of life and the mechanisms that regulate the oxygenation process in pulmonate Hbs.

MATERIALS AND METHODS

Specimens of the freshwater snail B. glabrata (strain Puerto Rico; albino 770302 I-01) were reared in aquariums with water plants at the location at 20°C. The animals were fed 3–4 times per week on a mixed diet that included trout pellets (Biomar, Ecolife 19).

Animals with a shell diameter of 1–2 cm, weighing 0.4–1.5 g, were used for experiments. The hemolymph was sampled directly from the pericardial sinus after piercing of the shell and body wall with a needle and imbuing it into thinly drawn-out glass capillaries. Samples from individual animals were stored separately on ice without freezing. Those that showed no met-Hb formation were pooled and used in experiments within at most 3 days.

In vivo hemolymph pH values were measured using a BMS 2 Mk 2 meter coupled to a PHM72 milli volt meter (Radiometer, Copenhagen, Denmark) after fitting the sharp end of a hypodermic needle to the capillary tube of the pH electrode, which was then used to pierce the animals, allowing hemolymph to be drawn directly into the pH electrode without contact with air. Hemolymph osmolality was measured in five individual snails using a Knauer semi-micro osmometer (Berlin, Germany).

Gel filtration of the native hemolymph was carried out using a 40 x 1.6 cm (height x diameter) Sephadex G200 column, eluted with 0.1 M Tris buffer, pH 7.1. Absorbances of eluted fractions were read at 280 and 415 nm (for identifying proteins and heme components, respectively). Isoelectric focusing was performed on hemolymph dialyzed against 0.01 M Tris buffer in a 110-ml column (LKB, Bromma, Sweden), using ampholines of pH 3–6 (0.6%), 5–7 (0.07%), and 3.5–10 (0.07%). After focusing (at 400 V), 1.1-ml fractions were collected for absorbance and pH measurements (at 23°C).

O2 binding measurements were performed on native hemo-lymph and on samples that had been dialyzed to remove possible cofactors to Hb-O2 binding. Dialyses were carried out in preboiled, semipermeable tubing (molecular weight cutoff, 101-fold dilution in 0.1 M Tris buffer (pH 7.5 at 25°C).

O2 equilibria were determined on 3-µl Hb samples using a modified gas diffusion chamber (32, 33) linked to cascaded Wösthoff gas mixing pumps (Bochum, Germany), where O2 tensions were increased stepwise by mixing air or O2 with highly pure (>99.998%) N2, while absorption was recorded continuously. O2 saturations were evaluated from absorbances relative to those values for the fully oxygenated and fully deoxygenated samples, which were obtained after equilibration with pure O2 and N2, respectively. Reductions in the absorption difference between the fully oxygenated and deoxygenated Hb during the recordings (that indicate met-Hb formation) were usually absent and always ≤5%.

The equilibria were recorded at different pH values, which were obtained either by adding Tris buffer to a final concentration of 0.1 M (to assess the “fixed-acid Bohr effect”) or by varying the CO2 tension in the equilibrium gases ("CO2 Bohr effect"). Half-saturation O2 tension (P50) values at specific pH values were interpolated from the P50-to-measured pH relationship. pH measurements were carried out in duplicate, at the same temperature as the O2 equilibrium measurements, on 50-µl subsamples after a minimum of 6 min of equilibration at 90–95% O2 saturation, using the pH meter described above.

Oxygenation data involving at least four equilibrium steps between 30 and 70% saturation were converted to Hill plots [log(Y/1–Y)] vs. log P, where Y is the fractional O2 saturation [log (Y/1–Y)] and P the O2 tension for estimation of P50 and Hill’s cooperativity coefficient at half-saturation (nH). The heat of oxygenation (ΔH) was calculated as ΔH = -2.303 R(log P50/ΔT), where R is the gas constant and T the absolute temperature. The effects of inorganic ions on O2 binding were examined by adding accurate volumes of 1-M solutions of CaCl2, MgCl2, KCl, or NaCl and was checked by measuring CO2 concentrations using a CMT 10 chloride titrator (Radiometer, Copenhagen, Denmark).

