Physical Studies of Hemocyanins. V. Characterization and Subunit Structure of the Hemocyanin of *Cancer magister**

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ABSTRACT: The hemocyanin from the Dungeness crab *Cancer magister* has been studied by sedimentation techniques. At pH 7, sucrose gradient centrifugation yielded two separate components of the protein. The major component was found to have $s^0_{20,w}$ = 25.6 S and mol wt 940,000; the minor component was found to be a half-molecule with $s^0_{20,w}$ = 18.0 S and mol wt 480,000. On increasing pH, the amount of 25S particle diminished, so that at pH 8.8 the half-molecules were at a maximum. Another component also appeared at this pH, havine $s^0_{20,w}$ = 5.7 S. At pH values above 10, this 5S component is the sole species. While most of the material, at pH 10.6, has a molecular weight of about 75,000–80,000, there are some smaller particles present in all solutions. Furthermore, studies at pH 11 or in 6 M guanidine hydrochloride plus mercaptoethanol yield heterogeneous mixtures of lower molecular weight. In the presence of 0.01 M Mg^{2+}, the 25S component is stable to pH values above 10. Reassociation of the 5S component upon bringing the pH back to neutrality proceeds quantitatively to the half-molecule stage in the absence of Mg^{2+}. In solutions containing 0.01 M Mg^{2+}, a small amount of 25S component is also formed, but complete recovery of the 25S component has not been achieved.

Previous papers in this series have dealt with the structure and function of the hemocyanin from a mollusc, the squid *Loligo pealei* (Van Holde and Cohen, 1964; Cohen and Van Holde, 1964; Van Holde, 1967; DePhillips *et al.*, 1969). Some arthropods also utilize hemocyanins as oxygen carriers, and these proteins differ from their molluscan counterparts in a number of interesting ways. For example, while the molecular weight per oxygen binding site is about 50,000 daltons for the molluscan hemocyanins, the value is roughly 75,000 in the crustacean group (Ghiretti, 1962). Further, sedimentation analysis (Eriksson-Quensel and Svedberg, 1936) and electron microscopy (Levin, 1963a–c; van Bruggen *et al.*, 1963; Fernandez-Moran *et al.*, 1966) indicate that the pattern of subunit aggregation is entirely different in the two groups of proteins. The relationship between molluscan and crustacean hemocyanins is thus obscure. For this reason, we have begun a series of detailed physical studies of the hemocyanin from a typical crustacean, the Dungeness crab, *Cancer magister*.

A number of crustacean hemocyanins were investigated in the pioneering ultracentrifuge studies by Svedberg and coworkers (see, for example, Eriksson-Quensel and Svedberg, 1936). In most cases, three components were observed in the diluted hemolymph under various conditions of pH. These components usually had sedimentation coefficients of about 25, 16, and 5 S. The latter is of particular interest, for it is of the right order of magnitude to correspond to a hypothetical "protomer" bearing one O_2 binding site.

The 25S component of the *C. magister* hemocyanin has been investigated by Thomson *et al.* (1959) and shown to have a molecular weight (by sedimentation and diffusion) of about 950,000.

It was our purpose in this initial study to examine the various states of aggregation of the *C. magister* hemocyanin with respect to molecular weight, homogeneity, and conditions for stability.

Experimental Section

Preparation of the Hemocyanin. Live Dungeness crabs, obtained from the Pacific coast, were bled by puncture at a leg joint. About 25–40 ml of blood was collected from each animal; this contained approximately 25 mg/ml of hemocyanin. The blood was allowed to clot, decanted, and then centrifuged for 10 min at 10,000 rpm at 4° to remove remaining particulate matter. After filtration through a 0.8 μ Millipore filter, it was centrifuged for 8 hr at 30,000 rpm at 4° in a Beckman Model L preparative ultracentrifuge, using a no. 30 rotor. The clear supernatant was discarded, and the blue hemocyanin pellet was dissolved in 0.1 ionic strength Tris buffer at pH 7.0. Velocity sedimentation in the ultracentrifuge showed that the hemocyanin thus obtained contained two peaks in the schlieren pattern, a major component with $s_{20,w} \simeq 25$ S, and a minor component with $s_{20,w} \simeq 16$ S, constituting about 80 and 20%, respectively, of the total protein present, as shown in Figure 1. The hemocyanin was recentrifuged for 2 hr at 50,000 rpm in a no. 50 rotor, the supernatant was removed and stored, and the protein pellet was redissolved in Tris buffer at pH 7.0. The centrifugation at 50,000 rpm was repeated four times, the hemocyanin thus obtained containing 92% or more of 25S particles. The supernatants from the successive centrifugations were combined and recentrifuged for 8 hr at 30,000 rpm; the hemocyanin pellet so obtained contained 55% of the 25S component and about 45% of the 16S component. This sample was used as a source of 16S material.

