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Analysis of the Effect of Three Different Allosteric Ligands on Oxygen Binding by Hemocyanin of the Shrimp, *Penaeus setiferus*†

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ABSTRACT: The hemocyanin of the shrimp *Penaeus setiferus* is present in the hemolymph as a high molecular weight aggregate with a sedimentation coefficient of 16 S. This value is characteristic for hexameric arthropodan hemocyanins. Only one band, corresponding to the 16S component, is observed on regular disc gel electrophoresis. Sodium dodecyl sulfate gel electrophoresis shows that the hexamer contains two molecular weight species in a ratio of 1 to 2.6, with estimated molecular weights of 82 000 and 77 000, respectively. The 16S aggregate is extremely stable. Complete dissociation into its constituent polypeptide chains can only be achieved under conditions where the protein loses its oxygen binding capacity. The oxygen binding properties of *Penaeus* hemocyanin have been studied. Analytical ultracentrifugation verified that the sedimentation coefficient of the oxy- and deoxyhemocyanin was 16 S in all of the conditions used in the binding studies. The oxygen affinity of *Penaeus* hemocyanin can be modulated by three different allosteric effectors: hydrogen ions, calcium ions, and chloride ions. Hydrogen ions decrease the oxygen affinity of *Penaeus* hemocyanin. There is a very strong positive Bohr effect. Calcium and chloride ions increase the oxygen affinity of *Penaeus* hemocyanin, opposing the effect of hydrogen ions. The possible physiological significance of these effects is discussed. The oxygen binding data could not be described by the allosteric two state model of J. Monod et al. ((1965) *J. Mol. Biol. 12,* 88–118). The introduction of one symmetrical hybrid state, R_3T_3, resulted in an excellent fit between theory and experiments.

Hemocyanins have been studied extensively, as elaborate models of cooperative and allosteric interactions, over the past few years. Information about the oxygen binding properties of these high molecular weight proteins is rapidly increasing (Van Holde & van Bruggen, 1971; Bonaventura et al., 1977). Hemocyanins fall into two classes, molluscan and arthropodan hemocyanins, both of which bind 1 O_2 per 2 copper atoms. The molluscan hemocyanins have a functional unit with a minimum molecular weight of 50 000. Their subunits contain multiple oxygen binding domains and the multisubunit aggregate may contain as many as 180 binding sites. Arthropodan hemocyanins have 75 000–80 000 dalton subunits. In arthropodan hemocyanins the multisubunit aggregate commonly contains 6, 12, 24, or 48 oxygen binding sites (Van Holde & van Bruggen, 1971; Bonaventura et al., 1977). One of the interesting problems in studying the oxygen binding by these multisubunit proteins concerns the question of how many sites are involved in the cooperative interactions. Do these interactions involve all the binding sites, or are they confined to subgroups or functional constellations each containing a fixed number of strongly interacting sites, responsible for most of the cooperativity shown by the entire system. Do secondary interactions exist between these functional constellations? The answer to these questions seems to depend upon the class of hemocyanins under study.

Molluscan hemocyanins can be easily dissociated into 1/10 molecules which have about 18 binding sites. The 1/10 molecules of *Helix pomatia* α-hemocyanin at pH 8.2, ionic strength 0.1, have oxygen binding properties corresponding to those of the high affinity state of the undissociated molecules at pH 8.2 in the presence of 10 mM $CaCl_2$. However, when these 1/10 molecules are surrounded in the whole molecule by 1/10 molecules whose oxygen binding sites have been made inactive

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by removing the copper, their oxygen behavior is similar to that of the low affinity state (van Driel, 1973). This clearly indicates that information concerning the conformational state of the whole molecule is passed over the interfaces between the 1/10 molecules. Moreover, at pH 8.2, ionic strength 1.1, cooperative oxygen binding by *Helix pomatia* α-hemocyanin is observed which seems to be based on the oxygen-linked dissociation of low affinity, noncooperative 1/2 molecules into high affinity, noncooperative 1/10 molecules. The 1/2 molecules seem to be the smallest units which can occur in the low affinity state, a prerequisite for cooperative oxygen binding. Therefore the 1/2 molecule, containing 90 binding sites, is regarded as the smallest cooperative oxygen binding unit in the intact *Helix pomatia* α-hemocyanin aggregate (van Driel & van Bruggen, 1974). However, the oxygen binding behavior of *Helix pomatia* α-hemocyanin at pH 8.2 in the presence of 10 mM $CaCl_2$ can be described within the framework of a two state model (Monod et al., 1965), taking 12 as the number of sites involved in the homotropic interactions (Colosimo et al., 1977). Under these conditions ligand-linked dissociation events are negligible. In another case, Klarman et al. (1975) have reported that 1/10 molecules of the mollusc *Levantina hierosolima* can bind oxygen cooperatively. Oxygen binding by *Helix pomatia* β-hemocyanin can basically be described by a two state model with the variation that K_T, the oxygen dissociation constant of the T state, is pH dependent. However, in order to describe the cooperativity as a function of pH, the minimum number of interacting binding sites has to be varied between 8 and 15, implying that the number of interacting sites is not fixed, but variable (Zolla et al., 1978). Multiple T states and R states are found in *Levantina hierosolima* hemocyanin as a function of the calcium concentration (Shaklai et al., 1975). The picture emerging from the studies described above seems rather complex. There is no clear cut and well-defined allosteric unit, with a fixed number of sites, that applies to molluscan hemocyanins in general. The number of sites involved in allosteric interactions seems to depend on the conditions to which the molecule is exposed. This complexity may be related to the complex structure of the molluscan hemocyanins, whose smallest subunits are uncommonly large polypeptide chains made up of 7–8 oxygen binding domains (Siezen & van Bruggen, 1974; Brouwer et al., 1976; Gielens et al., 1977).