Precise equilibria measurements for extended Hill plots that emphasize extreme O2 saturation values near 1–5 and 95–99% were carried out as previously described (35). Errors resulting from possible incomplete saturation of the Hb after equilibration with pure O2 at atmospheric pressure were corrected by end-point extrapolation as described (35). The data were analyzed in terms of the parameters of the two-state Monod-Wyman-Changeux (MWC) equation (17)

\[ S = \frac{K_T P (1 + K_T P)^{(q-1)} + K_R P (1 + K_R P)^{(q-1)}}{L (1 + K_T P)^q + (1 + K_R P)^q} \]

where S denotes saturation, L the allosteric constant, and q the number of interacting O2 binding sites. Additional analyses were carried out with q fixed at 6 (the approximate value obtained in analyses with “free” q, cf Table 1).

Derived parameters were calculated from the fitted parameters as follows. P50 was obtained by solving for (PO2) the equaition

\[ \log \frac{S}{1 - S} = 0 \]

The median O2 tension (Pm) was calculated as

\[ P_m = \frac{1}{K_R} \left[ (L + 1)/(Lc^i + 1) \right]^{1/q} \]

where c = K_T/K_R.

The maximum slope of log [S/(1 – S)] vs. log Po2 [i.e., maximum cooperativity coefficient (nmax)] was calculated by first solving (for Po2) the equation

\[ d^2 \left( \frac{\log [S/(1 - S)]}{d \log (P_O2)} \right)^2 = 0 \]

and then calculating Δlog[S/(1 – S)]/Δlog Po2 at that Po2.

The free heme-heme energy of interaction (ΔG) was calculated as

\[ RT \ln \left[ ((L + 1)(Lc^i + 1))/((Lc^i + 1)(Lc^{i-1} + 1)) \right] \]

Heme concentrations were determined from the α- and β-absorption peak values of oxygenated Hb after 25- to 101-fold dilution in 0.1 M Tris buffer (pH 7.5 at 25°C). Oxygenated Hb was obtained by flushing with O2, deoxy Hb by flushing with N2 and adding a trace of solid sodium dithionite, and the carboxy Hb by CO flushing of the deoxygenated sample. Addition of potassium ferricyanide (for obtaining met-Hb spectra) resulted in protein denaturation.

RESULTS

Whole hemolymph. The in vivo pH value in the hemolymph of B. glabrata measured at 25°C was 7.78
Hemolymph osmolality was measured to be 57 mosmol/kg (SD = 29.4, n = 5).

Heme concentrations in the pooled hemolymph samples varied greatly, from 0.31 to 0.87 mM (n = 20). Gel filtration of whole hemolymph samples and isoelectric focusing of dialyzed Hb (Fig. 1) showed that the Hb constitutes 86% of the total hemolymph protein and consists of a single component with an isoelectric point (pI) of 4.7 (Fig. 1).

The native hemolymph displayed a moderately high O2 affinity (P50 at 25°C = 6.1 and 2.8 mmHg at pH 7.7 and 8.4, respectively; Fig. 2A). Although a Bohr effect was observed in the entire pH range measured, the Bohr factor decreased from −0.50 at alkaline pH values (7.4–8.2) to −0.22 at pH 6.8–7.1 (Fig. 2A). n50 increased from 1.1 at pH 7.0 to 2.0 at pH 8.0, exhibiting maximum values at alkaline conditions where the Bohr effect is greatest (Fig. 2A). CO2 exerted no significant, specific (pH independent) effect on O2 binding as judged from similar O2 affinity and cooperativity when the pH was varied by adding either CO2 or buffers (resulting in the same CO2 and fixed-acid Bohr effects; Fig. 2B).

Extended Hill plots at different pH values are shown (Fig. 3). Analysis of the data in terms of the two-state MWC model and the derived parameters (Table 1) showed that in the pH range of 7.8 to 6.9 that spans physiological conditions, changes in proton concentration have a much greater effect on the O2 affinity of the oxygenated state (K_R), than the deoxygenated state (K_T) (Fig. 3, A and B). Whereas K_T increased from 0.27 mmHg⁻¹ at pH 6.8 to 0.99 mmHg⁻¹ at pH 8.4, reflecting a Bohr factor for binding the last O2 molecule of −0.41 (Fig. 4B), K_T showed virtually no pH dependence between 6.8 to 7.7, but increased from 0.087 to 0.23 mmHg⁻¹ between pH 7.7 and 8.4. Similar pH-induced variation in K_T and K_R values (not illustrated) was obtained in analyses with q fixed at 6, except for the lowest pH tested (6.86), where fixing q markedly increased K_R and L values.