For most of the experiments described here, the starting

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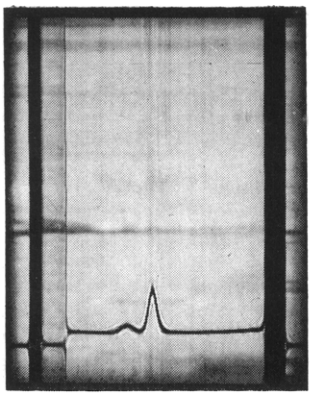

FIGURE 1: Sedimentation diagram of *C. magister* hemocyanin. The blood had been filtered through a 0.8 μ Millipore filter, centrifuged for 8 hr at 30,000 rpm at 4°. The blue pellet was redissolved in 0.1 ionic strength Tris buffer, pH 7.0. Sedimented for 32 min at 40,133 rpm.

material was a solution containing 92% or more of 25S particle. However, for accurate sedimentation equilibrium molecular weight studies of 25S and 16S particles it was desirable to have more homogeneous samples. Separation of the components was effected by sucrose gradient sedimentation. Gradients were prepared by mixing 2.55 ml of 10% and 2.25 ml of 30% sucrose solutions containing Tris buffer at pH 7.0 in a gradient-forming device (Gerhart, 1967). Then 0.2 ml of a solution containing ~25 mg/ml of the protein in Tris buffer at pH 7.0 was carefully added to the top of the gradient with a syringe. Three such samples prepared in this manner were then centrifuged for 5.75 hr at 50,000 rpm and 4° using a Spinco SW50 rotor. Each tube was then punctured at the bottom, and about 24 fractions were collected in each case.

For studies with the 25S particle, the preparation described above containing 92% 25S was used as a starting material in the sucrose gradient separation; for studies of the 16S subunit, the sample containing 45% 16S was used. The separation profiles for the two samples are shown in Figure 2. Fractions from the shaded areas were combined in each case, and the combined samples were dialyzed for at least 3 days against Tris buffer at pH 7.0 to remove the sucrose.

Preparation of Solutions. Buffers were prepared according to standard recipes (Long, 1961) using analytical grade reagents. The following pH ranges were covered: pH 5.2–5.8, sodium acetate–acetic acid; pH 6.0–6.9, potassium dihydrogen phosphate–disodium hydrogen phosphate; pH 7.0–9.0, Tris-HCl; pH 9.1–11.0, sodium carbonate–sodium bicarbonate. The buffers used were all 0.1 ionic strength, except for experiments involving magnesium, where 0.01 M $MgCl_2$ was also added to the buffer. In experiments using 6 M Gd·HCl[1] solutions, Eastman grade chemicals were used; solutions were filtered before use.

Concentrations of hemocyanin solutions were determined from dry weights of samples. Aliquots of 5–10 ml of both protein and dialysate were lyophilized to near dryness under vacuum at 0°, followed by heating at 100–110° to constant weight. Dilution of the same dialyzed protein sample in

[1] Abbreviation used is: Gd·HCl, guanidine hydrochloride.

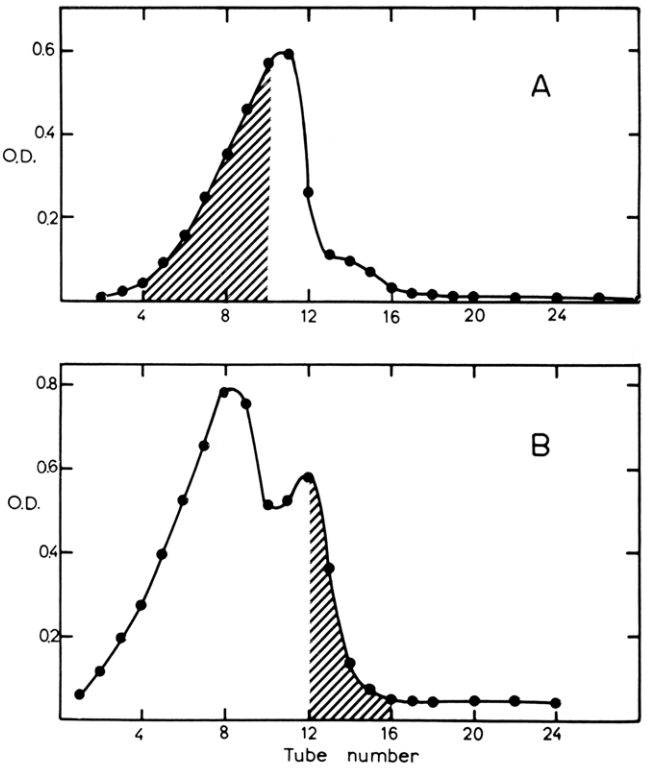

FIGURE 2: Separation of 25S and 16S hemocyanin by sucrose gradient centrifugation. In part A, the hemocyanin sample contained initially about 92% 25S particle; the contents of tubes in the shaded area were combined for molecular weight determinations on the 25S unit. In part B, the starting material contained approximately 45% 16S subunit; contents of tubes in the shaded area were combined for molecular weight studies on the 16S particle.

Tris buffer at pH 7.0 for optical density measurements yielded an $\epsilon_{1\text{ cm}}^{1\%}$ value of 14.7 at λ 279 nm for the 25S particle; in bicarbonate buffer at pH 10, an $\epsilon_{1\text{ cm}}^{1\%}$ value of 14.1 was determined for the 5S particle.

