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Equilibrium and kinetic studies of oxygen binding to the haemocyanin from the freshwater snail *Lymnaea stagnalis*

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The binding of oxygen by the haemocyanin of the gastropod *Lymnaea stagnalis* was studied by equilibrium and kinetic methods. The studies were performed under conditions in which the haemocyanin molecule was in the native state. Over the pH range 6.8–7.6, in the presence of 10mM-$CaCl_2$ the haemocyanin bound O_2 co-operatively. Over this pH range the haemocyanin molecule displayed a normal Bohr effect whereby the O_2 affinity of the molecule decreased with a fall in the pH of the solution. The maximum slope of the Hill plot ($h_{max.}$) was 3.5, obtained at pH 7.5. An increase in the $CaCl_2$ concentration from 5 to 20mM at pH 6.8 resulted in a slight increase in the oxygen affinity, with $h_{max.}$ remaining virtually unchanged. At constant pH and $CaCl_2$ concentration, an increase in NaCl concentration from 0 to 50mM resulted in a small decrease in O_2 affinity, but a significant increase in the value of $h_{max.}$ from 3.5 to 8.6. Temperature-jump relaxation experiments over a range of O_2 concentrations produced single relaxation times. The dependence of the relaxation time on the reactant concentrations indicated a simple bimolecular binding process. The calculated association and dissociation rate constants for this process at pH 7.5 are $29.5 \times 10^6 M^{-1} \cdot s^{-1}$ and $49 s^{-1}$ respectively. The association rate constant k_{on} was found to be essentially independent of pH and $CaCl_2$ concentration. The dissociation rate constant, k_{off}, however, increased with a decrease in the pH, but was also independent of $CaCl_2$ concentration. These results indicate that the stability of the haemocyanin–O_2 complex is determined by the dissociation rate constant.

The blood O_2 carriers of many arthropods and molluscs are high-molecular-weight copper-containing haemocyanins (Wood, 1980). The molecular architectures of the haemocyanins from these two phyla differ, but a common feature is that all bind a maximum of one molecule of O_2 for each pair of copper atoms. Typically the affinity with which O_2 is bound is under allosteric control. It is well established for a number of haemocyanins that their O_2-binding characteristics are markedly altered by the pH, by the ionic strength and by the presence of alkali-metal ions (Zolla *et al.*, 1978). In addition to these heterotropic interactions, haemocyanins also show homotropic interactions resulting from the influence of one O_2-binding site upon neighbouring sites. As is the case with mammalian haemoglobins, the heterotropic and homotropic interactions have an important functional role in facilitating the loading and unloading of O_2 over a comparatively narrow range of O_2 tensions.

Abbreviations used: SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

The haemocyanins of the gastropod molluscs have approx. 160 O_2-binding sites, and the question arises of whether co-operative interactions involve the entire molecule or whether functionally independent allosteric units exist within the molecule. Since the molecule can be made to dissociate partly or completely to polypeptide chains (each believed to contain eight covalently linked O_2-binding domains), or can be dissected, by using gentle digestion with different proteolytic enzymes, into fragments of various sizes (Lontie *et al.*, 1973; Gullick *et al.*, 1979), there exists the possibility of answering questions of this type. We have therefore initiated a study of the haemocyanin of the freshwater snail *Lymnaea stagnalis* in which the O_2-binding equilibria and kinetics of the whole molecule and of fragments of the molecule are investigated. In the present paper we report a detailed study of the O_2-binding properties of the whole or native haemo-

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cyanin molecule under different conditions of pH, NaCl and $CaCl_2$ concentrations. We have attempted to correlate functional properties with the physiological role of the haemocyanin. A preliminary report of some of this work has appeared (Dawson *et al.*, 1981*a*).

Materials and methods

Isolation and purification of haemocyanin

Specimens of *L. stagnalis*, obtained locally, were cleaned in distilled water and dried with tissue. A microspatula was used to scrape the foot of the animal gently causing it to retract into the shell, expelling haemolymph. The haemolymph was collected, through a small funnel packed with glass wool, into 0.1 M-sodium acetate/acetic acid buffer, pH 5.7, containing 1 mM-benzamidine and 0.005% thiomersalate as bacteriostats and 1 mM-phenylmethanesulphonyl fluoride as a proteinase inhibitor. The haemocyanin was purified by ultracentrifugation for 2h at 40000rev./min in a Beckman L2-65B instrument with a type 50Ti rotor. The blue pellet obtained was resuspended in the acetate buffer, re-sedimented, and finally suspended in the acetate buffer.

Characterization of the haemocyanin

Absorption spectra were recorded with a Pye–Unicam SP. 1800 instrument equipped with a Unicam AR 25 chart recorder. Protein concentrations were determined from the A_{280} by using the specific absorption coefficient $A^{1\%}_{1cm}$ of 15.6 obtained from dry-weight measurements (Gullick *et al.*, 1979). Analytical ultracentrifugation of samples of the protein after dialysis against an appropriate buffer was performed in a Beckman model E instrument equipped with a schlieren optical system and a rotor-temperature-control system (Wood & Peacocke, 1973).

SDS/polyacrylamide-gradient-gel electrophoresis was performed with the buffer system of Laemmli (1970) on 1-mm-thick slab gels. The gels were stained with Coomassie Brilliant Blue R250.