The oxygenation process exhibited strong temperature dependence. At pH 7.7, hemolysate P50 values were 6.1, 3.6, and 1.8 mmHg at 25, 17.5, and 10°C (Fig. 4A). The Bohr effect was little affected by temperature (Bohr factor = −0.43, −0.38, and −0.44 at 10, 17.5, and 25°C, respectively). Extended Hill plots at different tempera-
Fig. 4. Temperature (T) dependence of oxy-
tures (Fig. 4C) and the MWC parameters (Table 1, 
values with decreasing temperature underlie the tem-
perature insensitivity of cooperativity and ΔG values.

Table 1. MWC and derived parameters for the whole hemolymph and dialysed Hb of Biomphalaria glabrata

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Values for O2 association equilibrium constants of hemoglobin (Hb) in low-affinity (K_T) and high-affinity (K_R) states are ±SE. MWC, Monod-Wyman-Changeux; WH, whole hemolymph; [Ca^2+] concentration; P_m, median O2 tension; P_50, half-saturation O2 tension; n_max, Hill's cooperativity coefficient at half-saturation; q, number of interacting O2 binding sites.

ΔH, which includes the heat of solution of O2 and other oxygenation-linked processes, was not linear but decreased with increasing temperature (Fig. 4B); for the CO2-buffered hemolymph, it was −63 and −49
kJ/mol below and above 17.5°C, respectively (interpolated at pH = 7.7). Similar nonlinearity of ΔH values with respect to temperature was evident under fixed-acid buffering conditions (Fig. 4B).

Astrup titrations carried out to examine the Haldane effect and differences in the buffer capacities of oxygenated and deoxygenated whole hemolymph (Fig. 5) revealed higher pH values in deoxy than in oxy hemolymph at CO₂ tensions below P CO₂ = 12 mmHg (i.e., above pH 7.3), the difference increasing with increasing pH (as did the Bohr effect; Fig. 2A). At P CO₂ = 3.7 mmHg, pH values were 7.79 and 7.69 in deoxy and oxy hemolymph, respectively.

Dialyzed Hb solutions. While displaying similar O₂ affinity and cooperativity values as the whole hemolymph at pH < 7.3, the dialyzed Hb showed distinctly lower affinities at higher pH values (P₅₀ values at 25°C were 7.4 and 6.0 mmHg at pH 7.7 and 8.1, respectively, compared with 6.1 and 3.9 mmHg in native hemolymph; Fig. 2). Dialysis, moreover, decreased the Bohr factor (from ~0.50 in the whole hemolymph to ~0.32 at pH 8.0), but had no marked effect on cooperativity.

Extended Hill plots of dialyzed Hb showed similar pH dependence as in the whole hemolymph (Fig. 6, A and B; Table 1), K₉ varying strongly (0.71 and 0.25 mmHg⁻¹ at pH 8.1 and 7.0, respectively) and K₇ changing only slightly with proton concentration. At pH 8.1, where cooperativity is maximal (n₂₀ = 1.96), the K₇ and K₉ values (0.085 and 0.71 mmHg⁻¹) reflect an 8.4-fold greater O₂ affinity in the oxy than in the deoxy state and a ΔG value of 5.06 kJ/mol. Analyses in terms of the MWC model with q fixed at 6 gave similar values for the K₉-to-K₇ affinity ratio (8.1) and ΔG (4.99 kJ/mol).

The decrease in O₂ affinity on dialysis prompted examination of the effects of inorganic cations on Hb oxygenation. In contrast to the effects of increasing proton and salt concentrations in vertebrate Hbs (3), cations increased Hb-O₂ affinity of B. glabrata Hb.