Sedimentation Velocity. These experiments were conducted with a Spinco Model E analytical ultracentrifuge, equipped with phase-plate schlieren optics. The temperature was kept at 20° with the RTIC unit. Single-sector centerpieces (4°), made of either aluminum or filled epoxy, were used. For experiments in the concentration range 1–7 mg/ml, 12-mm centerpieces were used in an AN-D rotor, and in the range of 0.25–1-mg/ml 30-mm centerpieces were used in an AN-E rotor. Speeds used were 40,000 rpm for the 25S particle, and 48,000 or 56,000 rpm for the 16S and 5S subunits. The photographic plates (Kodak Metallographic) were measured with a Nikon two-way comparator. A computer program was used to calculate the sedimentation coefficients from a least-squares analysis; values were corrected to water at 20°. Relative viscosities of the buffers used were measured in a Ubbelohde viscometer at 25.00°.

Sedimentation Equilibrium. Experiments reported here were made by the high-speed technique (Yphantis, 1964). A 12-mm six-channel Kel-F centerpiece was used, with sapphire windows. Three solutions of different concentration of the same subunit were used in three channels; the other three contained dialysate buffer as reference. Initial protein concentrations used were generally in the range 0.3–0.9 mg/ml,

TABLE I: Densities, Partial Specific Volumes, and Viscosities.

Species	Solvent	Protein Concn (mg/ml)	Soln Density, (g/cc)	ϕ_v (ml/g)	η_{sp}/c (ml/g)
25 S	Tris, pH 7.0, ρ = 1.00248	6.5	1.00423	0.729	4.88
		13.0	1.00599	0.728	4.73
		26.0	1.00951	0.728	5.13
5 S	Bicarbonate, pH 10.0, ρ = 1.00129	5.9	1.00286	0.733	7.39
		11.8	1.00445	0.731	7.71
		23.6	1.00766	0.729	8.37
Gd·HCl subunits[a]	6 M Gd·HCl + 0.1 M β-mercaptoethanol + 0.02 *I* Tris, ρ = 1.14338	2.1	1.14384	0.681	50.0
		4.2	1.14420	0.703	54.5
		8.3	1.14505	0.699	58.8
		16.6	1.14669	0.700	69.7
		33.2	1.15005	0.699	96.0

[a] Concentrations used for computation of ϕ_v in Gd·HCl were estimated from optical density measurements. From the linear plot of ρ *vs.* c, $1 - (\partial\rho/\partial c) = 0.799$.

with the most concentrated solution in the innermost channel. Speeds used (with the AN-D rotor) were 8000 rpm for the 25S particle, 11,000 rpm for the 16S; for the 5S subunit studies, 30,000 rpm was used at pH 10.1 and 10.6, 32,000 rpm at pH 11.0, and 40,000 rpm in Gd·HCl. The time to reach equilibrium was reduced by overspeeding and underspeeding.

The concentration distribution at equilibrium was measured by Rayleigh optics. Interference photographs were taken (using Kodak IIG plates) as soon as possible after the machine had reached the desired speed, and then again later when the equilibrium had been reached. A "solvent–solvent blank" run was also made after the equilibrium run had been completed, using identical speed and alignment of the cell components, in order to check base-line corrections. White-light photographs were taken to help identify the monochromatic interference fringes. The fringes were measured on the Nikon comparator at 0.05-mm intervals, starting near the meniscus and working toward the bottom of the cell, until the fringes became indistinguishable. Displacements near the meniscus of less than 100 μ were not considered.

The base-line corrected data was analyzed using a CDC 3300 computer, utilizing a program written by Dr. R. D. Dyson and Mr. M. Johnson of this Department. The program enables us to obtain molecular weight from a point-by-point least-squares quadratic fit of ln c *vs.* r^2 (logarithm of concentration against square of radial position in cell), and it also calculated M_w, M_n, and M_Z at each value of r. The number of points used to fit the data at a given level in the cell could be varied. Usually about four to seven points on either side of the point in question were used, except near the ends of the column.

Partial specific volumes were calculated from density measurements of the protein solutions; single stem pycnometers, each of which had a volume of approximately 30 ml, were used. The pycnometers were cleaned before use with chromic acid, rinsed thoroughly, and then calibrated with glass-distilled water. The density measurements were made at 25.00 ± 0.01°, and weights were corrected for the buoyancy of air using a sealed glass tare. Solutions to be measured were dialyzed for 3 or more days, with several changes of dialysate. A sample of the final dialysate was used to determine the solvent density, and the apparent specific volumes were calculated in the usual manner (Creeth and Pain, 1967). The results are summarized in Table I.

Viscosity Measurements. A Ubbelohde viscometer, with a flow time of 246 sec for glass-distilled water at 25°, was used. The viscometer was cleaned with chromic acid and rinsed thoroughly with glass-distilled water before use. All solutions were filtered through sintered glass before flow times were measured at 25.00 ± 0.01°. Values for η_{sp}/c are also listed in Table I.

Results

pH Dependence of the Hemocyanin Structure. Since it was found that the conditions for dissociation of *C. magister* hemocyanin depend upon whether or not magnesium ion is present in the solutions, we shall consider these results separately.