O_2-equilibrium measurements

O_2-equilibrium curves were determined by tonometric (Riggs & Wolbach, 1956) and diffusion chamber (Sick & Gersonde, 1969) techniques. The tonometer (capacity 240ml) was constructed from a standard 1cm cuvette heat-fused to a glass bulb. The bulb was fitted with a tap by means of which the tonometer was evacuated of air, and a Suba-Seal-plugged side arm through which known volumes of air were injected. The haemocyanin solution was allowed to equilibrate for 5min with rotation of the tonometer after each addition of air. The degree of oxygenation of the protein was determined from the absorbance at 346nm. Values of A_{OXY} (the absorbance of fully oxygenated haemocyanin) were obtained after equilibrating the solutions in air. Values of A_{DEOXY} (the absorbance of fully deoxygenated haemocyanin) were obtained after repeated evacuations.

The second O_2-equilibrium technique made use of a modified O_2-diffusion chamber. In this procedure a N_2/O_2 gas mixture was used to equilibrate a thin film of deoxygenated haemocyanin solution. The oxygen tension of the gas mixture was increased in a stepwise fashion using a Wosthoff model 201/a-F gas-mixing pump. The changes in A_{365} were recorded with an Eppendorf model 1100M photometer fitted with a RCA 931-A photomultiplier tube. Values of A_{OXY} and A_{DEOXY} were obtained after equilibrating the solution in O_2 and N_2 respectively. This technique is rapid and only requires small amounts of protein (e.g. 50μg). This results in very gentle treatment of the protein, which minimized denaturation.

The fractional saturation (y) of the haemocyanin at each O_2 pressure (pO_2) was calculated by using the following equation:

$$y = [(A_p - A_{DEOXY})/(A_{OXY} - A_{DEOXY})] \quad (1)$$

where A_p is the absorbance at each pO_2.

Hill-plot calculations were made by using the University of Leeds Amdahl V7 computer, with a program written in ALGOL 68 based on the method of Marquardt (1963).

Kinetic measurements

Temperature-jump relaxation measurements were made by using an Eigen–De Maeyer instrument built by Messanlagen Gesellschaft, Gottingen, Germany (Eigen & De Maeyer, 1963; Eigen, 1968). The cell used had a heated volume of 1ml with a 10mm light path and a specially modified air-tight cap.

The haemocyanin solutions under investigation were allowed to reach equilibrium at different partial pressures of O_2 inside a glove bag that contained a mixture of air and N_2. The temperature-jump cell was filled inside the glove bag after the O_2 concentration of the solution had been measured with a Rank oxygen electrode.

A discharge of 20kV through the cell raised the temperature of the solutions by approx. 3°C to a final temperature of 20°C. The kinetics of the reaction were followed by monitoring the signal change at 346nm. In all cases a signal change corresponding to a decrease in absorbance with time was observed. It was assumed that the signal change was directly proportional to the absorbance change in the cell. Several experimental traces were obtained for each solution. The reciprocal relaxation time was obtained in the usual manner, from the slope of a plot of log(absorbance change) against time. Data

analysis was performed manually and by computer (Booth & Dawson, 1982). The concentration of free binding sites was calculated from the total protein concentration and the O_2-binding equilibrium curve. The concentration of O_2 was obtained from known solubility coefficients. In all the temperature-jump experiments the oxygen concentration was at least five times greater than the concentration of the free binding sites.

In view of fairly recent observations (Dawson *et al.*, 1981*b*) that spurious results may be obtained from temperature-jump studies of charged macromolecules (due to the presence of a joule-heating induced electric field), the authenticity of the relaxation spectra was checked by performing a series of experiments under conditions of varying ionic strength. The ionic strength was controlled by varying both the buffer and the NaCl concentrations to a maximum of 0.1M. Since, under all of the conditions studied, the relaxation spectra produced were single uni-directional processes, the absence of an electric-field effect was established.

Stopped-flow measurements were made by using an instrument designed and built in the Department of Biophysics of The University of Leeds. The kinetics of O_2 dissociation from *Lymnaea* haemocyanin were monitored by mixing the oxygenated protein with an equal volume of buffer containing sodium dithionite. The time course of the reaction was monitored by the signal change at 400nm. In all cases a signal change corresponding to a single exponential decrease in absorbance with time was observed. The apparent first-order rate constant of the reaction ($k_{app.}$) was assumed to be equivalent to the dissociation rate constant (k_{off}) of the *R*-state and was obtained from the slope of a plot of log (absorbance change) against time.

Results

O_2-equilibrium studies

Oxygen-equilibrium curves for native *Lymnaea* haemocyanin were determined under conditions of varying pH, $CaCl_2$ and NaCl concentration. Under all the conditions used in the present work the binding curves were sigmoidal, indicating co-operative binding of O_2. The binding data are presented as Hill plots in Figs. 1–3. The characteristic shape of the Hill plots reflects a conformational transition from a low-affinity *T*-state to a high-affinity *R*-state of the protein. Fig. 1 shows Hill plots for native haemocyanin at three pH values. In the presence of 10mM-$CaCl_2$ and over the pH range 6.8–7.6, *Lymnaea* haemocyanin displayed a normal Bohr effect. The Hill coefficient, $h_{max.}$, obtained from the slope of the Hill plot at 50% saturation had an average value of 2.2, and was virtually independent of pH. The equilibrium constants for the high- and

