Subunits and Association Equilibria of *Callianassa californiensis* Hemocyanin†

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ABSTRACT: The hemocyanin of the ghost shrimp, *Callianassa californiensis*, exists in the hemolymph of the organism in two forms, which we shall designate C and I. These have the following properties. (1) *Hemocyanin C* is present in the hemolymph as particles with a sedimentation coefficient ($s^0_{20,w}$) of about 39 S. When the isolated material is dialyzed into buffer (pH 7.6) containing no divalent ions, it dissociates completely into 17S particles. This component is competent to completely reassociate to the 39S form upon dialysis into buffers containing 0.05 M or more Mg^{2+} or Ca^{2+}. (2) *Hemocyanin I* is present in the hemolymph as 17S particles. It is incompetent to associate to the 39S form under any conditions tested, including high levels of divalent ions. The 17S and 39S forms of hemocyanin C are in a monomer–tetramer relationship. The former has a molecular weight of 4.3×10^5; for the latter a value of 17×10^5 was found. At levels of divalent ion concentrations between 0.01 and 0.05 M, these two species are in dynamic equilibrium. The 17S particles of hemocyanin C are each made up of six polypeptide chains, which appear to be homogeneous in weight, with a chain weight of 74,000. Hemocyanin I shows similar chains in sodium dodecyl sulfate gel electrophoresis, but also contains small amounts of smaller chain fragments.

In previous papers we have described physical investigations of a number of hemocyanins (see, for example, Van Holde and Cohen, 1964, DePhillips *et al.*, 1969, Ellerton *et al.*, 1970, and Carpenter and Van Holde, 1973). Throughout these studies, we have been fascinated by the multiplicity of aggregated states that these molecules are capable of forming. It has been our intent to investigate, in such systems, the interrelation between association–dissociation equilibria and the binding of small ligands, particularly the oxygen ligand. Unfortunately in many cases a completely satisfactory study has been blocked because of the apparent irreversibility (or quasi-reversibility) of association–dissociation reactions.

In this paper we report the behavior of a hemocyanin which appears to exhibit truly reversible, dynamic equilibrium between two distinct states of aggregation. This material is obtained from the hemolymph of the ghost shrimp, *Callianassa californiensis*. We report herein first studies of this hemocyanin in its various states of aggregation, and some of the factors which influence equilibria between these aggregation states. The following paper (Miller and Van Holde, 1974) describes studies of the oxygen binding by this protein.

Materials and Methods

Isolation and Purification of Hemocyanin. Shrimp were dug from muddy sand in Yaquina Bay, near the Oregon State University Marine Science Center, Newport, Ore. Hemolymph was withdrawn from the live shrimp by inserting a capillary into the ventral abdominal sinus, near the base of the tail. It was then cleared of debris by centrifugation at low speed, and filtered through a 0.8 μ Millipore filter. Gel filtration (see below) on Bio-Gel A5m (Bio-Rad) was then routinely carried out on the whole blood, using a 4×90 cm column. In most preparations, the eluent used was 0.1 ionic strength Tris buffer (pH 7.65) containing 0.05 or 0.1 M $MgCl_2$.

We find that the hemolymph of this animal usually contains about 70–100 mg/ml of protein, at least 95% of which is hemocyanin (see Figure 1). There is a small amount of a reddish pigment in the hemolymph. This is always found in the last fractions off the Bio-Gel column. In early experiments, we took hemolymph separately from males and females. However, we could observe no difference in sedimentation behavior between the samples, and thereafter pooled samples.

The two leading components shown in Figure 1 are both forms of hemocyanin, as judged from their absorption spectra and other properties. While they will be described in detail in the Results section, we remark here for purposes of identification that they correspond to particles with sedimentation coefficients ($s^0_{20,w}$) of approximately 39 and 17 S, respectively, and that these two components represent hemocyanin C and hemocyanin I, as defined below.

Preparation of Solutions. Solutions were prepared using 0.1 ionic strength buffers, as described by Long (1961). Doubly distilled water was used. Unless otherwise indicated, all studies were at pH 7.65 in Tris buffer. The hemocyanin concentrations were obtained from absorbance measurements at 280 nm. The extinction coefficient of purified 39 S form of hemocyanin C was determined by measuring the number of Rayleigh fringes produced in a synthetic boundary experiment in the ultracentrifuge, using a sample of known absorbance. Using the previously determined value for hemocyanins, 0.2324 (mg/ml)/fringe (Nickerson and Van Holde, 1971), we find an $E^{1\%}_{1\,cm}$ of 14.0. This is in good agreement with values obtained for other arthropod hemocyanins (Nickerson and Van Holde, 1971).

Since the 17S form of hemocyanin C is of lower molecular weight than the 39S form (see Results), we would expect it to exhibit less light scattering and hence a different value of the extinction coefficient. The difference was determined by adding solid $MgCl_2$ to a solution of 17 S particles of known optical density to increase the magnesium concentration from

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0 to 0.05 M. This direct addition of Mg^{2+} resulted in 70% of the protein associating to the 39S form.[1] From the change in absorbance that resulted, the extinction coefficient of pure 17 S material was calculated to be $E_{1\,cm}^{1\%} = 13.3$.