Results obtained in solutions to which no Mg^{2+} has been added are illustrated in Figures 3 and 4A. At pH 7, the hemocyanin shows a main peak in the schlieren pattern with a sedimentation coefficient of approximately 25 S, and a smaller component with a sedimentation coefficient of about 16 S, as in Figure 3A. On increasing the pH from near neutrality, the behavior illustrated in Figure 4A is observed. The 25S particle remains the principal sedimenting component until pH 8.6; thereafter, the proportion of this component diminishes, so that at pH 8.8 the 16S particle becomes a major component, as shown in Figure 3B. At pH 8, a third component appears, with a sedimentation coefficient of approximately 5 S. At pH 9.0 the 25S particle disappears,

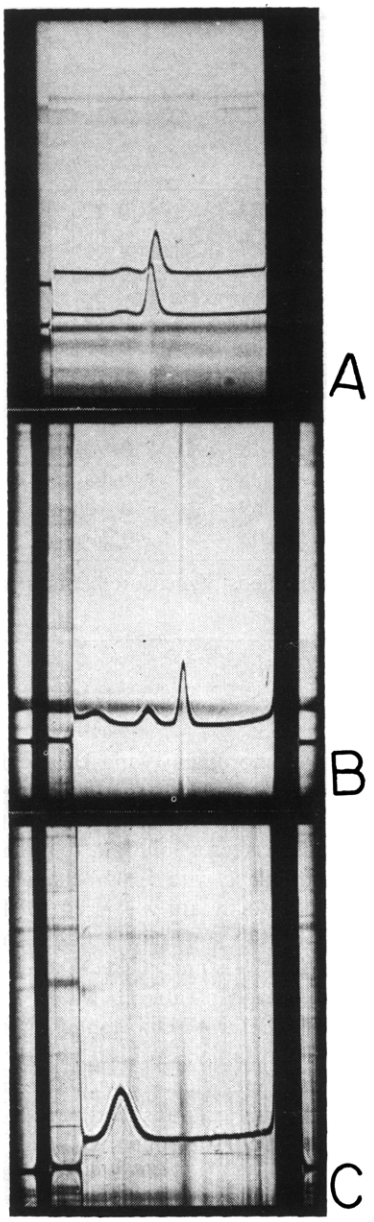

FIGURE 3: Sedimentation diagrams of *C. magister* hemocyanin under various conditions. (A) Blood samples in both regular and wedge cells had been centrifuged five times for 2 hr at 50,000 rpm in Tris buffer, pH 7.0. Sedimented for 32 min at 40,108 rpm. (B) Blood dialyzed against Tris buffer, pH 8.8, for 3 days. Sedimentation has proceeded for 32 min at 48,764 rpm. (C) Blood dialyzed against bicarbonate buffer at pH 10.0 for 3 days. Centrifuged for 24 min at 48,352 rpm.

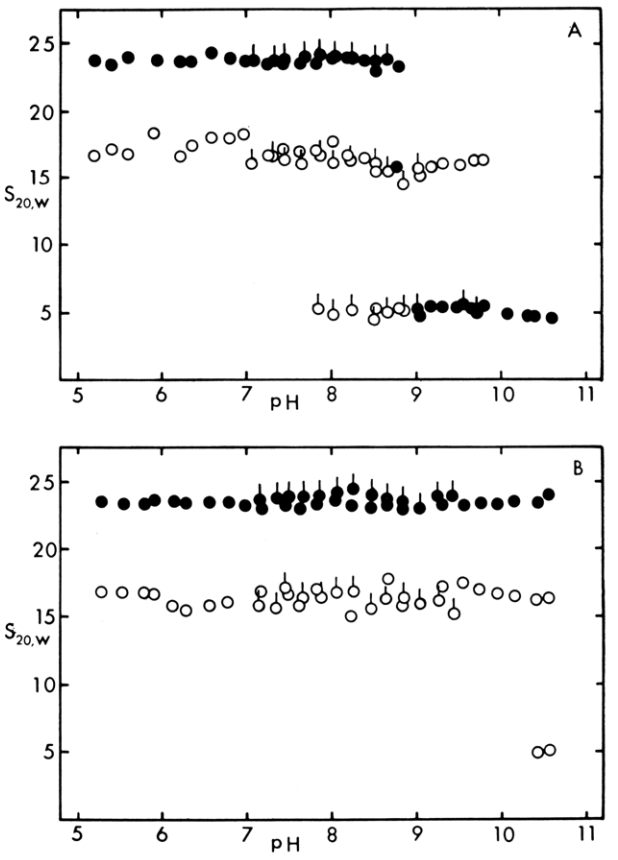

FIGURE 4: Diagrams of sedimentation coefficient *vs.* pH in the absence (A) and presence (B) of 0.01 M $MgCl_2$ in the buffer. In each case the solid circles represent the sedimenting boundary present in the greatest amount. Total protein concentration at each point was about 3.5 mg/ml. Experiments without 0.01 M $MgCl_2$ were run at 40,000 rpm in the pH range 5.2–8.8, and 48,000 rpm at pH greater than 8.8. Experiments with 0.01 M $MgCl_2$ were run at 48,000 rpm. The points with "flags" correspond to solutions prepared by simply diluting the stock solution into buffer at the appropriate pH at room temperature. All other solutions were prepared by dialysis of diluted stock to the appropriate pH.

leaving 16S and 5S particles in approximately equal proportions. On further increase in pH, the proportion of 16S unit decreases, so that above pH 9.8 only the broad 5S component is present. Between pH 10.2 and 11, the sedimentation coefficient gradually decreases, and above pH 10.6 the characteristic blue color of the hemocyanin solution disappears, indicating that oxygen binding capacity has been lost, or greatly diminished. The experiments illustrated in Figure 4A were made at a protein concentration of approximately 3.5 mg/ml; in most cases the solutions were dialyzed to the required pH for 3 days, using a stock solution initially at pH 7. Some experiments were performed using solutions prepared by direct dilution of stock solution into the appropriate buffer; as Figure 4 shows, no significant difference was found in results.