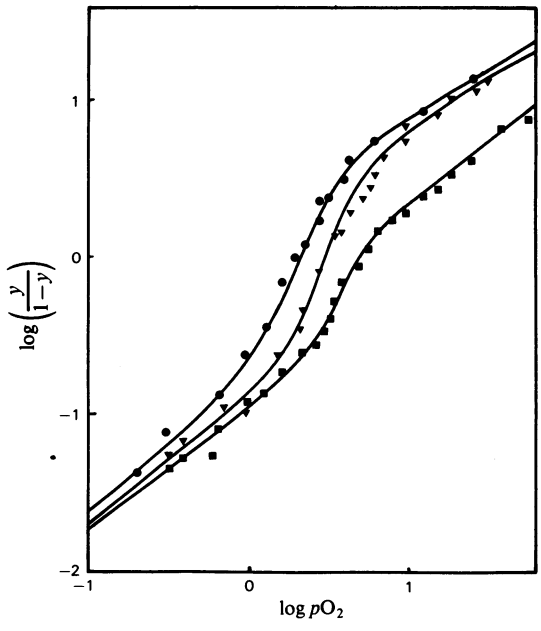

Fig. 1. *Hill plot of O_2 equilibrium for L. stagnalis haemocyanin in the presence of 10mM-$CaCl_2$/10mM-Hepes buffer, pH6.8* (■), *pH7.2* (▼) *or pH7.6* (●)
The temperature was 20°C and the haemocyanin concentration was approx. 1.0mg/ml.

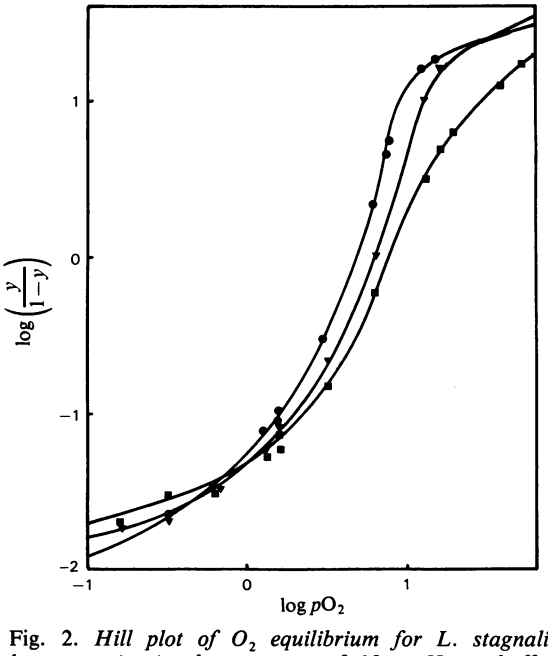

Fig. 2. *Hill plot of O_2 equilibrium for L. stagnalis haemocyanin, in the presence of 10mM-Hepes buffer, pH6.8, and 5mM* (■), *10mM* (▼) *and 20mM* (●)*-$CaCl_2$*
The temperature was 20°C and the haemocyanin concentration was approx. 1.0mg/ml.

low-affinity states of the protein, K_R and K_T, were determined from the value of $\log pO_2$ at the asymptotic intercepts of the Hill plot at 50% saturation. Both K_R and K_T increased with an increase in the pH, indicating that the O_2 affinity of both conformational states of the protein is enhanced.

The effect of $CaCl_2$ on the O_2 equilibrium is shown in Fig. 2. An increase in the concentration of $CaCl_2$ from 5 to 20mM resulted in a small increase in the O_2 affinity with the value of the Hill coefficient and the equilibrium constants remaining virtually unchanged. The effect of NaCl is shown in Fig. 3. An increase in the NaCl concentration from 5 to 50mM in the presence of 10mM-$CaCl_2$ at pH7.5 resulted in a decrease in the O_2 affinity, and a significant increase in $h_{max.}$ from 3.5 to 8.6. The results of the equilibrium studies are summarized in Table 1.

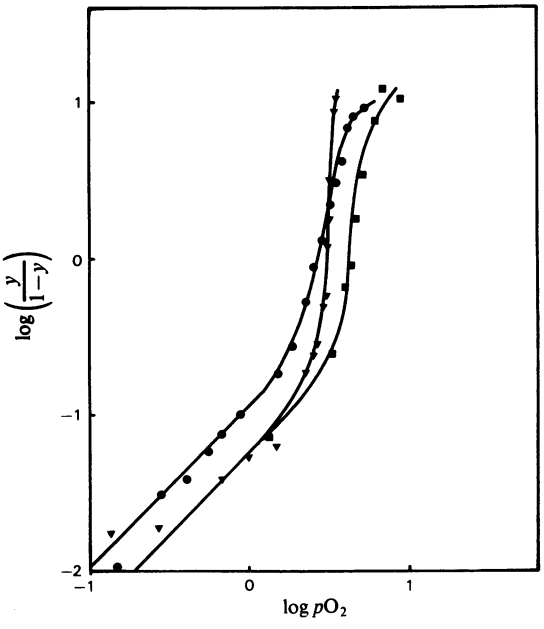

Fig. 3. *Hill plot of O_2 equilibrium for L. stagnalis haemocyanin in the presence of 10mM-$CaCl_2$/10mM-Hepes buffer, pH7.5, containing 0mM (●), 10mM (▼) and 50mM (■)-NaCl*

The temperature was 20°C and the haemocyanin concentration was approx. 1.0mg/ml.