The picture emerging from oxygen binding studies on arthropod hemocyanins seems to be more simple, as is their structure. The simplest arthropodan hemocyanins occur as hexamers and bind oxygen cooperatively (Kuiper et al., 1975; this study). Multiples of the hexameric structure are commonly found. *Limulus polyphemus* hemocyanin contains eight hexamers (48 binding sites). Its oxygen binding behavior can be described satisfactorily with a two state model modified in such a way that the allosteric effector changes the allosteric equilibrium constant and the oxygen affinity of the T state (Brouwer et al., 1977). Miller & Van Holde (1974) inferred from the dependence of the Hill coefficient on log p_{50} that the oxygen binding of *Callianassa californiensis* hemocyanin, containing 4 hexamers (24 binding sites), could be described by an extension of the two state model by allowing the occurrence of the symmetrical hybrid state R_3T_3. The number of interacting sites was again inferred to be 6 (Miller & Van Holde, 1974). A more detailed study, taking into account the effect of oxygenation on the 24-mer $\rightleftharpoons$ hexamer equilibrium of *Callianassa* hemocyanin, has recently been published (Arisaka, 1977). Hemocyanin of the shrimp, *Penaeus setiferus,* is a very stable, electrophoretically pure, hexamer. Here we report on the effect of three different allosteric ligands on its oxygen binding behavior. The observed binding curves can be described very well, using a saturation function derived for a hexameric molecule, taking into account the presence of one R state, one T state and the hybrid state R_3T_3 (Buc et al., 1973; Arisaka, 1977). The increase in oxygen affinity with increasing pH and calcium and chloride concentrations is kinetically associated with a decrease of the oxygen dissociation rate.

Materials and Methods

Specimens of *Penaeus setiferus* were collected offshore in the vicinity of Beaufort, N.C. Hemolymph was withdrawn from the live shrimp by dorsally inserting a needle between the cephalothorax and the abdomen. The coagulated hemolymph was dissolved in 0.1 ionic strength Tris buffer, pH 7, containing 10 mM $CaCl_2$, with the aid of a tissue homogenizer. The solution was cleared of debris by centrifugation at 12 060*g* for 15 min. Gel filtration on Sepharose CL-6B was carried out on the whole blood, using a 2.5 × 50 cm column. The eluent was 0.1 ionic strength Tris buffer, pH 7, containing 10 mM $CaCl_2$. Sedimentation velocity experiments were carried out at 20 °C using a Beckman Spinco Model E analytical centrifuge with mechanical speed control and Schlieren optics.

Oxygen equilibrium experiments were performed using a tonometric method (Riggs & Wolbach, 1956). Values for the percent saturation with oxygen were determined at 340 nm. In cases where the hemocyanin was not fully saturated at 1 atm of oxygen, the absorbance value at 340 nm of the fully oxygenated protein was obtained from the experimentally determined relationship: $\Delta A_{340nm}/A_{280nm} = 0.201$. For the binding studies, Tris-HCl buffers made up to the desired ionic strength with NaCl (Bates, 1973) were used in a pH range from 6.5 to 9. In studying the effect of calcium ions, the protein was first dialyzed extensively against 50 mM Tris, pH 8.9, containing 10 mM EDTA in order to remove all divalent cations. Subsequently the samples were dialyzed vs. buffer containing the desired amount of $CaCl_2$. When the effect of NaCl was studied the salt was added directly to the samples. The rate of dissociation of oxygen from the oxyhemocyanin was measured by rapid mixing of air equilibrated protein with dithionite-containing buffers in a Durrum stopped-flow spectrophotometer. The photomultiplier output was processed by an Aminco analogue digital converter and transient recorder (DASAR) coupled to a PDP-11 computer and Tektronix graphics terminal. Polyacrylamide disc gel electrophoresis was carried out using a gel concentration of 4% or 7.5%, with 2.5% cross-linking. Gel phase buffer and electrophoresis buffer were the same: 50 mM Tris, pH 8.9, containing 10 mM EDTA. Sodium dodecyl sulfate gel electrophoresis was carried out on 7.5% polyacrylamide gels with 2.5% cross-linking, according to Weber & Osborn (1969). Gels were calibrated with chymotrypsinogen A, ovalbumin, serum albumin, and transferrin.