Figure 4B shows the behavior of the protein as a function of pH in the presence of 0.01 M $MgCl_2$. Even at pH 10.6, the 25S material is still the major component, and the schlieren patterns closely resemble the photographs obtained at pH 7 or 8 in the absence of magnesium (*cf.* Figure 3A). It is evident that the presence of Mg^{2+} retards the dissociation of the 25S component. This observation is similar to the results obtained by Van Holde and Cohen (1964) who found that the dissociation of the larger component of *Loligo pealei* hemocyanin was prevented up to pH 10.0 by the presence of magnesium.

Reversibility of the Dissociation. In order to test whether the dissociation of the hemocyanin at higher pH was reversible, several solutions were dialyzed from pH 7 to various pH values between 9 and 11, and then redialyzed back to pH 7.

Velocity sedimentation experiments showed that, in the absence of magnesium, reassociation is possible only as far

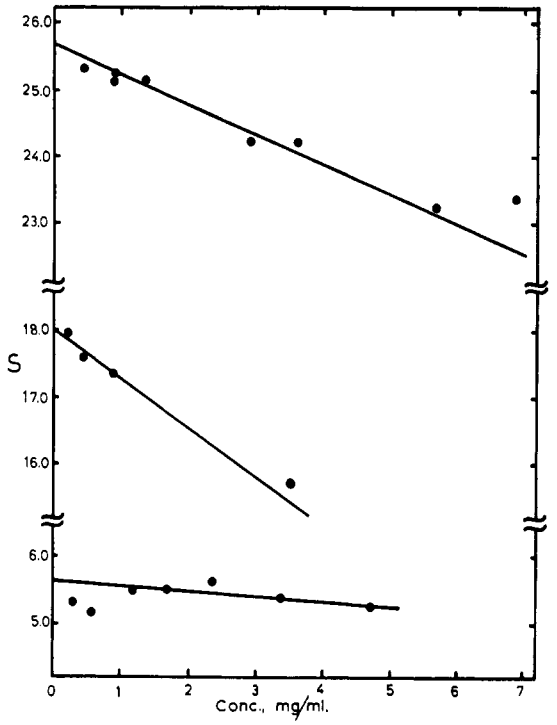

FIGURE 5: Sedimentation coefficient *vs.* concentration for 25S, 16S, and 5S components.

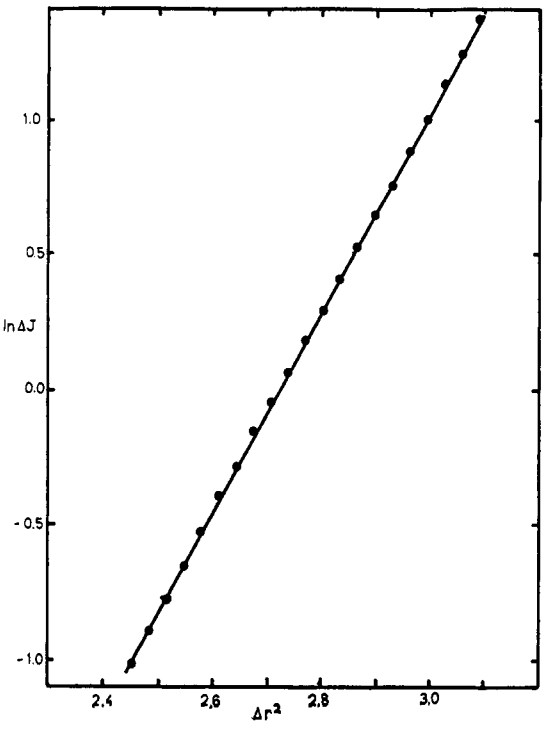

FIGURE 6: Logarithmic plots of the fringe displacements as a function of radial position in the cell, equilibrium experiment with a 25S particle. Centrifuged for 18 hr with overspeeding and underspeeding; speed at equilibrium was 8122 rpm.

as the 16S component. Furthermore, the protein obtained upon reconstitution at pH 7 in the absence of magnesium was almost entirely 16S subunits, with very little of the 5S component remaining. Although all reconstitution experiments were performed with protein concentrations of approximately 3.5 mg/ml, more variation than usual was observed in the measured sedimentation coefficients from one experiment to the next. However, the average value was 15.7, compared with approximately 15.5 S obtained from Figure 5.