![Fig. 5. Astrup titration of CO₂ buffered deoxygenated (●) and oxygenated (○) hemolymph at 15°C. Heme concentration = 0.64 mM.](http://ajpregu.physiology.org/)

![Fig. 6. A: Hill plots at 25°C of dialyzed Hb at different pH values (fixed-acid Bohr effect measured in 0.1 M Tris); heme concentration = 0.41 mM. B: pH dependence of K₉, K₇, and P₅₀⁻¹ parameters derived from A.](http://ajpregu.physiology.org/)

![Fig. 7. Effects of K⁺, Na⁺, Mg²⁺, and Ca²⁺ on O₂ affinity at 25°C. Fixed-acid conditions, 0.1 M Tris, pH 7.5. Heme concentration = 0.52 mM.](http://ajpregu.physiology.org/)
pH 8.0; Fig. 2A). Significantly, Ca\(^{2+}\) almost completely obliterated cooperativity (n\(_{50}\) decreased from 1.96 to 1.12 at pH 8.1).

The effects of cations on O\(_2\) affinity can be expressed in terms of the basic linkage equation

\[
\Delta \log P_{50} = \Delta \log \text{cation concentration} = \Delta X,
\]

where X is the amount of cations bound to the Hb on O\(_2\) binding (3, 33). The overall correspondence between the P\(_{50}\) and P\(_m\) values, and between n\(_{50}\) and n\(_{max}\) values (Table 1; see also Figs. 3B and 9B), indicates symmetrical O\(_2\) binding curves, permitting analysis of the P\(_{50}\) data in terms of linkage equations. In the cation concentration range of 20–100 mM, the \(\Delta \log P_{50}/\Delta \log \text{cation concentration}\) relationship indicates oxygen-linked binding of 0.04 Na\(^+\) or K\(^+\) ions and 0.17 Ca\(^{2+}\) ions per heme group oxygenated.

At constant pH, the shift in K\(_T\) and K\(_R\) values in the absence (cf Fig. 6A) or presence (cf Fig. 8A) of Ca\(^{2+}\) indicates that cations increase O\(_2\) affinity primarily by raising K\(_T\) (Fig. 8B). As interpolated at pH 7.8, Ca\(^{2+}\) increased K\(_T\) 2.7-fold (from 0.083 to 0.22 mmHg\(^{2+}\)) and K\(_R\) 1.3-fold (from 0.50 to 0.65 mmHg\(^{2+}\)). This trend was confirmed in analyses with q fixed at 6.

The effect of varying Ca\(^{2+}\) concentrations on dialyzed Hb was examined at pH 8.1, where cooperativity is maximal (Fig. 9A). With increasing Ca\(^{2+}\) concentration, n\(_{50}\) decreased (from 2.0 to 1.1 at 100 mM Ca\(^{2+}\)), and affinity increased to a relatively stable value at Ca\(^{2+}\) concentrations above 10 mM. These effects are attributable to a marked increase in K\(_T\) (that also was evident when the MWC model was fitted with q fixed at 6) with increasing Ca\(^{2+}\) levels (Fig. 9B; Table 1) and a transient increase in K\(_R\). Significantly, addition of Ca\(^{2+}\) to concentrations up to 0.1 M did not significantly affect the pH of the Hb solution at pH 8.1 and only slightly decreased pH at lower pH values. This was confirmed in separate experiments that showed 1) \(\Delta \text{pH} = -0.026 (\text{Ca}^{2+}) + 0.0017 (r = 0.121)\) at pH 8.1 and heme concentration = 0.41 mM, where [Ca\(^{2+}\)] is the molar calcium concentration and 2) \(\Delta \text{pH} (0–100) = 0.026 \text{pH} - 0.216 (r = 0.906)\), where \(\Delta \text{pH} (0–100)\) is the pH induced by 100 mM Ca\(^{2+}\).

Spectrophotometric characteristics. Spectrophotometric analysis (Fig. 10) showed \(\alpha\)- and \(\beta\)-absorption maxima at 574 and 539 nm, respectively (compared with 577 and 542 nm for human Hb A), and an \(\alpha\)-to-\(\beta\)-absorption ratio of 1.01 (compared with 1.07 and 1.04 in human Hb A (27) and extracellular annelid Hb...
from the giant earthworm Megascolides australis (34), respectively. The lack of a distinctive absorption peak at 630 nm reflected absence of Met-Hb. With this in view, the absorptions near 630 nm and the low α-to-β-absorption ratio (0.94) earlier recorded for P. corneus (37) likely represent met-Hb formation rather than a pulmonary character. The carboxy compound showed α- and β-absorption peaks at 568 and 539 nm, thus differing only at the α-peak compared with oxy Hb, and an α-to-β-absorption ratio of 0.94. The span between the α-peaks for oxy and carboxy Hb (that reflect the affinity difference of Hb for the two ligands in vertebrate Hbs) is 6 nm. Deoxy Hb showed a single broad band with a peak at 555 nm. The Soret band maxima for oxy, deoxy, and carboxy Hb were at 413, 430, and 419 nm, and exhibited an absorption ratio of 1:1.35:0.94.