Sedimentation Velocity. All sedimentation experiments were carried out on a Beckman Model E ultracentrifuge. In velocity studies either schlieren optics or the photoelectric scanner were used. If not stated otherwise, the instrumental data were analyzed conventionally; that is, by following the migration rate of the maximum ordinate of the derivative curve or of the integral boundary. Many of the sedimentation velocity experiments were analyzed by determining the second moment of the boundaries and the scanner was used exclusively to obtain these data. This method gives a weight-average sedimentation coefficient for the mixture of components as a whole. Data were corrected to water at 20° in the conventional way, except for some comparative studies, which were simply corrected to 20°.

Sedimentation equilibrium experiments to determine the molecular weights of the 17S and 39S forms were carried out utilizing the high-speed technique of Yphantis (1964). Solutions of initial concentrations between 0.5 and 1.0 mg per ml were run using 3-mm solution column heights in a six-channel centerpiece in an AN-J rotor. Each solution was shown to be homogeneous by means of sedimentation velocity experiments, within the limitations of that technique. Rayleigh optics were used in the equilibrium experiments, and the interference patterns were photographed on Kodak II-G spectroscopic plates. Fringe positions were measured by averaging the vertical position of five successive fringes at spacings of 50–100 μ using the Nikon microcomparator with a 50X objective. Equilibrium was assumed when no fringe change occurred over a several-hour period. These data were then analyzed by a computer program written by Dr. Robert Dyson which gave number-, weight-, and z-average molecular weights at any point in the solution column, using a quadratic fit of two to four points on either side of the central point to obtain these averages. The subunits of the hemocyanin were studied by similar high-speed equilibrium experiments in 6 M Gdn·HCl.[2] Densities of these solutions were calculated from the data of Kawahara and Tanford (1966). For these experiments conventional double-sector cells were used.

Partial Specific Volumes. The apparent specific volumes (ϕ_v) of the 17S and 39S forms of hemocyanin C were determined in 0.1 *I* Tris-HCl (pH 7.65) and 0.1 *I* Tris-HCl–0.05 M $MgCl_2$ (pH 7.65), respectively. We used the relation

$$\phi = \frac{1}{\rho_0}\left(1 - \frac{\rho - \rho_0}{c}\right) \qquad (1)$$

where ρ is the density of the solution, ρ_0 is the density of the dialysate, and c is the concentration of the solution in grams per milliliter. The densities of solutions and dialysates were determined at 25.000 ± 0.002° with 25-ml calibrated pycnometers. All weighings to determine the densities were obtained on a Mettler H-16 balance to a precision of 50 μg. The 17S hemocyanin solutions were obtained by dialysis of purified 39S hemocyanin C against the proper buffer for 24 hr. The solutions of 39S hemocyanin were obtained by dialysis of 17S material against the proper buffer for 24 hr. The samples were analyzed by means of sedimentation velocity experiments. The 17S hemocyanin solution was found to be homogeneous, while the 39S solution was found to have 10% 17S present. The concentrations of the solutions were determined spectrophotometrically with a Cary 15, using the extinction coefficient of 14.0 for the 39S particles, and 13.3 for the 17S particles as previously described.

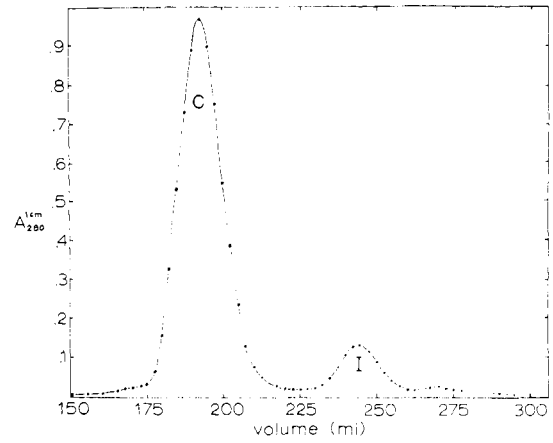

FIGURE 1: Gel filtration chromatography of *Callianassa* hemolymph on Bio-Gel A5m in Tris buffer (pH 7.6) in the presence of 0.1 M Mg^{2+}. The components C and I are indicated.

Four experiments with the 17S component gave values ranging from 0.720 to 0.730 ml per g, with an average of 0.724. There was no systematic variation of ϕ_v with concentration. Two experiments with the 39S component gave values of 0.726 and 0.722 ml per g. Accordingly, we adopt the value $\bar{v} = 0.724$ ml/g for both components at 25°.

Sodium Dodecyl Sulfate Gel Electrophoresis. These experiments were carried out in 19 cm × 7 cm × 0.35 cm slabs of 8% acrylamide gel, containing 0.2% bisacrylamide, 0.06% Temed, 0.05% ammonium persulfate, 0.2% sodium dodecyl sulfate, and 0.001 M EDTA. The tank buffer contained 0.03 M Tris-acetate, 0.2%, sodium dodecyl sulfate, and 0.001 M EDTA. Proteins were dissolved in 0.03 M Tris-sulfate, 40% sucrose, 1% sodium dodecyl sulfate, and 1% mercaptoethanol, and heated 10 min at 65°. All solutions were at pH 8.0. Electrophoresis was for about 6 hr, at 150 mA, and a voltage varying from 50 to 80 V. The apparatus was cooled with water at 12°. Methylene Blue was used as a tracking dye, and the gels were stained with Coomassie Blue, by the technique of Fairbanks *et al.* (1971). Bovine serum albumin, ovalbumin, chymotrypsinogen A, and myoglobin were used as standards. Sample loadings were generally in the range of 5–20 μg/1 cm wide channel.