In similar experiments in the presence of 0.01 M Mg^{2+}, it is possible to obtain some (up to about 10%) of the 25S component as well. However, in all experiments conducted to date the 16S component has been the major product of reassociation. The use of 0.01 M Ca^{2+} in a few experiments gave the same result. On the other hand, addition of an equal ionic strength of KCl was not effective.

In one experiment, a hemocyanin solution in Tris buffer (pH 6.97, no Mg^{2+}) was titrated to pH 9.49. Dissociation into 5S subunits was observed. Back-titration to pH 6.92 led to reassociation to 16S material. Subsequent addition of $MgCl_2$ to 0.01 M led to the formation of a small amount of 25S component.

Finally, we have observed that nearly complete reassociation of 5S to 16S component is produced by dialyzing magnesium-free subunits at pH 10.1 against pH 10.1 buffer containing 0.01 M Mg^{2+}. Only a trace of 25S hemocyanin is formed.

Characterization of the Components. It has been shown above that *C. magister* hemocyanin exists under different conditions in three distinct states of aggregation with sedimentation coefficients of approximately 25, 16, and 5 S. The sedimentation behavior of these three species as a function of concentration is shown in Figure 5. Extrapolation to infinite dilution yields values of 25.6, 18.0, and 5.7 S, respectively, for $s^0_{20,w}$. We shall continue to refer to the species as the 25S, 16S, and 5S components, using the approximate values of sedimentation coefficients in the concentration range around 3.5 mg/ml.

In order to understand the structural relationships between such a series of components, molecular weight values are required. The sedimentation equilibrium technique was used. Values of the partial specific volume, $\bar{v}$, were taken from the data in Table I. Since for the 25S and 5S components the apparent partial specific volume, ϕ_v, did not show significant variation with concentration, the average of each set of values was taken as $\bar{v}$ for the corresponding components. Purified 16S material was not available in sufficient quantity to allow ϕ_v determination. However, the values of $\bar{v}$ for 25S and 5S hemocyanin are very nearly the same, so that the value for the 16S intermediate would not be expected to differ greatly. We have chosen to use the value for 25S hemocyanin for the 16S component since the latter structure should more nearly resemble the 25S structure, and has been studied at more nearly the same pH.

In the case of the 25S particle, initial molecular weight studies by the high-speed equilibrium method using the purified hemocyanin at pH 7 yielded inconclusive results, due to the presence of a small amount of the 16S component (Figure 3A). This problem was overcome by using a sample of 25S particles which had been separated by velocity centrifugation in a sucrose gradient (Figure 2A). A typical graph of the logarithm of the fringe displacement *vs.* the radial position in the cell is shown in Figure 6. The slope of a linear fit to such a fringe displacement plot yields the molecular

TABLE II: Physical Data for *C. magister* Hemocyanin.

Component	$s^0_{20,w}$ (S)	Mol Wt	$\bar{v}$ (ml/g)	[η] (ml/g)	f/f_0
25 S	25.6	938,000 ± 20,000	0.728	4.67	1.35
16 S	18.0	480,000 ± 4,000	(0.728)		1.22
5 S	5.7	65,000–80,000[a]	0.731	7.05	
Gd·HCl subunits		Less than 75,000; see text	0.700	47.4	

[a] Heterogeneous.

weight for a homogeneous sample. Five such experiments, analyzed in this way, gave a mean value for the molecular weight of 938,000 ± 20,000. This is in good agreement with the value of 950,000 reported by Thomson *et al.* (1959). In order to test for homogeneity, point-average M_w values were computed as a function of concentration in the cell at equilibrium, and are plotted in Figure 7. Homogeneity of the sample was further demonstrated by the computer-calculated point-average values of M_w, M_n, and M_z, which, on the average, for any particular experiment, agreed to within ±2.5% or better. The intrinsic viscosity and frictional coefficient ratio (Table II) indicate that the particles are fairly compact.

Molecular weight determinations on the 16S subunit were also made by the high-speed equilibrium method using purified fractions obtained by sucrose gradient centrifugation (Figure 2B). The slope of the linear graph of fringe displacement *vs.* radial position in the cell yielded a mean value from three experiments of 480,000 ± 4,000. Point-average molecular weights were computed, and weight averages for the three experiments are shown in Figure 7. Again M_n, M_w, and M_z were in good agreement. From these results it is clear that the 16S subunit is homogeneous, and represents a half-molecule of the 25S particle.