Results

Gel chromatography of the hemolymph of *Penaeus setiferus* on Sepharose CL-4B shows the presence of two peaks absorbing at 280 nm. Only the first peak, corresponding to 95% of the total absorbance at 280 nm, absorbs at 340 nm, characteristic of the copper–oxygen complex in hemocyanins. This material sediments as a single boundary with a sedimentation coefficient of 16 S, a value typical of hexameric arthropodan hemocyanins. The hexamer appears to be extremely stable. Complete dissociation into its constituent polypeptide chains takes place only at pH 10.8 in the absence of divalent cations. Unfortunately, the protein loses its oxygen binding capacity under these conditions. Regular gel electrophoresis in 50 mM

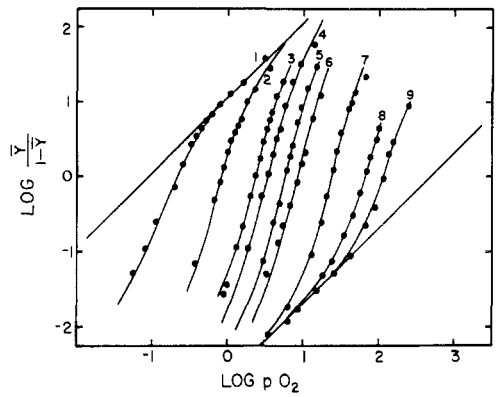

FIGURE 1: Hill plots for oxygen binding at 20 °C in a 50 mM Tris buffer, ionic strength 0.1. Curves 2, 4, 5, 7, and 8 measured in the presence of 10 mM $CaCl_2$ at pH 8.8, 8.2, 7.85, 7.6, and 6.9, respectively. (1) With 10 mM $CaCl_2$, 2 M NaCl, pH 8.2; (3) 10 mM $CaCl_2$, 0.5 M NaCl, pH 8; (6) 5 mM $CaCl_2$, pH 7.85; (9) no $CaCl_2$, pH 6.5. This figure shows only part of the measured binding data. Most of the measured binding curves are omitted for clarity. Lines are computed based on eq 7 of the text, taking $c = 1.90 \times 10^{-4}$, $q = 6.61$, and L' as given in Table IV. Circles represent experimental results (see also Figure 2).

Tris, pH 8.0 or 9.5 containing 10 mM EDTA gives a single sharp band. This band corresponds to the one electrophoretically pure hexameric hemocyanin species present in *Penaeus setiferus*. As no conditions have been found under which the hemocyanin could be dissociated without damaging the protein, regular gel electrophoresis of the native polypeptide chains could not be carried out. However, sodium dodecyl sulfate gel electrophoresis shows the presence of two molecular weight species, with estimated molecular weights of 82 000 and 77 000, respectively, in a ratio of 1:2.6. The hemocyanin seems therefore not to be homogeneous on the polypeptide chain level.

Effect of pH on Oxygen Binding. Figure 1 and Table I show the effect of pH on the oxygen binding by *Penaeus setiferus* hemocyanin. There is a very strong positive Bohr effect with p_{50} values ranging from 0.35 to 112 mm of mercury as a function of pH (Table I). The Hill coefficient has a maximum at intermediate p_{50} values around pH 8 and decreases at lower and higher pH values.

Effect of NaCl on Oxygen Binding. Figure 1 (curves 1 and 4) and Table II show the effect of NaCl on the p_{50} and the Hill coefficient of *Penaeus* hemocyanin. The oxygen affinity increases considerably with increasing NaCl concentration, concomitant with a decrease of the value of the Hill coefficient. At pH 8.2, in the presence of 2 M NaCl, the slope of the Hill plot is unity at fractional saturation values equal to or greater than 0.9. This allows us to draw the hypothetical binding curve of the R state (see Discussion and Figure 1).

Effect of Ca^{2+} on Oxygen Binding. The effect of Ca^{2+} on the oxygen binding by *Penaeus* hemocyanin is shown in Figure 1 (curves 5 and 6), and Table III. Calcium has a small but distinct effect on the oxygen affinity at pH 7.85. Increasing calcium concentrations increase the oxygen affinity. At pH 6.5 in the absence of $CaCl_2$ the slope of the Hill plot is unity at fractional oxygen saturation values equal to or less than 0.1. This allows us to draw the hypothetical binding curve of the T state (Figure 1 and Discussion).

Oxygen Dissociation Kinetics. The time course of oxygen dissociation from *Penaeus* hemocyanin is autocatalytic; that is, the dissociation rate constant increases as the reaction proceeds. This is observed under all the conditions studied. Tables I–III give the apparent overall oxygen dissociation rate constants, as calculated from the slopes of the first-order plots between 75 and 25% of the reaction, as a function of the concentration of the three allosteric effectors. In all three cases an increase in the oxygen affinity is accompanied by a decrease of the dissociation rate constants.

TABLE I: Oxygen Binding Parameters for *Penaeus* Hemocyanin as a Function of pH, in the Presence of 10 mM $CaCl_2$, 20 °C.

pH	p_{50} (mm)	k_{off}[a] (s^{-1})	n_H
6.63	112		3.1
6.90	65		3.2
7.12	54		3.7
7.60	24	374	4.0
7.80	5.6	148	4.2
8.20	3.3	131	3.3
8.90	0.8	57	3.1
9.10	0.3	43	2.8

[a] Calculated from the slope of the first-order plots between 75 and 25% of the reaction.