Copper Analysis. The concentration of copper in purified *Callianassa* hemocyanin was determined by atomic absorption analysis. Standards were prepared in identical Tris buffers to those used for the solution. Some standards included added protein (bovine serum albumin) at concentrations comparable to those of the hemocyanin. Such addition did not affect the results.

Results

Hemocyanin Components in the Hemolymph; Separation and Properties. In whole and seawater-diluted *Callianassa* hemolymph three boundaries are seen in sedimentation velocity experiments. Two of these represent approximately 85 and

[1] At first glance, this result appears to conflict with our assertion (see Results) that the competent hemocyanin is almost entirely in the 39S form at 0.05 M Mg^{2+}. However, as we shall describe later, there is some dissociation at low concentrations (<2 mg/ml). Furthermore, the addition of solid Mg^{2+} to solutions may not be as effacious in producing reassociation as dialysis, the procedure used in our reversibility tests.

[2] Abbreviations used are: Gdn·HCl, guanidine hydrochloride; Temed, *N,N,N',N'*-tetramethylethylenediamine.

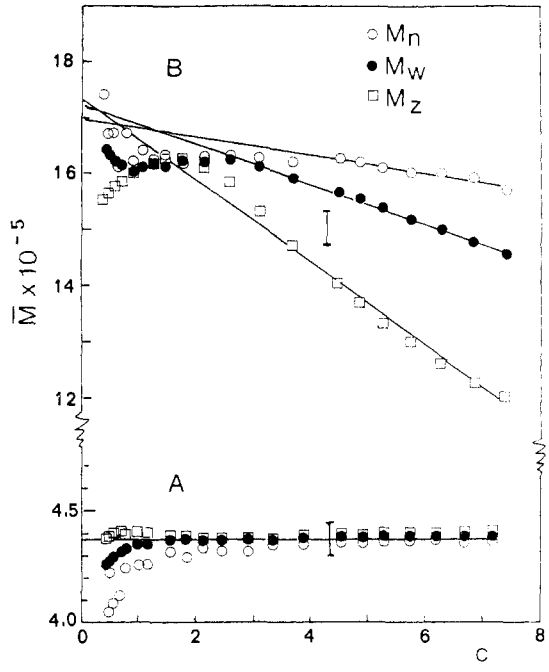

FIGURE 2: Plots of average molecular weights against protein concentration, expressed as fringe numbers, for hemocyanin C at pH 7.65. Conditions are: (A) 0.0 M Mg^{2+}, 1.0 mg/ml, 26.6°, 10,000 rpm; (B) 0.1 M Mg^{2+}, 0.7 mg/ml, 20.0°, 5,000 rpm. The vertical bar represents ±2% error.

15% of the total material, and have sedimentation coefficients (at a concentration of about 3 mg/ml) of about 35 and 16 S, respectively.[3] These are evidently both hemocyanins, since both display the typical absorption spectrum, and both are capable of reversible oxygenation. The third, a 6S component present in very small amounts, is detectable with schlieren optics at high total concentration. It does not show the absorption bands characteristic of hemocyanin. We have not further investigated this component, nor the red pigment (see Materials and Methods) which may or may not be associated with it.

The two hemocyanin components are evidently not in equilibrium in the hemolymph or in seawater-diluted hemolymph, for their proportions do not vary appreciably upon 20-fold dilution with filtered seawater. A slight increase in the proportion of 17S component is seen at 100-fold dilution, corresponding to concentrations of 1 mg/ml or less. This is consistent with results seen in sedimentation equilibrium (see below). Initial attempts to remove the 6S material by gel filtration in Tris buffer containing no added Mg^{2+} were successful; but the hemocyanin emerged from the A5m column entirely in the 17S form. Addition of Mg^{2+} to 0.05 M restored the proportions of 39S and 17S components originally observed in the serum; further addition of Mg^{2+} had no apparent effect. We have found that the hemolymph contains about 0.05 M Mg^{2+} and Thompson and Pritchard (1969) report the presence of about 0.01 M Ca^{2+}.

The 39S and 17S components presumably represent different association states of the hemocyanin. The combined observations that the 17S material eluted from the Mg^{2+}-free A5m column associates readily, but to a limited extent, upon addition of Mg^{2+} and that the 39 S which is formed does not dissociate readily upon dilution of the protein at Mg^{2+} levels of 0.05 M or greater suggest that the mixture present in the serum does not represent an equilibrium system. Evidently, two types of molecule are present which can be distinguished on the basis of their ability (or lack of ability) to associate to the 39S form. Gel filtration experiments under associating conditions (0.05–0.1 M Mg^{2+}) support this conclusion. Gel permeation chromatograghy of serum in the presence of 0.1 M Mg^{2+}, illustrated in Figure 1, resolves two major peaks in respective proportions of 85 and 15%. Sedimentation analysis shows the first band to elute to be pure 39S component, the second to be pure 17 S. No reequilibration is observable within a period of many days. Removal of Mg^{2+} from the fractionated samples by dialysis, furthermore, leads to dissociation of the 39S component into 17S particles. Readdition of Mg^{2+} to this fraction to greater than 0.05 M by dialysis causes *quantitative* reassociation to the 39S form. We designate as *hemocyanin C* this component, which is competent to associate from the 17S to the 39S form. On the other hand, the 17S material obtained from the second peak in Figure 1 is entirely insensitive to changes in Mg^{2+} concentration. We shall henceforth refer to this as the "incompetent" 17S component, hemocyanin I.