Kinetic studies

Temperature-jump experiments were performed with solutions of haemocyanin at fractional O_2 saturation levels between 0.75 and 0.99. At these saturation levels the protein is in the high-affinity *R*-state. The relaxation process observed involved a decrease in absorbance with time at the oxyhaemocyanin absorbance wavelength (346nm), and was characterized by a single relaxation time (Figs. 4 and

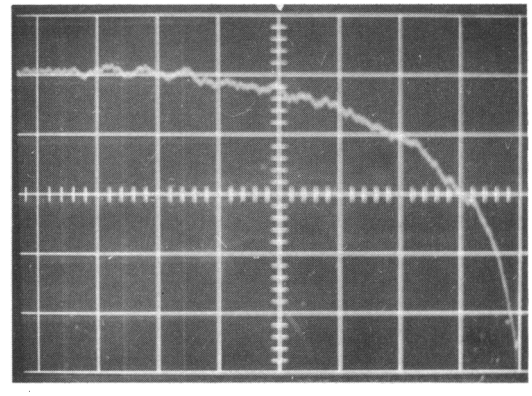

Fig. 4. *Temperature-jump reaction curve for L. stagnalis haemocyanin*

Conditions: buffer, 10mM-Hepes, pH7.5, containing 5mM-$CaCl_2$; haemocyanin concentration, 1.1mg/ml; free-O_2 concentration 125μM; temperature, 20°C; horizontal axis, 0.2ms/division.

Table 1. *Equilibrium O_2-binding data for L. stagnalis haemocyanin*

The conditions were as follows: buffer, 10mM-Hepes; protein concentration, 1mg/ml; temperature, 20°C. p_{50} is the partial pressure of O_2 at half saturation, $h_{max.}$ is the maximum slope of the Hill plot, K_R and K_T are the equilibrium constants for the high- and low-affinity states of the protein respectively.

pH	$[CaCl_2]$ (mM)	[NaCl] (mM)	p_{50} (kPa)	p_{50} (mmHg)	$h_{max.}$	K_R ($\times 10^5 M^{-1}$)	K_T ($\times 10^5 M^{-1}$)
6.8	5	0	0.966	7.24	2.3	1.32	0.436
6.8	10	0	0.668	5.01	2.0	1.32	0.436
6.8	20	0	0.609	4.57	2.3	1.62	0.436
7.2	10	0	0.412	3.09	2.2	4.36	0.676
7.5	10	0	0.367	2.75	3.5	8.13	0.549
7.5	10	5	0.273	2.05	3.5	—	0.309
7.5	10	50	0.593	4.45	8.6	6.92	0.309
7.6	10	0	0.265	1.99	2.4	6.61	0.871

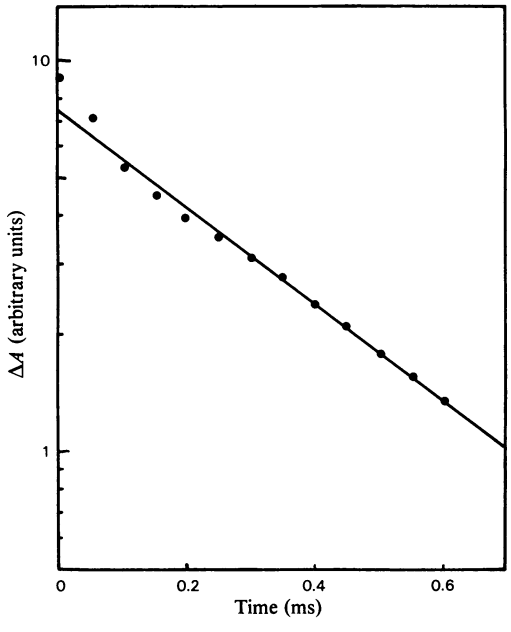

Fig. 5. *Logarithmic plot of temperature-jump reaction curve in Fig. 4* [τ^{-1} *(reciprocal relaxation time)* $= 2853\,s^{-1}$]

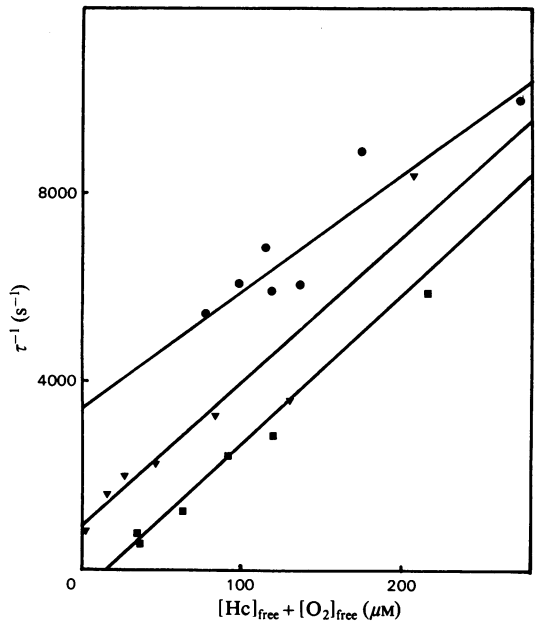

Fig. 6. *Concentration-dependence of reciprocal relaxation time (τ^{-1}) at different pH values*
Conditions: buffer, 10mM-Hepes/10mM-$CaCl_2$, pH 6.8 (●), pH 7.2 (▼), or pH 7.5 (■); temperature, 20°C. Abbreviation used: Hc, haemocyanin.