Discussion

The hemocyanin of the shrimp, *Penaeus setiferus*, is present in the hemolymph as an aggregate with a sedimentation coefficient of 16 S. It is fairly well established that the 16S molecule corresponds to an oligomer containing 6 subunits of about 75 000–80 000 molecular weight (Carpenter & Van Holde, 1973; Ellerton et al., 1970; Roxby et al., 1974; Kuiper et al., 1975; Hamlin & Fish, 1977; Ellerton et al., 1977). The 16S molecule of *Penaeus* is electrophoretically pure, as has been found for the *Cancer magister* hexamer (Carpenter & Van Holde, 1973). In contrast, the hemocyanin hexamer of the fresh water crayfish *Cherax destructor* consists of four electrophoretic components (Jeffrey et al., 1976). The subunits of *Penaeus* hemocyanin are heterogeneous as judged by sodium dodecyl sulfate gel electrophoresis which shows both 82 000 and 77 000 molecular weight species. Molecular weight heterogeneity of the polypeptide chains in arthropodan hemocyanins seems to be common (Loehr & Mason, 1973; Carpenter & Van Holde, 1973; Kuiper et al., 1975; Murray & Jeffrey, 1974; Hamlin & Fish, 1977). To our knowledge all arthropodan hemocyanins previously studied could be dissociated into their constituent polypeptide chains by raising the pH to 9–10 and removing divalent cations. In this respect *Penaeus* hemocyanin behaves rather exceptionally. At pH 10 in the absence of divalent cations only the hexameric species is observed. Dissociation is complete at pH 10.8 where the protein loses its oxygen binding capacity. Therefore the oxygen binding properties of the subunits or their electrophoretic behavior could not be studied and our experiments were confined to the study of the properties of the hexamer of *Penaeus* hemocyanin. Sedimentation analysis verified that the oxy- and deoxyhemocyanin was 16 S in all of the conditions used in our oxygen binding experiments. The binding of oxygen proceeds cooperatively (Figure 1) and reaches a maximum value of n_H of 4.2 (see Figure 3). There is a marked positive Bohr effect, i.e., an increase of the oxygen affinity with increasing pH (Table I), as has been reported for a number of other arthropodan hemocyanins (Miller & Van Holde, 1974; Kuiper et al., 1975; Loewe & Linzen, 1975; Ellerton et al., 1977).

The effect of NaCl on oxygen binding by *Penaeus* hemocyanin (Table II) might be due to the sodium ion, the chloride ion, or the ionic strength. Using a 0.1 ionic strength Tris buffer pH 8, containing 10 mM $CaCl_2$ and either 1 M Na_2SO_4 or 2 M NaCl we found the oxygen affinities of *Penaeus* hemocyanin to be 1.2 and 0.35 mm of mercury, respectively. This rules

TABLE II: Effect of Chloride on the Oxygen Binding Parameters of *Penaeus* Hemocyanin, in the Presence of 10 mM $CaCl_2$, 20 °C.

[Cl] (M)	p_{50} (mm)	k_{off}[d] (s^{-1})	n_H
0.1[a]	4.5	140	4.2
0.5[a]	2.2	100	4.1
1.0[a]	1.0	50	3.9
2.0[a]	0.4	28	3.2
0.1[b]	3.3	131	4.2
0.5[b]	1.1	60	3.7
2.0[b]	0.2	23	2.2
0.1[c]	2.5		4.0
2.0[c]	0.3		2.8
3.0[c]	0.2		2.2

[a] pH 8.0. [b] pH 8.2. [c] pH 8.3. [d] Calculated from the slope of the first-order plots between 75 and 25% of the reaction.

TABLE III: Effect of Calcium on the Oxygen Binding Parameters of *Penaeus* Hemocyanin, pH 7.85, 20 °C.

[Ca^{2+}] (mM)	p_{50} (mm)	k_{off}[b] (s^{-1})	n_H
0	10.5	198	4.0
2	10.5	186	4.0
5	8.3	167	3.7
10	5.6	150	4.2
20	3.2	105	4.0
0[a]	117		2.9

[a] pH 6.5. [b] Calculated from the slope of the first-order plots between 75 and 25% of the reaction.