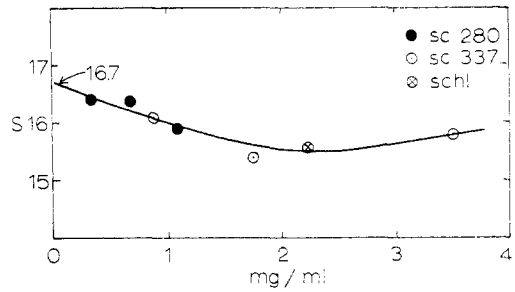

FIGURE 3: A graph of $s_{20,w}$ *vs.* concentrations for *Callianassa* hemocyanin at pH 7.65, 0.0 M Mg^{2+}. The open and filled circles represent scanner experiments using 337 and 280 nm light, respectively. The circle with a cross represents a schlieren experiment.

Almost all of the studies described herein (unless otherwise indicated) were carried out with hemocyanin C purified by gel filtration of serum in the presence of 0.05 or 0.1 M Mg^{2+}.

Characterization of the 39S and 17S Forms of Hemocyanin C. The ratio between the sedimentation coefficients of the aggregated and dissociated species, with the assumption that the protein is roughly globular, suggests that they bear a monomer–tetramer relationship to one another. Molecular weight measurements made on hemocyanin C in the presence and absence of Mg^{2+} confirm this relationship.

A number of sedimentation equilibrium experiments with the 17S material at pH 7.6 in the absence of Mg^{2+} gave log c *vs.* r^2 graphs which were linear to a close approximation. Calculating weight average molecular weights at points along their curves, one finds that these values are almost independent of concentration (see Figure 2A). The same result was found at four different initial loading concentrations. Values of M_w, extrapolated to $c = 0$, ranged from 4.18×10^5 to 4.37×10^5 (four experiments), with an average of 4.31×10^5. There was no systematic dependence of M_w on loading concentration between 0.4 and 1.0 mg per ml. No tendency to associate is observed under the conditions of these experiments.

The studies with the 39S form of hemocyanin C presented greater difficulties, for reasons that are not entirely clear. As the example shown in Figure 2B indicates, most of the experiments indicated a significant positive virial coefficient. In

[3] As we shall show below, the $s_{20,w}$ values, when extrapolated to zero concentration, yield values of about 39 and 17 S for these two components. Therefore, we shall refer to these two states as the "17S form" and "39S form," even though they may exhibit somewhat smaller $s_{20,w}$ values at finite concentration in the hemolymph or in buffer.

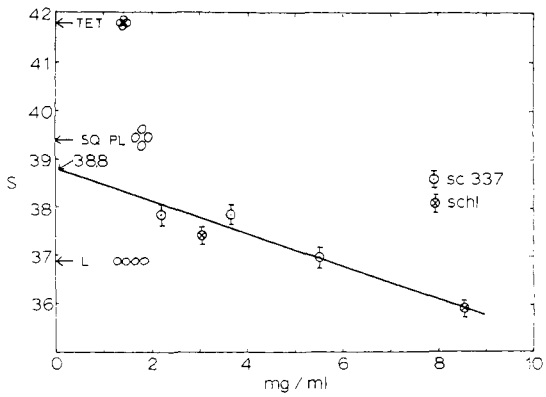

FIGURE 4: A graph of $s_{20,w}$ *vs.* concentration for *Callianassa* hemocyanin in 0.05 M Mg^{2+}. Symbols are as in Figure 3. The arrows near the ordinate labeled L, SP, and T are the predicted values for $s^0_{20,w}$ for linear, square-planar, and tetrahedral arrays of subunits, respectively.

addition, however, the data gave evidence for partial dissociation at concentrations less than about two to three fringes (0.5–0.75 mg/ml) and in a two experiments there was evidence for further association, exhibited by an upturn in the M^a *vs.* c data near the bottom of the solution column. However, the results, obtained by extrapolating the linear region of the M^a *vs.* c curve to $c = 0$, were quite reproducible. Four experiments, at loading concentrations ranging from 0.4 to 1.0 mg per ml gave values for M_w from 1.70×10^6 to 1.72×10^6. This demonstrates quite conclusively that the 39S particles are tetramers of 17S particles, for the average result for the former (about 1.71×10^6) is very nearly equal to four times the 17S value, that is, 1.73×10^6.