5). (In cases where the haemocyanin sample was partially degraded, a multi-relaxational process was observed, suggesting the presence of more than one kind of O_2-binding species). The presence of a single relaxation time indicates a single-step chemical process. A simple scheme representing a single-step bimolecular process for the binding of O_2 to a single haemocyanin (Hc below) site may be described by:

$$Hc + O_2 \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} HcO_2 \qquad (2)$$

The relaxation equation for such a process is given by:

$$1/\tau = k_{on}([Hc] + [O_2]) + k_{off} \qquad (3)$$

where τ is the relaxation time and [Hc] and [O_2] represent the equilibrium concentrations of binding sites and O_2 respectively. Eqn. (3) implies a linear concentration-dependence of the reciprocal relaxation time. Plots of $1/\tau$ versus the sum of the equilibrium concentrations of the reactants were indeed linear. Fig. 6 shows the concentration-dependence of the reciprocal relaxation time at three pH values. Fig. 7 shows the same plot with respect to $CaCl_2$ concentration. Values of k_{on} and k_{off} were determined from the slope and ordinate intercepts respectively. However, in view of the relatively large error involved in the determination of the dissociation rate constant by this procedure, k_{off} was

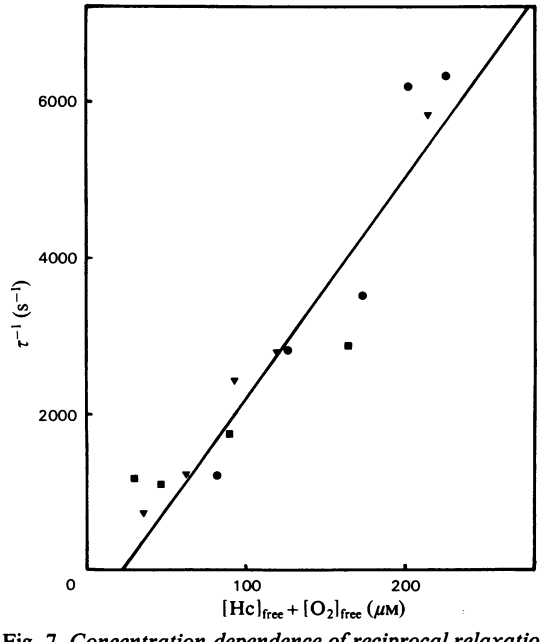

Fig. 7. *Concentration-dependence of reciprocal relaxation time (τ^{-1}) at different $CaCl_2$ concentrations*
Conditions: buffer, 10mM-Hepes, (pH 7.5)/$CaCl_2$ [5mM (●), 10mM (▼) and 20mM (■)]; temperature 20°C.

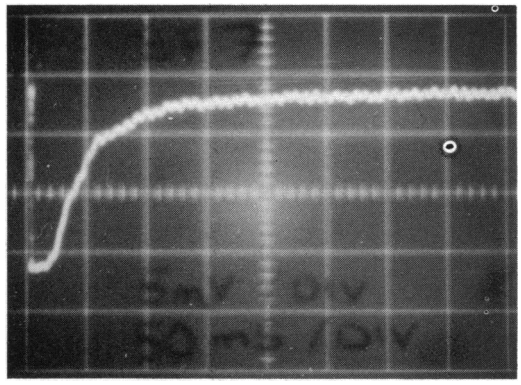

Fig. 8. *Stopped-flow trace for reaction of L. stagnalis haemocyanin with dithionite*
Conditions: buffer, 10mM-Hepes (pH 7.0)/10mM-$CaCl_2$; temperature, 20°C; horizontal axis, 50ms/division.

Table 2. *Kinetic O_2-binding data for L. stagnalis haemocyanin*
The conditions were as follows: buffer, 10mM-Hepes; protein concentration, ~1mg; temperature, 20°C. k_{on} and k_{off} are the association and dissociation rate constants respectively. Values of k_{off} in parentheses were determined from stopped-flow measurements.

pH	$[CaCl_2]$ (mM)	k_{on} ($\times 10^6 M^{-1}\cdot s^{-1}$)	k_{off} (s^{-1})
6.8	10	24.75	187.79
7.0	10	—	(25.67)
7.2	10	30.80	70.57
7.25	10	—	(17.93)
7.5	5	~26.00	~44.00
7.5	10	29.51	49.18 (16.09)
7.5	20	~26.00	~44.00

estimated by using k_{on} and the independently determined equilibrium constant for the *R*-state, K_R.

Stopped-flow experiments were performed by mixing oxygenated haemocyanin with buffer containing dithionite. The deoxygenation reaction observed involved a single exponential decrease in absorbance with time at 400nm (Fig. 8). A summary of the kinetic results is shown in Table 2.

The results in Table 2 show that the association rate constant is essentially independent of pH and $CaCl_2$ concentration. The dissociation rate constant also appears to be independent of $CaCl_2$ concentration, but shows a marked dependence on the pH. Values of k_{off} determined from stopped-flow measurements were somewhat smaller than those determined by the temperature-jump method, but showed a similar trend with respect to pH.