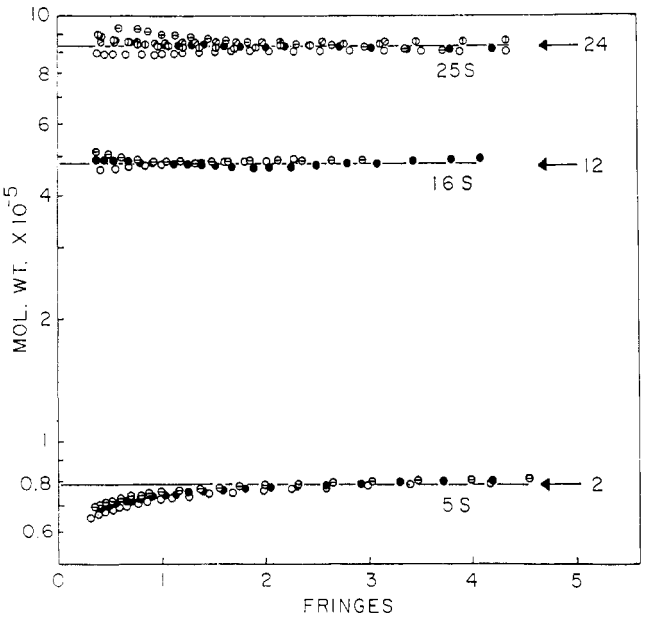

FIGURE 7: Weight-average molecular weights as a function of concentration for the 25S, 16S, and 5S particles. Concentration is expressed as fringe displacement, ΔJ. One fringe represents a concentration of approximately 0.25 mg/ml. Each set of points represents a separate experiment. The numbers (2, 12, and 24) on the right indicate the molecular weights corresponding to 2, 12, and 24 copper atoms (1, 6, and 12 O_2 binding sites), respectively.

Sedimentation equilibrium studies of the 5S component were first made at pH 10.1, in bicarbonate buffer. The results, however, were inconclusive. While average values tended to be around 70,000, it was clear that both lower molecular weight and higher molecular weight material was present.

Further equilibrium studies were made at pH 10.6 in the hope that greater homogeneity of the subunit might be achieved. The point-average M_w from three separate experiments at this pH are plotted as a function of concentration in the solution column in Figure 7. It is apparent that the solution is heterogeneous, but it also seems reasonable to suggest, from the leveling off of M_w values at higher concentration, that a considerable fraction of the protein exists as a subunit with a molecular weight of approximately 75,000–80,000. The intrinsic viscosity of this unit (7.05, Table II) indicates that the molecule is relatively compact, though more asymmetric and/or hydrated than the 25S particle.

High speed equilibrium experiments at pH 11 yielded very little useful information. All three experiments performed yielded point-average molecular weights which varied continuously between 31,000 and 64,000. Likewise, equilibrium studies on a hemocyanin sample which had been exhaustively dialyzed against 6 M Gd·HCl gave continuously varying molecular weights between about 30,000 and 55,000 but with a notable tendency to "level off" between 50,000 and 54,000. The experiments in Gd·HCl and at pH 11 were not as reproducible as other experiments. Since the reason for this variance is not known, we shall interpret them only to indicate that material smaller than 75,000 is present under these circumstances.[2] The hemocyanin lost its characteristic blue color both at pH 11.0 and in Gd·HCl; this suggests that unfolding of the protein has taken place under these conditions, accompanied by a loss of oxygen binding properties. Further indication of the unfolding in Gd·HCl solution is given by the value of the intrinsic viscosity (47.4 ml/g). If the empirical equation of Tanford *et al.* (1967) is used, this will correspond to a viscosity-average molecular weight of about 63,000.

[2] While the possibility of the slow cleavage of especially sensitive peptide bonds at low temperature at pH 11 cannot be entirely discounted, this explanation could not account for the lower molecular weights also found in Gd·HCl solutions.

The copper content of the hemocyanin was determined for us by Dr. Joann Loehr of the University of Oregon Medical School, using the method of atomic absorption analysis. Three different preparations yielded 24.0, 23.9, and 24.3 moles of copper per mole of protein, based on a molecular weight of 938,000. This was in agreement with earlier determinations (Thomson *et al.*, 1959), and also more recent determinations from that same laboratory (J. Loehr and H. S. Mason, personal communication, 1969).

Discussion

From the sedimentation velocity, sedimentation equilibrium, and viscosity studies presented herein, the results summarized in Table II are obtained. The molecular weight of the 25S particle is almost exactly twice that of the 16S component. This conclusion is in accord with electron microscopy studies of other crustacean hemocyanins, which show particles in 25S preparations which appear to be dimers of the particles observed in 16S preparations. (See, for example, Fernandez-Moran *et al.*, 1966.)

It is evident that the 16S and 25S components are not readily interconvertible in the magnesium-free buffer. This is demonstrated, in the first place, by the fact that these components can be separated by sucrose gradient sedimentation, and then characterized as homogeneous components. Furthermore, reassociation of hemocyanin disassociated into the 5S subunits proceeds (in the absence of Mg^{2+}) only as far as the 16S stage. To obtain *any* 25S hemocyanin, magnesium ion is required.

However, it should be noted that we have not as yet been able to obtain reassociation to 25S hemocyanin to the extent to which that component is present in the solution before disassociation. Divalent ions appear to be necessary, but are not sufficient to allow complete reassociation. Further, some 16S units appear to be incompetent for dimerization in the original serum. We may suggest that either: (1) some low molecular weight substance, in addition to divalent cations, is required for dimerization; or (2) the 5S subunits are of two or more types, and a particular arrangement of these in the 16S particles is required to allow dimerization. That the former is not likely the case is indicated by the experiment in which the hemocyanin was simply titrated to high pH and back again. The results obtained were very similar to those in dialysis experiments. It should be noted that this kind of behavior is not unique to *Cancer* hemocyanin; very similar results were reported by Di Giamberardino (1967) with the hemocyanin from another crustacean, *Eriphia spinifrons*.