**DISCUSSION**

Invertebrate Hbs display an intriguing diversity in structure and function, correlating with extraordinary variations in the physicochemical conditions under which they operate in vivo, and the diversity may compensate for a lesser-developed organization at the molecular level compared with vertebrates (31). Compared with vertebrates, little is known about the relation between physiological function and molecular characteristics in invertebrate Hbs, particularly in planorbid Hbs that form a distinctive class as regards quaternary structure. These Hbs may be compared with the larger (3–4 × 10⁶ Da) extracellular annelid pigments that exhibit highly characteristic, two-tiered hexagonal structures (hexagonal bilayers), may be dissociated into 12 submultiples, and may contain non-heme “linkers” in addition to heme-carrying chains (28).

O₂ affinity and cooperativity and their pH dependence. The relatively high O₂ affinities (P₅₀ = 6.1 mmHg at pH 7.7; Fig. 2A) and low cooperativities (n₅₀ = 2.0 and 1.1 at 25°C and pH 8.4 and 6.9, respectively) are in general agreement with previous data for planorbid snails (Table 2).

The Bohr effect of B. glabrata hemolymph is markedly pH dependent (Bohr factor = −0.50 between pH 7.4 and 8.4, decreasing markedly at lower pH; Fig. 2A). This is at variance with earlier findings (7, 18) but confirms those of Van Aardt and Naude (26). A pH dependence of affinity and cooperativity has also been recorded in the Hbs of P. corneus Hb (38) and H. trivolvis (22). The pH dependence cannot be attributed to molecular dissociation, as follows from observations that P. corneus Hb only dissociates into submultiples at pH below 2 and above 9.9 (37).

Planorbid Hbs appear not to have been analyzed earlier in terms of the MWC model. Our data (Fig. 3, A and B) indicate that proton concentrations affect Hb-O₂ affinity primarily through modulation of the affinity of the (almost fully) oxygenated Hb, whereas that of deoxy Hb only changes at high pH values that exceed physiological conditions. This implies preferential proton binding to the Hb late in the oxygenation process and that the Bohr effect of B. glabrata is dependent both on pH range and percentage O₂ saturation. This character may be adaptive in favoring O₂ loading as pH increases at the respiratory surfaces.

The proton binding effects are analogous to those found in the extracellular annelid Hbs (Arenicola marina (32) and Lumbricus terrestris (8)), where pH changes primarily affect Kₐ. These characteristics contrast with the tetrameric vertebrate Hbs, where increases in the concentrations of protons and anionic organic phosphates decrease O₂ affinity by lowering Kₐ values, which may represent adaptations favoring O₂ unloading in the tissues.

**Table 2. Oxygenation characteristics of planorbid hemolymph**

<table>
<thead>
<tr>
<th>Species</th>
<th>P₅₀, mmHg</th>
<th>n₅₀</th>
<th>Temperature, °C</th>
<th>pH</th>
<th>Bohr Factor</th>
<th>ΔH₂, kJ/mol</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Planorbus corneus</td>
<td>5.0</td>
<td>2.3</td>
<td>21.3</td>
<td>7.3</td>
<td>0</td>
<td>−0.29</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>2.7</td>
<td></td>
<td>20</td>
<td>8.50</td>
<td>−0.29</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Helisoma trivolvis</td>
<td>2.5</td>
<td>1.3</td>
<td>20</td>
<td>7.6</td>
<td>−0.37</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Biomphalaria glabrata</td>
<td>4</td>
<td>1.08</td>
<td>25</td>
<td>6.7–7.6</td>
<td>0</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.4</td>
<td>1.7</td>
<td>25</td>
<td>6.8–7.6</td>
<td>0</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.3</td>
<td>1.8</td>
<td>25</td>
<td>8.0</td>
<td>−0.29</td>
<td>−77</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>6.1</td>
<td>2.0</td>
<td>25</td>
<td>7.75</td>
<td>−0.50</td>
<td>−49</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>10.2</td>
<td>1.3</td>
<td>25</td>
<td>7.16</td>
<td>−0.2</td>
<td>Present study</td>
<td></td>
</tr>
</tbody>
</table>