Further information is obtained concerning the relationship between the 17S and 39S forms of hemocyanin C extrapolating the s values to zero concentration, as shown in Figures 3 and 4. So long as concentrations greater than 2 mg/ml are used in 0.05 M Mg^{2+} (pH 7.6), no evidence for dissociation of the 39S particles is seen, and a good limiting value can be obtained. While 17S particles (in 0.0 M Mg^{2+}, pH 7.6) show some evidence of association at high concentrations, it is easy to obtain $s^0_{20,w}$ by extrapolation of the low concentration data. Given the value of the sedimentation coefficient of the 17S particles, it is possible to estimate the sedimentation coefficient of a tetramer, using the Kirkwood approximation for hydrodynamic interactions (Kirkwood, 1954; Bloomfield *et al.*, 1967; Van Holde, 1974). One obtains for s^0_4, the sedimentation coefficient of a tetramer of monomers with sedimentation coefficients s^0_1

$$s^0_4/s^0_1 = 1 + \frac{1}{4}\sum_i\sum_{\substack{j \\ i\neq j}}(1/\alpha_{ij}) \qquad (2)$$

where $\alpha_{ij} = R_{ij}/R$, R_{ij} being the center-to-center distances between subunits of radius R. On this basis, values can be predicted for tetramers that have linear, square-planar, and tetrahedral arrangements of their subunits. As can be seen in Figure 4, the results obtained for $s^0_{20,w}$ for the tetramer are consistant with a rather compact tetrameric structure, and would appear to exclude a linear arrangement.

Analysis of the 17S–39S Equilibrium. Having established that the forms of hemocyanin C that are stable in 0 M Mg^{2+} and 0.05 M (or greater) Mg^{2+}, respectively, bear a monomer–tetramer relationship, we next ask what factors determine the mixture of species present.

It is clear that the tetramerization is reversible with respect

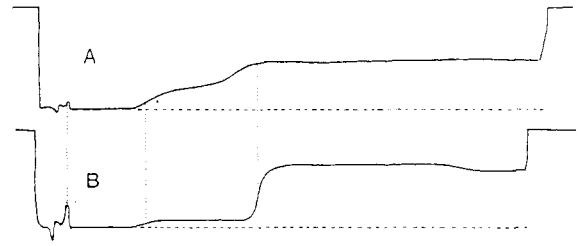

FIGURE 5: (A) A scanner trace recorded during sedimentation of an equilibrating mixture of 17S and 39S forms of *Callianassa* hemocyanin C in 0.02 M Mg^{2+} at 40,000 rpm. (B) A comparable scan (at 0.1 M Mg^{2+}) for a nonequilibrating mixture of hemocyanin C and hemocyanin I. This shows that the 17S and 39S boundaries are cleanly resolved in the absence of equilibration.

to Mg^{2+}, at least near neutral pH. At Mg^{2+} concentrations intermediate between 0.0 and 0.05 M, broad, partially resolved boundaries are found (see Figure 5). The shapes of these boundaries are generally representative of what might be expected for a rapidly equilibrating monomer–tetramer system. However, we cannot assert from this data that the reaction is *very* rapid, or that no intermediate species (such as dimer) are present. Such complications could give a wide variety of boundary shapes. We can only say that qualitatively, the boundary shape is consistant with a rapidly equilibrating monomer–tetramer system.

The system is clearly reversible. Figure 6 shows the weight average sedimentation coefficient (obtained from the second moment of the boundary curves) graphed *vs.* Mg^{2+}. It is evident that solutions prepared either by increasing or decreasing Mg^{2+} lie on the same curve.

The effect is not specific to Mg^{2+}. While monovalent cations, at comparable ionic strength, have no effect, Ca^{2+} seems to serve quite as effectively to shift the equilibrium to the tetramer form. Figure 7 shows a series of schlierien patterns obtained at different calcium ion concentrations; the behavior is qualitatively very similar to that in comparable concentrations of Mg^{2+}.

A further test of equilibration of the system can be obtained by observing the effect of dilution on the sedimentation velocity pattern. As Table I shows, solutions 0.04 M in Mg^{2+} show

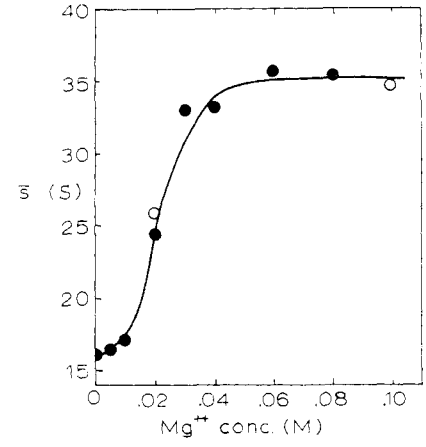

FIGURE 6: Dependence of weight-average sedimentation coefficient, $\bar{s}_{20}$, on Mg^{2+} concentrations at pH 7.6 and a protein concentration of 3.5 g/l. Solutions represented by filled circles were prepared by dialyzing purified hemocyanin C originally in 0.1 M Mg^{2+} against buffer containing lower Mg^{2+} concentrations. Open symbols represent solutions reversed from zero to 0.1 M Mg^{2+} and from 0.001 to 0.02 M Mg^{2+}. Corrected to 20°, but not for buffer viscosity or density.