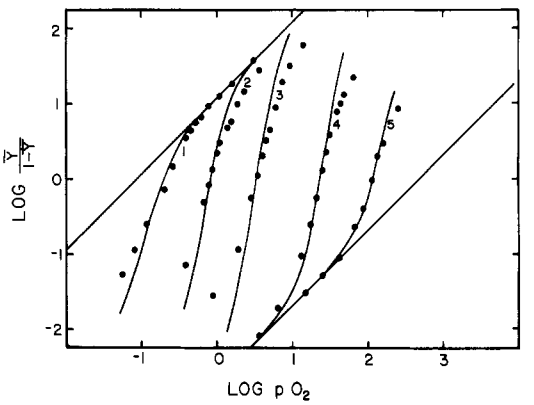

FIGURE 2: Hill plots for oxygen binding at 20 °C in a 50 mM Tris buffer ionic strength 0.1. (1) With 10 mM $CaCl_2$, 2 M NaCl, pH 8.2; (2) 10 mM $CaCl_2$, pH 8.8; (3) 10 mM $CaCl_2$, pH 8.2; (4) 10 mM $CaCl_2$, pH 7.6; (5) no $CaCl_2$, pH 6.5. Lines were computed based on eq 1 of the text, taking $c = 1.90 \times 10^{-4}$ and L' as given in Table IV. Compare with Figure 1, in which the lines are calculated with eq 7 of the text, using the same values for c and L'. Circles represent experimental results.

out the possibility that the sodium ion is the allosteric effector. The oxygen affinity of *Penaeus* hemocyanin in a Tris, 10 mM $CaCl_2$ buffer, pH 8 made up to ionic strength 3 with either Na_2SO_4 or NaCl is 1.2 and 0.27 mm of mercury, respectively. This rules out the possibility that the changes in oxygen affinity are due primarily to changes in ionic strength. Therefore we conclude that the chloride anion is the allosteric effector, as has been found for *Limulus polyphemus* hemocyanin (Brouwer et al., 1977).

The effect of $CaCl_2$ on the oxygen binding by *Penaeus* hemocyanin (Table III) is less than that of the pH and chloride ions. Since calcium is added as $CaCl_2$, it might be asked if the observed changes in oxygen affinity are due to chloride instead of calcium. Comparing Tables II and III we see that $CaCl_2$ affects the oxygen binding in the range of 0–20 mM $CaCl_2$, whereas chloride is effective in the range of 0–2000 mM chloride. The contribution of chloride to the observed $CaCl_2$ effect is therefore negligible. An increase in oxygen affinity in the presence of calcium has been reported for a number of arthropod hemocyanins (Larimer & Riggs, 1964; Pickett et al., 1966; Hwang & Fung, 1970; Miller & Van Holde, 1974). However, the oxygen affinity of *Panulirus interruptus* hemocyanin decreases in the presence of calcium (Kuiper et al., 1975).

The three allosteric effectors described in this study change the rate of oxygen dissociation from *Penaeus* hemocyanin in accord with their effects on oxygen equilibria (Tables I–III). Under most conditions examined by the rapid-mixing method, the complete dissociation reaction was observed. The exceptions were those experiments done in 0.1 ionic strength buffers with a pH lower than 7.6 where the oxygen dissociation rate becomes too fast and part of the reaction is lost in the dead time of the stopped-flow apparatus. Therefore no k_{off} values are presented in Tables I–III at pH values lower than pH 7.6. It can be seen that an increase in oxygen affinity is accompanied by a decrease of the overall oxygen dissociation rate. The same phenomenon has been described for the hemocyanins of *Limulus polyphemus* (Sullivan et al., 1974; Brouwer et al., 1977) and *Murex fulvescens* (Brouwer et al., 1978). Oxygen binding curve 1 in Figure 1 shows that only the R state is populated in oxygenated *Penaeus* hemocyanin in a pH 8.2 buffer containing 10 mM $CaCl_2$ and 2 M NaCl. The initial rate of oxygen dissociation under these conditions is 10 s^{-1} and corresponds to the oxygen dissociation rate constant of the R state. Similar values have been reported for the oxygen dissociation rate constants of the high affinity state of *Limulus polyphemus* hemocyanin, 8 s^{-1} (Brouwer et al., 1977), *Helix pomatia* α-hemocyanin, 10 s^{-1}, (van Driel et al., 1974), and *Murex fulvescens* hemocyanin, 10 s^{-1} (Brouwer et al., 1978). Somewhat faster dissociation rates have been observed for the high affinity state of *Panulirus interruptus* hemocyanin, 35 s^{-1} (Kuiper et al., 1977) and *Buccinum undatum* hemocyanin, 80 s^{-1} (Wood et al., 1977).

Analysis and Interpretation of the Oxygen Binding Data. In terms of the allosteric transition model proposed by Monod et al. (1965), there are two conformations of the hemocyanin molecule, referred to as T and R. Each form of the *Penaeus* hemocyanin hexamer can independently bind six oxygen molecules. The T state has a lower affinity for oxygen than the R state. This difference in affinity is responsible for the cooperative oxygenation, as during the course of ligation the equilibrium between the T and R states is shifted toward the latter. We now proceed to examine to what extent the data presented above may be interpreted quantitatively in terms of the two state model with the extensions of the model as given by Rubin & Changeux (1966) and Blangly et al. (1968). Inspection of Figure 1 shows that all the oxygen binding curves are enclosed between two limiting curves which, according to the theory, correspond to the binding of oxygen to the R state and T state. Both curves have slope unity which indicates that the oxygen binding sites in *Penaeus* hemocyanin are equivalent. The dissociation constant or p_{50} of the R state is 0.089 mm of Hg and that of the T state is 467.7 mm of Hg. Therefore the nonexclusive binding coefficient, $p_{50,R}/p_{50,T}$ is 1.90×10^{-4}. The Hill plots can now be calculated with

$$\frac{\bar{Y}}{1-\bar{Y}} = \frac{\alpha(1+\alpha)^5 + L'\alpha c(1+c\alpha)^5}{(1+\alpha)^5 + L'(1+\alpha c)^5} \quad (1)$$