Discussion

The freshwater snail *Lymnaea stagnalis* has haemocyanin as its respiratory pigment. Previous reports that no haemocyanin (or other pigment) was present (see Jones, 1972) are likely to have resulted from the examination of snails that had been disturbed and had ejected a considerable proportion of their haemolymph via the 'hemal pore' (Bekius, 1972). This blood loss, probably serving a defensive function, is made good over the subsequent week or so (Wood *et al.*, 1981). However, such observations do raise the question of to what extent the haemocyanin is essential for life in these animals or whether it serves some other function. The present work shows that the haemocyanin of *Lymnaea stagnalis* has O_2-binding properties, measured by both equilibrium and kinetic methods, which allow it to act as a respiratory pigment. Thus the haemolymph typically has a pH near 7.5 and the Ca^{2+} concentration is about 6mM (Hall *et al.*, 1975), and, under these conditions, the haemocyanin binds oxygen co-operatively and shows a normal Bohr effect. Our data confirm what has been found with other haemocyanins, which individually show a great variation in their O_2-binding properties, namely that the kinetic origin of the Bohr effect is on k_{off} rather than k_{on}. This has been shown to be the case for haemocyanins with a normal Bohr effect, such as the haemocyanin from the lobster *Panulirus interruptus* (Kuiper *et al.*, 1975), as well as those with a reverse Bohr effect, such as that from the whelk *Buccinum undatum* (Wood *et al.*, 1977).

In all of the experiments performed in the present work the haemocyanin would exist in the 'whole' or native state (mol.wt. 9×10^6), when the molecule has a cylindrical structure, the walls of which are formed by an assembly of twenty multidomain polypeptide chains in a helical arrangement (Mellema & Klug, 1972). It seems very unlikely that there is interaction between all 160 O_2-binding sites in the native molecule. Probably there is an 'allosteric unit' of interacting centres, but it is not possible to say at present whether this resides in, say, one polypeptide chain (= eight O_2-binding units) or is formed by the juxtaposition of O_2-binding domains from different polypeptides. The oxygen-equilibrium curves presented as Hill plots show the general features for haemocyanins of slopes of unity at the extremes with a higher slope in the intermediate region. To a first approximation it appears to be possible to describe the O_2-binding behaviour in terms of the Monod–Wyman–Changeux model for ligand binding to an allosteric protein, which assumes the existence of two conformations, a high-affinity *R*-state and a low-affinity *T*-state (Monod *et al.*, 1965). In order to attempt to determine the 'size' of this allosteric unit,

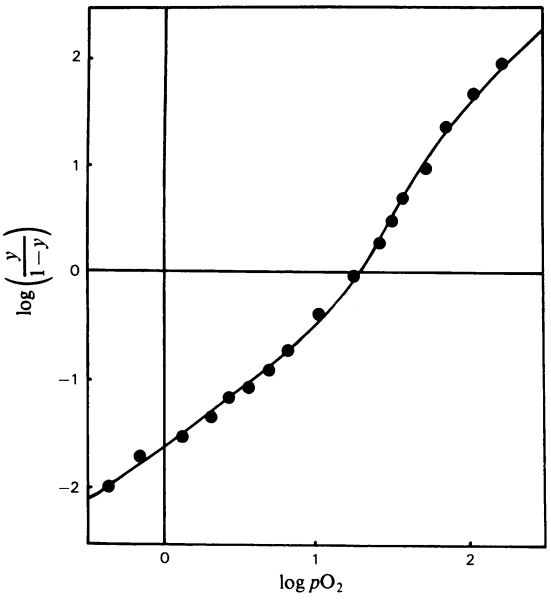

Fig. 9. *Theoretical Hill plot computed on the basis of eqn. (4) in the text*

The line is calculated and points (●) are experimental results. Conditions: buffer, 10mM-Hepes (pH 7.5)/5mM-$CaCl_2$; temperature, 20°C; haemocyanin concentration, approx. 1mg/ml; $L_0 = 1.6 \times 10^6$; $\alpha = 0.026$, $r = 5.155$.

Colosimo *et al.* (1974) computer-fitted the Hill plots by using different values for L_0, the allosteric constant, α ($= K_R/K_T$), and r, the number of interacting sites. We followed a similar fitting procedure for *Lymnaea* haemocyanin under conditions approximating those *in vivo*, i.e. pH 7.5 and 5mM-$CaCl_2$. By using a non-linear least-squares method (Marquardt, 1963), the following values were obtained when eqn. (4) was fitted to the experimental data: $L_0 = 1.5957 \times 10^6$, $\alpha = 0.02585$ and $r = 5.1552$ (Fig. 9).

$$y = \frac{L_0\,\alpha x(1+\alpha x)^{r-1} + x(1+x)^{r-1}}{L_0\,(1+\alpha x)^r + (1+x)^r} \qquad (4)$$

where x is the ligand concentration.

The value obtained for r suggests that the co-operative unit is composed of approximately five O_2-binding domains.

As distinct from r, the Hill coefficient, $h_{max.}$ had a maximum value of 8.6 obtained at pH 7.5 in the presence of 10mM-$CaCl_2$ and 50mM-NaCl. This value is similar to reported values for the snail *Levantina hierosolima* haemocyanin (Er-el *et al.*, 1972) and the β-haemocyanin of the Roman snail, *Helix pomatia* (Zolla *et al.*, 1978), but is greater than the value of about 2 reported for *Buccinum undatum* (Wood *et al.*, 1977) and *Panulirus interruptus* (Kuiper *et al.*, 1977).