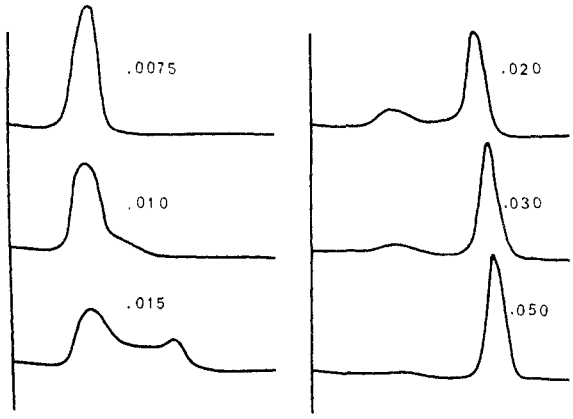

FIGURE 7: Tracings of schlieren patterns (each obtained at 40,000 rpm, about 13 min after reaching speed) for *Callianassa* hemocyanin C in solutions containing the indicated molarities of Ca^{2+}. The pH was 7.65, the concentrations approximately 4 mg/ml.

a progressive decrease in weight-average sedimentation coefficient as the concentration is decreased.

All of the previously described experiments were carried out at pH 7.65. The association of hemocyanins in general is pH dependent. Figure 8 shows the results of sedimentation velocity runs carried out over the pH range from 7 to 8, all at a protein concentration of 3.5 g/l. and a Mg^{2+} concentration of 0.02 M. The observed dependence on pH is unusual, but not unique for hemocyanins, in that higher pH (up to about pH 9.0), favors association. Like the Mg^{2+} dependence, the pH dependence is reversible.

Finally, we should point out that as Figure 9 shows, the weight-average sedimentation coefficient of the equilibrating mixture decreases somewhat with increasing rotor speed. If we assume the equilibrium to be a simple monomer–tetramer reaction, we can roughly estimate the volume change on tetramerization from the equation (Kegeles *et al.*, 1967)

$$K(P) = K(1 \text{ atm}) \exp\left[\frac{-\Delta v \rho (r^2 - a^2)\omega^2}{2RT}\right] \qquad (3)$$

Values of the equilibrium constant were estimated from the values of monomer and tetramer, and the weight-average sedimentation coefficients given in Figure 9, Assuming an average value of $(r^2 - a^2) = 2.5$ cm^2 during the period in which each of the runs was followed, the data yield a value of $\Delta v \cong 800$ ml/mol of 17S particles. This will correspond to an increase in $\bar{v}$ of about 0.002 ml/g upon formation of the tetramer. While the result is significant only as to order of magnitude, it shows that the error is within the uncertainty of our $\bar{v}$ measurements, and will not appreciably influence the molecular weight calculations given above.

Polypeptide Chains of Callianassa Hemocyanin. The results of our copper analysis show that the purified *Callianassa*

TABLE I: Dependence of s_{20} of Hemocyanin C on Protein Concentration at pH 7.65, 0.04 M Mg^{2+}.

Protein Concn (mg/ml)	$\bar{s}_{20,w}$ (S)[a]
3.5	33.2
0.5	31.0
0.2	26.5

[a] Weight-average sedimentation coefficients corrected to 20°.

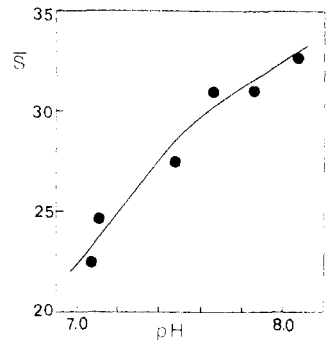

FIGURE 8: pH dependence of weight-average sedimentation rate, $\bar{s}_{20}$, of hemocyanin C. All solutions at a protein concentration of 3.5 mg/ml, Mg^{2+} concentration of 0.02 M. Solutions were prepared by dialysis of a stock to varying pH values at the same Mg concentration. Corrected to 20°, but not for buffer viscosity or density.

hemocyanin C contains 0.164 wt % of copper. This corresponds to a molecular weight of 38,700 per copper atom, or about 77,000 per pair, which constitute one oxygen binding site. This copper content is close to that reported for a number of other arthropod hemocyanins (see Van Holde and van Bruggen, 1971). Thus, one might expect the individual polypeptide chain weight to be either about 38,000 or 77,000. Values in the latter range have been reported for other arthropod hemocyanins (Di Gamberardino, 1967; Loehr and Mason, 1973; Carpenter and Van Holde, 1973).

We find that by raising the pH to 9.2 in the absence of magnesium, hemocyanin C may be dissociated into subunits with a sedimentation coefficient of approximately 5 S. This result is reminiscent of that obtained with *Cancer magister* hemocyanin, where such 5S subunits were shown to have a molecular weight of about 78,000 (Ellerton *et al.*, 1970). The *Callianassa* subunits appear to be less stable however, rapidly losing oxygen binding capacity and decreasing in sedimentation coefficient at pH values greater than 9.5.