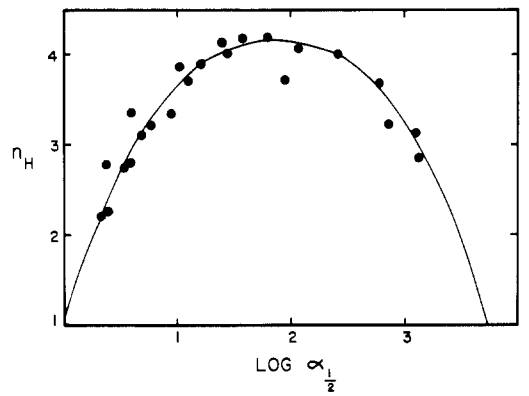

FIGURE 3: A graph of the maximum Hill coefficient n_H vs. log $\alpha_{1/2}$. Data were obtained as a function of the three allosteric effectors and are given in Tables I, II, and III.

in which $\alpha = pO_2/p_{50,R}$, $c = 1.90 \times 10^{-4}$, and L' is the apparent allosteric equilibrium constant, which is given by

$$L' = \frac{(\alpha_{1/2} - 1)}{(1 - \alpha_{1/2}c)} \left(\frac{1 + \alpha_{1/2}}{1 + c\alpha_{1/2}}\right)^5 \quad (2)$$

in which $\alpha_{1/2} = p_{50}/p_{50,R}$. The fit between the calculated Hill plots and the experimental results is poor (Figure 2), the calculated Hill plots being much steeper than the experimental ones. The decreased cooperativity apparent in Figure 2 can be explained by assuming that the allosteric transition is not fully concerted, but that hybrid states such as R_iT_{n-i} are present. If this is the case, the equation which relates the Hill coefficient to the two state model parameters becomes (Buc et al., 1973):

$$n_H = \left(n\frac{\sigma^2}{\sigma_{max}^2} - 1\right)\frac{(\alpha_{1/2} - 1)(1 - c\alpha_{1/2})}{(\alpha_{1/2} + 1)(1 + c\alpha_{1/2})} \quad (3)$$

The value of σ^2/σ_{max}^2 depends on the frequency of occurrence of hybrid states and is 1 if the transition is fully concerted. Figure 3 shows a plot of the Hill coefficient n_H vs. log $\alpha_{1/2}$. The n_H values were obtained in the presence of three allosteric effectors and are given in Tables I, II and III. The curve is fairly symmetrical and goes to unity for $\alpha_{1/2} = 1$ corresponding to $p_{50,R}$ and $\alpha_{1/2} = 1/c$ corresponding to $p_{50,T}$. The curve passes through a maximum which is given by $c\alpha_{1/2}^2 = 1$. The c value calculated from $\alpha_{1/2} = 1/c$ for $n_H = 1$ and from $c\alpha_{1/2}^2 = 1$ at maximum n_H is 1.90×10^{-4}, which is exactly the same value as calculated from the p_{50} values of the R and T state (Figure 1). From the height of the curve and eq 3, we obtain $n\sigma^2/\sigma_{max}^2 = 4.32$. Taking the number of interacting sites as 6, σ^2/σ_{max}^2 becomes 0.72. The curve in Figure 3 is accordingly calculated with eq 3 using $\sigma^2/\sigma_{max}^2 = 0.72$. The fit to the experimental data is satisfactory. The value of σ^2/σ_{max}^2 (less than unity) suggests that hybrid R–T states are involved. The symmetry of the plot of n_H vs. log $\alpha_{1/2}$ suggests that the symmetrical state R_3T_3 is predominant. This is supported by the structural data which show that a hexameric hemocyanin molecule consists of six monomers arranged in two parallel triangles that have a common threefold axis (Schepman, 1975). If a symmetrical hybrid state is present, we have the following allosteric equilibria:

$$T_6 \overset{L'}{\rightleftarrows} R_6; T_6 \overset{qH'/2}{\rightleftarrows} R_3T_3 \text{ and } R_6 \overset{2H'}{\rightleftarrows} R_3T_3$$

with $L' = T_6/R_6$, $qH'/2 = T_6/R_3T_3$, $2H' = R_3T_3/R_6$, and $H' = \sqrt{L'/q}$. The value of q was calculated according to Buc et al. (1973). At maximum n_H, $c\alpha_{1/2}^2 = 1$ and $\sigma^2 = \frac{1}{4}(1 - 1/(\sqrt{q} + 1))$. Using $\sigma^2/\sigma_{max}^2 = 0.72$ and $\sigma_{max}^2 = 0.25$, we obtain $q = 6.61$. The values of σ^2/σ_{max}^2 and q reported here are identical with the values found for pyruvate kinase (Johannes & Hess, 1973) and almost identical with the values found for *Callianassa* hemocyanin (Miller & Van Holde, 1974; Arisaka, 1977).