While the reassociation of 5S subunits to form 16S particles proceeds smoothly upon dialysis to neutral pH, reassociation at high pH requires the presence of divalent cations. Thus, under some conditions the stability of the 16S particles as well as the 25S particles requires the presence of such ions.

Attempts to prepare a wholly homogeneous 5S preparation have not been successful; a mixture is invariably obtained. From the fact that results of experiments at different initial concentrations and rotor speeds are not entirely superimposable, we conclude that the mixtures are not in rapid equilibrium. However, the data indicate that most of the material present has a molecular weight of approximately 75,000–80,000. This would be consistent with a subunit containing one O_2 binding site, since copper analyses (Thomson *et al.*, 1959, and also the data reported herein) indicate a weight of 74,000–80,000/2 copper atoms. It should be noted that solutions at pH 10.6 are still capable of oxygen binding. The data indicate, then, that a structure containing one binding site exists, but that smaller subunits can be obtained. This conclusion is supported by the experiments conducted at pH 11 and in Gd·HCl.

It is of interest to consider the subunit molecular weights in terms of structures proposed from electron microscopy, and in comparison with other hemocyanins. The value of about 480,000 for the 16S component seems well established, both from internal consistency of the data and by comparison with the 25S results. There must be six 78,000 dalton units in each 16S particle.

If we consider that the 78,000 dalton units are themselves made up of smaller subunits, there must then be twelve or more such subunits in a 16S particle. Levin (1963c) has proposed a twelve-subunit model for the 16S hemocyanins. On the other hand, van Bruggen *et al.* (1963) interpret their electron micrographs in terms of an eight-subunit particle. This model is difficult to reconcile with our data; if the subunits are of equal size, a molecular weight of 60,000 daltons for each would be required, and only six of the eight could carry O_2 binding sites.

Twelve-subunit models encounter another kind of difficulty. The subunit weight would have to be about 39,000 daltons. It has been shown (Ghiretti-Magaldi *et al.*, 1966) that the amino acid compositions of crustacean hemocyanins closely resemble those of molluscan hemocyanins. The weight per O_2 site for molluscan hemocyanins is uniformly about 50,000 daltons. Thus, we are faced with the problem of understanding how a unit of 50,000 daltons can closely resemble one of 78,000 daltons, or how one of 25,000 daltons can be very similar to one of 39,000 daltons.

We may suggest here a third model, which would resolve these difficulties. If there were eighteen subunits, each of about 26,000 daltons, six pairs of these could be oxygen binding units, accompanied by six "structural" subunits. A model based on this concept has been constructed. It has the O_2 binding subunits placed on the twelve edges of a cube, and the six structural units centered behind the faces in an octahedral "core." The model thus involves six symmetrically arranged protomers, each containing one "core" subunit and two subunits which contribute to an O_2 binding site. Styrofoam models of such a structure readily present either the square or hexagonal profiles always observed in electron microscope studies of 16S particles. (See, for example, Fernandez-Moran, *et al.*, 1966, or van Bruggen *et al.*, 1963.)

Resolution of the question of the ultimate subunits of crustacean hemocyanins must require more investigation of the products of disassociation. Such studies are currently in progress.

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References

Cohen, L. B., and Van Holde, K. E. (1964), *Biochemistry 3*, 1809.

Creeth, G. M., and Pain, R. H. (1967), *Progr. Biophys. Mol. Biol. 17*, 217.

DePhillips, H. A., Nickerson, K. W., Johnson, M., and Van Holde, K. E. (1969), *Biochemistry 8*, 3665.

Di Giamberardino, L. (1967), *Arch. Biochem. Biophys. 118*, 273.

Eriksson-Quensel, I. B., and Svedberg, T. (1936), *Biol. Bull. 71*, 498.

Fernandez-Moran, H., van Bruggen, E. F. J., and Ohtsuki, M. (1966), *J. Mol. Biol. 16*, 191.

Gerhart, J. C. (1967), *Methods Enzymol. 11*, 187.

Ghiretti, F. (1962), *in* Oxygenases, Hayashi, O., Ed., New York, N. Y., Academic, Chapter 12.

Ghiretti-Magaldi, A., Nuzzolo, C., and Ghiretti, F. (1966), *Biochemistry 5*, 1943.

Levin, O. (1963a), *Arkiv Kemi 21*, 1.

Levin, O. (1963b), *Arkiv Kemi 21*, 15.

Levin, O. (1963c), Abstracts of Uppsala Dissertations in Science, No. 24, Almqvist and Wiksells, Uppsala.

Long, C., Ed., (1961), *in* Biochemist's Handbook, Princeton, N. J., Van Nostrand, p 28.

Tanford, C., Kawahara, K., and Lapanje, S. (1967), *J. Am. Chem. Soc. 89*, 729.

Thomson, L. C. G., Hines, M., and Mason, H. S. (1959), *Arch. Biochem. Biophys. 83*, 88.

van Bruggen, E. F. J., Schuiten, V., Weibenga, E. H., and Gruber, M. (1963), *J. Mol. Biol. 7*, 249.

Van Holde, K. E. (1967), *Biochemistry 6*, 93.

Van Holde, K. E., and Cohen, L. B. (1964), *Biochemistry 3*, 1803.

Yphantis, D. A. (1964), *Biochemistry 3*, 297.