In order to determine more unambiguously the polypeptide chain weight, we have utilized both sedimentation equilibrium in concentrated guanidine hydrochloride solutions, and sodium dodecyl sulfate gel electrophoresis. Sedimentation equilibrium experiments were carried out in 6 M Gdn·HCl–0.1 M mercaptoethanol. A significant degree of nonideality was indicated by a downward concavity in the log c *vs.* r^2 plots so the results are presented as plots of $1/M_n^a$, $1/M_w^a$ and $1/M_z^a$, against concentration (Figure 10). These plots are linear and extrapolate to a molecular weight of 74,000 with the assumption that the apparent partial specific volume is 0.72. The slopes of the graphs are approximately in the ratio of 1:2:4. This is as expected for a homogeneous solute, where the values should be $B/2$, B, and $2B$, in graphs of $1/M_n^a$, $1/M_w^a$, and $1/M_z^a$, respectively, where B is the second virial coefficient. The value of B obtained is in the range expected for a flexible linear polymer in a good solvent (Tanford, 1961). The molecular weight found is close to the weight per oxygen binding site. Thus, the results demonstrate that the 17S form must contain six such subunits, as in the case of *Cancer magister*. The fact that effective specific volumes usually decrease somewhat upon transfer to Gdn·HCl solution (Hade and Tanford, 1967) suggests that the estimated subunit molecular weight might be revised downward by a few per cent. Thus, a best estimate for M would be about 72,000, which is exactly one-sixth of the weight of the 17S particle.

Polyacrylamide gel electrophoresis experiments were carried out in the presence of sodium dodecyl sulfate and mercaptoethanol (see Materials and Methods for details). Frac-

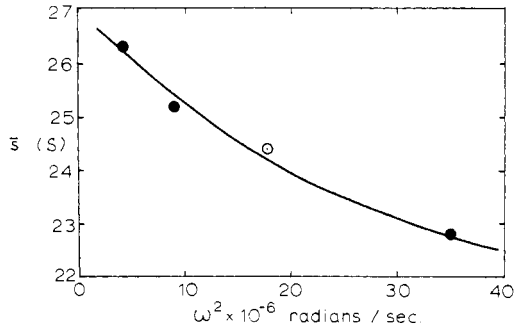

FIGURE 9: Dependence of $\bar{s}_{20}$ on rotor speed at pH 7.6, 0.02 M Mg^{2+} and 3.5 mg of protein/ml. The point represented by the open circle was taken from Figure 6. Corrected to 20°, but not for buffer viscosity or density.

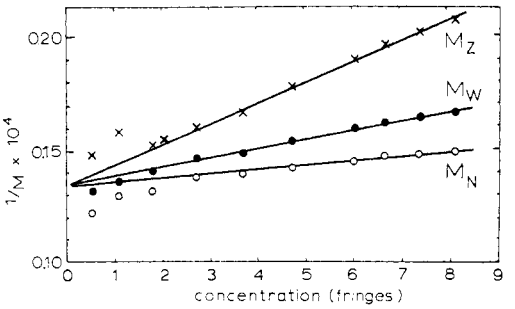

FIGURE 10: Plots of $1/M_n^a$ (○), $1/M_w^a$ (●), and $1/M_z^a$ (×) against concentration from a sedimentation equilibrium experiment with hemocyanin C in 6 M Gdn·HCl and 0.1 M mercaptoethanol. The intercepts of the straight lines yields a value of $M \cong 74{,}000$.

tions of hemocyanin C and hemocyanin I were examined in several experiments. The proteins used as standards gave a linear relation between log M and mobility. Hemocyanin C invariably gave a single band, with molecular weight corresponding to 70,000–75,000. No material was left at the origin, nor were faster bands seen even at high loading concentrations. On the other hand, hemocyanin I regularly gave, in addition to strongest band in the 70,000–75,000 dalton range, a series of faint faster bands. Bands corresponding to 60,000, 49,000, and 26,000 have been observed.

Discussion

Callianassa hemocyanin shares a number of features with other arthropod hemocyanins. The basic unit, as in most if not all of these proteins, is a hexamer of polypeptide chains, each of which has a molecular weight of about 75,000. However, in contrast to *Cancer* hemocyanin, these chains in *Callianassa* appear to be of only one size class. Whether or not there is heterogeneity or exact homogeneity in the polypeptide sequences remains to be answered.

Callianassa hemocyanin C appears to be unusual in that it does not exhibit, at least as a major species under conditions investigated so far, the 25S dimer of 17S particles so common in other arthropod hemocyanins (see Van Holde and van Bruggen, 1971). Rather, the dominant associated species is the tetramer. Monomer and tetramer appear to be in dynamic equilibrium. This equilibrium is influenced by the pH, by the presence of divalent cations, and as we shall show in the following paper (Miller and Van Holde, 1974), by the binding of oxygen. We should emphasize, however, that none of our results to date exclude the possibility of *some* dimer intermediate in the tetramerization. Sedimentation equilibrium studies to resolve this question are in progress.

Why, we may ask, does *Callianassa* hemocyanin prefer tetramer association? In a previous paper (Carpenter and Van Holde, 1973), it was argued that the limitation of *Cancer* hemocyanin to dimerization was possibly a consequence of the existence of two types of polypeptide chains; that the 16S particles might have distinguishable ends, and associate by isologous interactions on *one* of these ends. In this connection, it is important that we learn whether the *Callianassa* association is strictly monomer–tetramer, or whether appreciable quantities of dimer are involved. Matthews and Bernhardt (1973) argue that strict monomer-*n*-mer associations are to be expected only if the interactions are heterologous. Thus, *Callianassa* and *Cancer* hemocyanins may differ in that one has evolved a heterologous, the other an isologous mode of association. To fully understand this problem, however, we must learn whether or not the *Callianassa* association is *strictly* monomer–tetramer, and whether or not there is microheterogeneity in the *Callianassa* subunits.