Derivation of the Saturation Function (Arisaka, 1977). The binding polynomial or generating function for a hexamer in the two state model is given by:

$$P = (1 + \alpha)^6 + L'(1 + \alpha c)^6$$

The binding polynomial for the hexamer, taking into account the presence of the symmetrical hybrid state R_3T_3, is given by:

$$P = (1 + \alpha)^6 + L'(1 + \alpha c)^6 + 2\sqrt{L'/q}\,\{(1 + \alpha)^3(1 + \alpha c)^3\} \quad (4)$$

The fractional saturation of the hexamer with ligand is given by Wyman (1965):

$$\bar{Y} = \frac{1}{n}\frac{\mathrm{d}\ln P}{\mathrm{d}\ln \alpha} = \frac{1}{n}\frac{\alpha \mathrm{d}P}{P\mathrm{d}\alpha} \quad (5)$$

it follows that

$$\bar{Y} = \frac{[\alpha(1 + \alpha)^5 + L'\alpha c(1 + \alpha c)^5 + \sqrt{L'/q} \times \{\alpha(1 + \alpha)^2(1 + c\alpha)^3 + \alpha c(1 + \alpha)^3(1 + c\alpha)^2\}]}{[(1 + \alpha)^6 + L'(1 + \alpha c)^6 + 2\sqrt{L'/q} \times \{(1 + \alpha)^3(1 + c\alpha)^3\}]} \quad (6)$$

and

$$\frac{\bar{Y}}{1 - \bar{Y}} = \frac{[\alpha(1 + \alpha)^5 + L'\alpha c(1 + \alpha c)^5 + \sqrt{L'/q} \times \{\alpha(1 + \alpha)^2(1 + \alpha c)^3 + \alpha c(1 + \alpha)^3(1 + \alpha c)^2\}]}{[(1 + \alpha)^5 + L'(1 + \alpha c)^5 + \sqrt{L'/q} \times \{(1 + \alpha)^2(1 + \alpha c)^2(2 + \alpha + c\alpha)\}]} \quad (7)$$

Equation 7 enables us to calculate the oxygen binding curves of *Penaeus* hemocyanin as a function of L'. L' was calculated as a first approximation with eq 2. Using these values for L' (Table IV), the binding curves 2–9 (Figure 1) were calculated. The L' value used for calculating curve 1 was chosen so as to give the best fit to the data points. (L' calculated was 812, L' chosen 400). The agreement between theory and experiments is excellent (Figure 1). The thermodynamic parameters derived for the model are summarized in Table IV. The step of the transition from the T state to the R state as given by the ΔG values in this table is broken down into smaller transition steps.

Figure 4 shows the oxygen saturation function and the distribution of the T, R, and hybrid states as a function of the logarithm of the partial pressure of oxygen, under conditions corresponding to the Hill plots 1 and 9 in Figure 1. The saturation function was calculated with eq 6. The state functions were calculated with:

$$\bar{R} = \frac{(1 + \alpha)^6}{(1 + \alpha)^6 + L'(1 + c\alpha)^6 + 2\sqrt{L'/q}\,\{(1 + \alpha)^3(1 + c\alpha)^3\}} \quad (8)$$

$$\bar{T} = \frac{L'(1 + c\alpha)^6}{(1 + \alpha)^6 + L'(1 + c\alpha)^6 + 2\sqrt{L'/q}\,\{(1 + \alpha)^3(1 + c\alpha)^3\}} \quad (9)$$

TABLE IV: Thermodynamic Parameters Resulting from the Values of the Various Equilibrium Steps Involved in the Hybrid Model (ΔG, kcal/mol).

pH	$[CaCl_2]$ (mM)	[NaCl] (M)	L'	$-\Delta G^a$	$qH'/2$	$-\Delta G^b$	$2H'$	$-\Delta G^c$
6.50	0	0.05	2.29×10^{18}	24.6	1.94×10^{9}	12.5	1.18×10^{9}	12.1
6.90	10	0.05	8.94×10^{16}	22.7	3.80×10^{8}	11.5	2.33×10^{8}	11.2
7.60	10	0.05	2.98×10^{14}	19.4	2.22×10^{7}	9.8	1.34×10^{7}	9.6
7.85	10	0.05	6.46×10^{10}	14.5	3.27×10^{5}	7.4	1.98×10^{5}	7.1
7.85	5	0.05	6.48×10^{11}	15.8	1.03×10^{6}	8.0	6.26×10^{5}	7.8
8.20	10	0.05	2.86×10^{9}	12.6	6.87×10^{4}	6.5	4.16×10^{4}	6.1
8.80	10	0.05	1.05×10^{6}	8.0	1.32×10^{3}	4.2	7.97×10^{2}	3.9
8.00	10	0.50	2.90×10^{8}	11.3	2.19×10^{4}	5.8	1.32×10^{4}	5.5
8.20	10	2.00	4.00×10^{2}	3.5	2.57×10	1.9	1.56×10	1.6