ΔH₂, overall heat of oxygenation.
\( \Delta G \) in B. glabrata hemolymph increases with pH (from 1.74 kJ/mol at pH 6.9 to 4.94 kJ/mol at pH 7.7; Table 1 and Fig. 3A), resulting from the differential effects of proton concentrations on \( K_R \) and \( K_T \). The decrease in \( \Delta G \) values at high pH (from 4.94 kJ/mol at pH 7.7 to 3.52 kJ/mol at pH 8.4) follows from the marked increase in \( K_T \). The \( K_R \)-to-\( K_T \) ratio for the hemolymph at in vivo pH 7.7 (Table 1) reflects a 7.8-fold higher affinity in the oxy than the deoxy state. The latter ratio compares with a much higher value of 33 seen in A. marina Hb at the physiological pH of 7.37 (32).

In contrast to mammalian Hb, where \( n_{50} \) is invariant of pH (4), cooperativity in B. glabrata Hb is markedly pH dependent, showing a broad maximum at pH 7.6–8.4, indicating that it enhances O\(_2\) transport in vivo. A pH dependence of cooperativity has also been observed in vascular annelid and pogonophoran Hbs, but not in bivalve Hbs that lack marked cooperativity (21, 24). Hbs from the annelids L. terrestris (8, 15) and Perinereis albithusiensis (25) show pronounced cooperativity maxima at alkaline pH values (8.0 to 8.2) as here found for B. glabrata. The maximal cooperativity in B. glabrata Hb (\( n_{50} \sim 2.0 \) at pH 8.0; Fig. 3A) is low compared with that attained in annelids (6.5 and 9.5 in M. australis and L. terrestris, respectively (15, 34)).

Cation effects. Our experiments indicate that the effect of salts on O\(_2\) binding to B. glabrata Hb depends exclusively on cations, in contrast to vertebrate Hbs, where O\(_2\) affinity is decreased by organic and inorganic anions. Although the cations were added as chloride salts, the view that the measured effects may be due to Cl\(^{-}\) ions is rejected by the higher affinity seen in the presence of a given CaCl\(_2\) concentration than in NaCl at twice that concentration (Fig. 7). This difference and the greater effect of Ca\(^{2+}\) than Mg\(^{2+}\) (Fig. 7) shows that factors other than ionic strength govern O\(_2\) affinity. Pulmonate Hbs may exhibit variable responses; the O\(_2\) affinity of H. trivolvis Hb is only slightly increased in the presence of 0.25 M Ca\(^{2+}\) and appears to be unaffected by Na\(^{+}\), K\(^{+}\), Mg\(^{2+}\), and Cl\(^{-}\) (22).

The greater increase in \( K_T \) than in \( K_R \) in the presence of Ca\(^{2+}\) (Fig. 8B) reflects greater oxygenation-linked Ca\(^{2+}\) binding to the Hb in the deoxy state than the oxy state and explains the Ca\(^{2+}\)-induced reduction in cooperativity and \( \Delta G \) values (Table 1; Figs. 8B and 9A). These characteristics contrast with the control mechanism in the extracellular annelid Hbs, where cations bind preferentially to the oxy state, increasing \( \Delta G \) and cooperativity (11, 25, 32).