The values of p_{50} (the partial pressure of O_2 at half saturation) obtained in the present work varied from about 2.67 to 9.33kPa (2–7mmHg), indicating a high O_2 affinity. The observed values fall within the p_{50} range of most other mollusc haemocyanins studied so far. *Lymnaea* haemocyanin displays a pronounced positive Bohr effect whereby the O_2 affinity decreases with a fall in the pH of the medium. A similar effect of pH was observed with the α-haemocyanin of *Helix pomatia*, although some mollusc haemocyanins show a negative Bohr effect. In the case of *Lymnaea* haemocyanin the pH effect is more pronounced in the high-O_2-saturation region of the binding curves. This effect of pH is evident as a change in the value of the high-affinity equilibrium binding constant, K_R, and hence the allosteric constant, L_0. This means that, as the pH is increased, the high-affinity conformation of the protein is favoured. Other ions have been shown to be allosteric effectors of mollusc haemocyanin. Shaklai *et al.* (1975) have proposed that the influence of Ca^{2+} on the O_2-binding behaviour of *Levantina hierosolima* haemocyanin may be explained in terms of two O_2-linked calcium-binding sites, one promoting and the other inhibiting O_2 binding. In the same paper, Shaklai *et al.* reported that sodium ions do not bind to *Levantina* haemocyanin. Indeed, Brouwer *et al.* (1977) reported that the allosteric effect produced in the arthropod *Limulus polyphemus* (king crab) haemocyanin by NaCl was due to the binding of chloride ions, and other anions, such as sulphate or acetate, may produce a qualitatively similar allosteric effect. On the other hand, Norne *et al.* (1979), by using ^{23}Na n.m.r. spectroscopy obtained direct evidence of sodium binding to *Panulirus interruptus* haemocyanin. Moreover, they report evidence for competition between Na^+ and Ca^+ for protein binding sites. The competition for *Panulirus* binding sites between sodium and calcium was also observed by Kuiper *et al.* (1979). These workers showed that binding of calcium or sodium ions is associated with a release of protons.

In the present work we have investigated the effects of $CaCl_2$, and NaCl in the presence of $CaCl_2$, on the O_2-binding behaviour of *Lymnaea* haemocyanin. In the absence of NaCl and at pH 6.8, an increase in $CaCl_2$ concentration from 5 to 50mM resulted in a small increase in O_2 affinity as measured by the p_{50} and the equilibrium binding constants. An increase in NaCl concentration from 5 to 50mM in the presence of 10mM-$CaCl_2$ at pH 7.5 resulted in a small decrease in O_2 affinity. This NaCl-dependence, although small, has also been detected by temperature-jump measurements (A. Dawson & E. J. Wood, unpublished work). In

Table 3. *Rate constants for the reaction of O_2 with various haemocyanins in the R-state*

Species	pH	[$CaCl_2$] (mM)	k_{on} ($\times 10^6 M^{-1} \cdot s^{-1}$)	k_{off} (s^{-1})	Reference
P. interruptus	9.6	10	31	60	Kuiper *et al.* (1977)
H. pomatia	8.2	10	3.8	10	van Driel *et al.* (1978)
B. undatum	8.2	10	8.5	50	Wood *et al.* (1977)
Lim. polyphemus	8.0	10	7.0	8	Brunori *et al.* (1981)
Lym. stagnalis	7.5	10	29.5	49	The present work

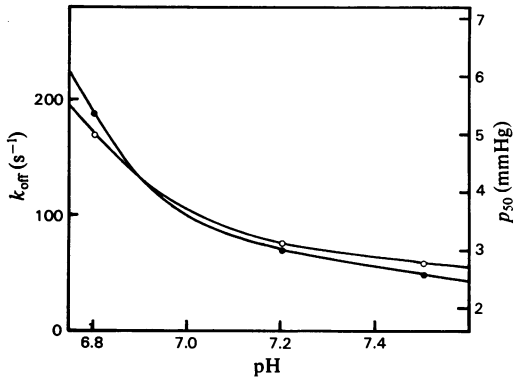

Fig. 10. *pH-dependence of O_2 binding to L. stagnalis haemocyanin*
●, k_{off}; ○, p_{50}; 1 mmHg ≡ 133.3 Pa.

contrast with the small salt effect in *Lymnaea* haemocyanin, the O_2-binding curves of haemocyanins from marine animals such as *Buccinum undatum* (Wood *et al.*, 1977) and *Limulus polyphemus* (Brouwer *et al.*, 1977) show a marked dependence on NaCl concentration. In both cases an increase in NaCl concentration lowers the O_2 affinity, with maximum effectiveness at an optimum pH. Wood *et al.* (1977) found that *Buccinum* haemocyanin achieved a maximum co-operativity and a minimum O_2 affinity in a medium very similar in composition to sea water, namely 0.05 M in $MgCl_2$ and 0.5 M in NaCl.