To our point of view, the chief attraction in working with the *Callianassa* hemocyanin C lies in the reversibility of the association of 17S particles. However, this raises the interesting question as to why about 15% of the hemocyanin found in the hemolymph (hemocyanin I) is incompetent for such association. A clue may lie in the fact that this incompetent material exhibits a different behavior in sodium dodecyl sulfate gel electrophoresis; a part of it is present as smaller units than the homogeneous mol wt 74,000 chains found for hemocyanin C. It seems possible to us that hemocyanin I represents "old" hemocyanin, material in the first stages of proteolytic degradation. After all, such processes are known to occur, and we are here dealing with a "tissue" (the hemolymph) from which we can quantitatively recover total protein with only the mildest of separation methods. It seems quite reasonable that we should observe such materials. The observation of such fragments in one component of our hemocyanin suggests that gel electrophoresis studies of whole hemocyanins, aimed at determination of chain size, should be treated with great caution.

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Oxygen Binding by *Callianassa californiensis* Hemocyanin†

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ABSTRACT: The oxygen binding by the hemocyanin of the ghost shrimp, *Callianassa californiensis*, has been studied as a function of pH, divalent ion concentration, and temperature. Most of the experiments were performed with the hemocyanin C component, which is competent to undergo an association from a 17S form to a 39S tetramer near neutral pH. It is demonstrated that the effects of pH and divalent ions on oxygen binding can be described by the theory of Monod *et al.* (*J. Mol. Biol. 12*, 88 (1965)) for allosteric transitions as modified by Buc *et al.* (*J. Mol. Biol. 76*, 199 (1973)). The 17S hexamer of polypeptide chains appears to be the allosteric unit. The T and R states of this 17S particle exhibit differing tendencies to associate. The 17S particles of hemocyanin I, which are not capable of association, have a T state identical with that of hemocyanin C, but cannot adopt the same R state. Possible significance of this behavior to the physiology of the shrimp is discussed.

As has been described in the preceeding paper (Roxby *et al.*, 1974), the ghost shrimp *Callianassa californiensis* has a hemocyanin capable of a completely reversible monomer–tetramer association. There exist normally in the blood two components which can be completely separated on a A5m column. One sediments as a 17S component in the ultracentrifuge and the other as a 39S component.[1] The 39S component shows the reversible dissociation, breaking down into 17S subunits when Mg^{2+} is removed, and reassociating to 39S subunits when Mg^{2+} is replaced to a level of 0.05 M or greater. We have designated this hemocyanin C. The 17S component normally present in whole blood is incompetant to associate into 39S subunits regardless of the Mg^{2+} levels. This we call hemocyanin I. At high pH ($\geq$9.2) the hemocyanin dissociates further into 5S subunits, which apparently represent individual polypeptide chains. We have shown that the 17S component is a hexamer of these subunits. (Roxby *et al.*, 1974).

We wished to investigate oxygen binding by *Callianassa* hemocyanin for a number of reasons. The background respiratory physiology of this shrimp has been investigated (Thompson and Pritchard, 1969; K. Miller and Pritchard, in preparation) and in particular preliminary measurements were made of the oxygen binding of whole blood. It seemed logical in this case to attempt to duplicate the conditions of divalent ion composition, temperature, and pH normally found in whole blood under completely controlled conditions in an attempt to fully understand the effect that varying physiological states might have on the oxygen binding properties of the hemocyanin. The normal variations in physiological pH have been measured (K. Miller and Pritchard, in preparation). Several researchers report a pronounced effect of Ca^{2+} ions on oxygen binding in other hemocyanins (Hwang and Fung, 1970; Larimer and Riggs, 1964) and Mg^{2+} ions have been shown to have the same effect (Larimer and Riggs, 1964). The blood calcium levels in *Callianassa* have been measured by Thompson and Pritchard (1969) but it was necessary for us to measure the normal hemolymph Mg^{2+} levels before composing a buffer system for dilute samples.

Since with this hemocyanin one can obtain several different stable aggregation states, as well as some equilibrium mixtures of these, it makes an ideal system for the study of the interrelation between aggregation state and oxygen binding. In this paper we describe binding studies with both hemocyanin C and hemocyanin I, as a function of pH, divalent ion concentration, and temperature.

Experimental Section

Preparation of Solutions. Shrimp were dug at Yaquina Bay and bled, and the blood was purified on a Bio-Gel A5m column as described previously (Roxby *et al.*, 1974). The eluent for the column was 0.1 *I* Tris buffer (pH 7.65) with

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[1] The actual sedimentation coefficients at protein concentrations normally used with schlieren studies, and in the presence of 0.1 *I* Tris buffer, are about 16 and 35 S, respectively. However, since we have shown (Roxby *et al.*, 1974) that the $s^0_{20,w}$ values are 16.7 and 38.8 S, respectively, we shall refer to these as 17S and 39S components.