[a] Free energy for the transition from T to R state. [b] Free energy for the transition from T to hybrid state. [c] Free energy for the transition from hybrid to R state.

$$\bar{H} = \frac{2\sqrt{L'/q}\,\{(1+\alpha)^3(1+c\alpha)^3\}}{(1+\alpha)^6 + L'(1+c\alpha)^6 + 2\sqrt{L'/q}\,\{(1+\alpha)^3(1+c\alpha)^3\}} \quad (10)$$

Differentiation of eq 10 with respect to α shows that $\bar{H}_{max}$, which corresponds to $1 - (\sigma^2/\sigma_{max}^2) = 0.28$, occurs at

$$\alpha = \frac{\sqrt[6]{L'} - 1}{1 - c\sqrt[6]{L'}} \quad (11)$$

Therefore the value of α, for which $\bar{H} = \bar{H}_{max}$, depends only on L' and c. The value of q, which is $(x/(1-x))^2$ with $x = \sigma^2/\sigma_{max}^2$, determines the abundancy of the hybrid state. From eq 8 and 9 we see that $\bar{R} = \bar{T}$ for the value of α as given by eq 11. In other words, if $\bar{H}$ reaches its maximum value, $1 - (\sigma^2/\sigma_{max}^2)$, then $\bar{R} = \bar{T} = \frac{1}{2}(\sigma^2/\sigma_{max}^2)$. Summarizing, we can say that the oxygen binding behavior of *Penaeus setiferus* hemocyanin can be described by an extended two state model which allows the occurrence of one symmetrical hybrid state R_3T_3. The same model has been used to describe the oxygen binding by *Callianassa* hemocyanin (Miller & Van Holde, 1974; Arisaka, 1977), suggesting a general validity of the "hybrid model" for oxygen binding by crustacean hemocyanins.

Finally, we would like to discuss the possible physiological significance of the binding data reported above. The decapod crustacean, *Penaeus setiferus,* is exposed to various environments during its life. The shrimps spawn in oceanic waters. Postlarvae are planktonic and live offshore. As they grow they move toward and finally enter inshore waters. *Penaeus setiferus* may be found as far inland from the coast as 200 km. The shrimps grow rapidly on the rich nursery grounds in the estuaries and return to the sea when they have attained a length of between 10 and 12 cm (Perez Farfante, 1969). A similar migration behavior is found for the decapod crustacean, *Callinectes sapidus,* the blue crab. After development and metamorphosis to the adult form the young crabs swim upstream, leaving behind the well-oxygenated saline ocean waters for the hypoxic dilute waters of the estuaries. In the autumn of each year the impregnated females migrate to more saline waters. The mechanism that permits the oxygen transport system of the blue crab to function effectively has been studied by Mangum & Towle (1977). During migration from waters of 35‰ to 1‰ salinity the reduction in total blood salt is about 37%, which is accompanied by a decrease in oxygen affinity of the hemocyanin. The respiratory stability in dilute waters is maintained by concomitant changes in blood pH, which oppose the salt effect. The pH change results from the ammonia produced in deamination of the intracellular pool of free amino acids as the cells conform to osmotic changes in body fluids. The ammonium ion in turn provides a counterion to facilitate salt absorption by the sodium potassium dependent ATPase in the gill (Mangum & Towle, 1977).

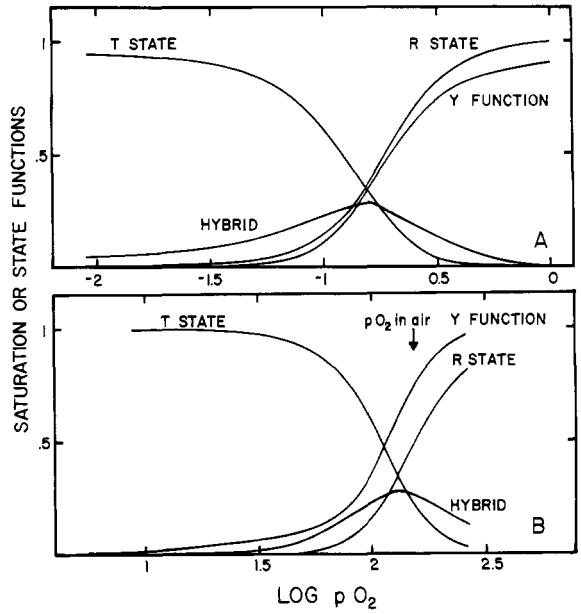

FIGURE 4: Saturation functions and the fraction of molecules in the R, T, and hybrid state as a function of pO_2, in 50 mM Tris, ionic strength 0.1. (A) pH 8.2, 10 mM $CaCl_2$ + 2 M NaCl (curve, 1, Figure 1). (B) pH 6.5 (curve 9, Figure 1).

Penaeus and *Callinectes* are closely related decapod crustaceans. Both show similar migrational behavior which exposes them to the same environment. Both have hemocyanins whose salt effects are opposed by the positive Bohr effect. Therefore it seems reasonable to suppose that the allosteric effectors, H^+ and chloride, provide *Penaeus setiferus* with a respiratory stability in an environment which is unstable in both ions and oxygen.

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