In A. marina Hb, which exhibits a high content of negatively charged amino acid residues and a low pl (4.6), addition of cations decreases the pH of the bulk solution, indicating displacement of protons that may form an electrical double layer at the negatively charged macromolecular surfaces (36). On the basis of the interplay between protons and cations, Santucci et al. (19) correspondingly attribute the Bohr effect in Hb of the earthworm Octolasion entirely to O\(_2\)-linked binding of the cationic allosteric effector. Measurements on L. terrestris Hb (8) suggest the release of one and two protons, respectively, on binding of monovalent Na\(^{+}\) and divalent Ca\(^{2+}\) and explain different effects of cations with similar valences in terms of differences in their ionic radii. Although pulmonate Hbs have similarly low pl values as in annelid Hbs (4, 7 in B. glabrata and P. corneus (this study and Ref. 37)), the absence of Ca\(^{2+}\)-induced decreases in pH with B. glabrata Hb indicates different binding sites for protons and metal cations and suggests a different mechanism controlling the cation and Bohr effects than in annelids. The differential effects of Ca\(^{2+}\) and Mg\(^{2+}\) indicate that, in addition to charge, other specific properties of the cationic effectors govern O\(_2\) affinity in B. glabrata Hb.

The present data show that variations in concentrations of divalent cations (particularly Ca\(^{2+}\)) concentrations will perturb hemolymph O\(_2\) binding characteristics, although their role in regulating O\(_2\) affinity in vivo remains questionable given that the cation (and total osmolality) levels appear not to be finely regulated in invertebrates (14).

Buffer capacity. The CO\(_2\) titrations show a greater capacity for proton binding in deoxy than in oxy hemolymph, the difference (the Haldane effect) increasing with pH (Fig. 5). Among vertebrates, an increased formation of carboxylic compounds in deoxygenated Hb contributes to the Haldane effect. In the absence of a specific effect of CO\(_2\) on O\(_2\) binding in B. glabrata Hb (Fig. 2B), the Haldane effect appears to be due solely to the increased ability of deoxy Hb to bind protons. This effect expresses the same heterotopic interaction as the Bohr effect (negative effect of protons on the oxygenation) (Figs. 2A and 5), and both increase in magnitude above pH 7.3.

The greater proton buffering in deoxy Hb will facilitate carboxylic acid dissociation and bicarbonate formation, increasing the transport and elimination of CO\(_2\) at the respiratory exchange surface, and may limit variations in the acid-base balance induced by acidic by-products of anaerobic metabolism (24).

Temperature effects. The oxygenation of Hb is exothermic, and increasing temperature lowers O\(_2\) affinity directly by weakening the bond between Hb and O\(_2\), and indirectly via the Bohr effect due to associated pH decrease. \( \Delta H \) in B. glabrata Hb decreased from \(-63\) to \(-49\) kJ/mol with increasing temperature intervals at pH 7.7 (Fig. 4B). This accords with the value (\(-59\) kJ/mol at pH 7.3) reported for P. corneus (31) but is lower than that (\(-77\) kJ/mol at pH 8.0) earlier observed in B. glabrata (26). Given the endothermic nature of Bohr proton release (cf. Ref. 33), the values may be expected to increase at low pH where the Bohr effect falls.

The equation used for calculating \( \Delta H \) (see MATERIALS AND METHODS) assumes it to be independent of temperature. Weber (29) argues for the use of a more complex equation that includes the heat capacity difference, which takes into account differences in the tendency of water to order itself around polar surface areas of the protein that may be greater in the deoxy (unfolded) molecules than in oxy (folded) molecules where these
surfaces are partly buried in the protein moiety. The nonlinearity of the van’t Hoff plots for B. glabrata Hb (Fig. 4B) is consistent with a change in the heat capacity of the system, as in some Antarctic fish Hbs (6). The nonlinearity at pH 7.7, where the Bohr effect and cooperativity are pronounced, indicates that the change in heat capacity may in part result from breakage of salt bridges and hydrogen bonds that attends the shift from the tense to the relaxed configuration of the molecules (cf Ref. 6).

It is generally considered that an inverse relationship between the ΔH value of a respiratory pigment and the temperature range in which it functions would be adaptive in securing O2 loading in poikilothermic animals living in thermally unstable environments. Although the habitats of planorbid snails are notoriously unstable and may vary from near freezing to 30°C in a single day (26), the present results show no evidence for an adaptive reduction in temperature sensitivity. On the contrary, the lower O2 affinities at high temperatures may favor O2 delivery to the tissues in synchrony with increased metabolic O2 demand, without significantly compromising O2 loading at their respiratory surfaces when the snails access atmospheric air.

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Address for reprint requests: R. E. Weber, Department of Zoophysiology, Building 131, Institute of Biological Sciences, University of Aarhus, DK-8000 Aarhus C, Denmark.

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