In order to understand the basis for the changes in the O_2 affinity of *Lymnaea* haemocyanin as observed by the equilibrium measurements, a number of kinetic experiments were performed. Temperature-jump relaxation measurements were performed at high O_2 saturation levels and showed a relaxational process which was assigned to the high-affinity (*R*) state of the protein. Temperature-jump experiments yield a value of k_{on} of the order of $26 \times 10^6 M^{-1} \cdot s^{-1}$, which was relatively independent of pH. In contrast, k_{off} values estimated from temperature-jump data varied considerably with pH, from approx. 40 to $200 s^{-1}$ over the pH range 7.5–6.8. Values of k_{off} determined by the stopped-flow method over a similar pH range were somewhat lower than those determined by the temperature-jump method (16–$26 s^{-1}$), but showed a similar trend with respect to pH. Possibly owing to the rather large errors involved in the temperature-jump method, we were not able to detect a change in the kinetic binding constants resulting from a change in the $CaCl_2$ concentration. However, with respect to the pH experiments, the kinetic results reflect the O_2-binding behaviour observed with the equilibrium measurements and indicate that the binding of O_2 is controlled by the dissociation rate constant (Fig. 10). The kinetic control of O_2 binding by the dissociation rate constant appears to be a common feature of haemocyanins. A comparison of the present kinetic parameters and those obtained from other haemocyanins is shown in Table 3. The data in Table 3 show that the association rate constants are generally very high, ranging between 10^7 and $10^8 M^{-1} \cdot s^{-1}$ and are within a factor of 30 for the different haemocyanins. Moreover, the association rate constant is very often independent of experimental conditions. In contrast, the dissociation rate constant varies considerably with the nature and origin of the protein. Future studies on the O_2-binding properties of individual isolated subunits should throw light on such problems as whether all the domains show a Bohr effect or not, and how the constraining of the domains in the native whole molecule affects this individual O_2-binding behaviour.

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References

Bekius, R. (1972) *Neth. J. Zool.* **22**, 1–58

Booth, A. G. & Dawson, A. (1982) *Biochem. Educ.* **10**, 65–67

Brouwer, M., Bonaventura, C. & Bonaventura, J. (1977) *Biochemistry* **16**, 3897–3902

Brunori, M., Kuiper, H. A. & Antonini, E. (1981) in *Invertebrate Oxygen-Binding Proteins* (Lamy, J. & Lamy, J., eds.), pp. 693–698, Marcel Dekker, New York

Colosimo, A., Brunori, M. & Wyman, J. (1974) *Biophys. Chem.* **2**, 338–344
Dawson, A., Baxendale, D. A. & Wood, E. J. (1981*a*) *Biochem. Soc. Trans.* **9**, 446–447
Dawson, A., Gormally, J., Wyn-Jones, E. & Holzwarth, J. (1981*b*) *J. Chem. Soc. Chem. Commun.* 386–387
Eigen, M. (1968) *Q. Rev. Biophys.* **1**, 3–33
Eigen, M. & De Maeyer, L. (1963) in *Techniques of Organic Chemistry* (Friess, S., Lewis, E. & Weissberger, A., eds.), part 2, vol. 8, pp. 895–1054, Interscience, New York
Er-el, Z., Shaklai, N. & Daniel, E. (1972) *J. Mol. Biol.* **64**, 341–352
Gullick, W. J., Herries, D. G. & Wood, E. J. (1979) *Biochem. J.* **179**, 593–602
Hall, R. L., Pearson, J. S. & Wood, E. J. (1975) *Comp. Biochem. Physiol.* **52B**, 211–218
Jones, J. D. (1972) *Comparative Physiology of Respiration*, Edward Arnold, London
Kuiper, H. A., Gaastra, W., Beintema, J. J., van Bruggen, E. F. J., Schepman, A. M. H. & Drenth, J. (1975) *J. Mol. Biol.* **99**, 619–629
Kuiper, H. A., Antonini, E. & Brunori, M. (1977) *J. Mol. Biol.* **116**, 569–576
Kuiper, H. A., Forlani, L., Chiancone, E., Antonini, E., Brunori, M. & Wyman, J. (1979) *Biochemistry* **18**, 5849–5854
Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
Lontie, R., Deley, M., Robberecht, H. & Witters, R. (1973) *Nature (London)* **242**, 180–182
Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math.* **11**, 431–441
Mellema, J. E. & Klug, A. (1972) *Nature (London)* **239**, 145–150
Monod, J., Wyman, J. & Changeux, J. (1965) *J. Mol. Biol.* **12**, 88–118
Norne, J. E., Gustavsson, H., Forsen, S., Chiancone, E., Kuiper, H. A. & Antonini, E. (1979) *Eur. J. Biochem.* **98**, 591–595
Riggs, A. F. & Wolbach, R. A. (1956) *J. Gen. Physiol.* **39**, 585–605
Shaklai, N., Klarman, A. & Daniel, E. (1975) *Biochemistry* **14**, 105–108
Sick, H. & Gersonde, K. (1969) *Anal. Biochem.* **32**, 362–376
van Driel, R., Kuiper, H. A., Antonini, E. & Brunori, M. (1978) *J. Mol. Biol.* **121**, 431–439
Wood, E. J. (1980) *Essays Biochem.* **16**, 1–48
Wood, E. J. & Peacocke, A. R. (1973) *Eur. J. Biochem.* **35**, 410–420
Wood, E. J., Cayley, G. R. & Pearson, J. S. (1977) *J. Mol. Biol.* **109**, 1–11
Wood, E. J., Corfield, G. C. & Siggens, K. W. (1981) *Comp. Biochem. Physiol.* **69B**, 877–880
Zolla, L., Kuiper, H. A., Vecchini, P., Antonini, E. & Brunori, M. (1978) *Eur. J. Biochem.* **87**, 467–473