Subunit Association and Heterogeneity of *Limulus polyphemus* Hemocyanin†

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ABSTRACT: The molecular weights of the 6S, 24S, 36S, and 60S components of *Limulus polyphemus* hemocyanin were determined by high speed sedimentation equilibrium to be 69 400, 856 000, 1 690 000, and 3 160 000. The behavior of this hemocyanin appears to be similar to that of other arthropod hemocyanins where the first aggregation step is the formation of a hexamer of the 6S monomer. Here the larger aggregated states (24S, 36S, and 60S) are successive dimers of an unobserved hexamer (16S). The 24S-36S-60S association was found to be heterogeneous, suggesting that 24S components of different composition may be present.

Each oxygen binding site of hemocyanin is associated with two copper atoms and no porphyrin ring. The molecular weight per oxygen binding site is approximately 50 000 for molluscan and 74 000 for arthropod hemocyanin (Redfield et al., 1928). The molecular weights of native hemocyanins range from a few hundred thousand to many million depending on the species and solvent conditions (Svedberg & Pederson, 1940).

Many techniques indicate that *Limulus* hemocyanin is unusual: The isoelectric point of *Limulus* hemocyanin is 6.4, higher than that of most other hemocyanins (Redfield, 1933). The extinction coefficient at 278–280 nm is lower than the values observed for other hemocyanins (Ghiretti-Magaldi et al., 1966; Nickerson & Van Holde, 1971). The position of the absorption maximum in the near UV is intermediate: 345 nm for molluscan, 335 nm for arthropod, and 340 nm for *Limulus* hemocyanin. Immunological studies have shown that *Limulus* hemocyanin does not cross-react with "any other hemocyanin" (Ghiretti-Magaldi et al., 1966). In an early paper Svedberg & Hedenius (1934) commented that the molecular size and pH stability of *Limulus* hemocyanin are "unique" among hemocyanins. Because of this uniqueness of the hemocyanin from *Limulus polyphemus* and because *Limulus* is a very old and primitive species, a study of the subunit structure of its hemocyanin was undertaken.

Svedberg & Pederson (1940) reported that *Limulus* hemocyanin exists in several different aggregated states, depending on the buffer conditions. Their values for the sedimentation coefficients are 56.6, 34.6, 24.0, 16.0, and 5.87 S. Other authors have reported 60, 40, 25, 16, and 5.2 S (Bancroft et al., 1966). For simplicity we refer to the aggregated states by their approximate sedimentation coefficients, namely, 60, 36, 24, 16, and 6 S, without implying that these are anything more than approximate.

Electron microscopic studies (Levin, 1963; Fernandez-Moran et al., 1966) suggested that the 16 S associated species of *Limulus* hemocyanin is cubical and approximately 95 Å on a side. This 16S species dimerizes to form the 24S component. The 24S component in turn dimerizes to form the 36S component which is seen as a flat ring structure approximately 240 Å in diameter with a hole in the center. Finally the 60S component appears as a stacked dimer of the 36S ring structure. The electron microscopic studies also suggested the 16S component to be an octamer of the 6S component. In ultracentrifugal studies of *Homarus americanus* and *Cancer magister*, the 16S component is found to be a hexamer of the 6S component (Morimoto & Kegeles, 1971; Ellerton et al., 1970). Because of the small dimensions of the 6S and 16S components, it is very difficult to determine with the electron microscope whether the 16S component is an octamer or a hexamer of the 6S component, as usually seems the case with arthropod hemocyanins. A study of the subunit molecular weights should resolve this ambiguity.

The average copper content of *Limulus* hemocyanin is 0.173% by weight (Allison & Cole, 1940; Ghiretti-Magaldi et al., 1966; Redfield et al., 1927; Hernler & Phillippi, 1933; Wharton & Rader, 1970). The two copper atoms per oxygen binding site would correspond to a molecular weight of 73 400 per oxygen binding site. If impure protein samples were used for these determinations, then the molecular weight per binding site would be overestimated. Thus this value is probably an upper limit for the monomer molecular weight. A similar procedure using the amino acid composition (Ghiretti-Magaldi et al., 1966; Roche & Jean, 1934; Sullivan et al., 1976) is not feasible since the amino acids are not present in small enough quantities and since *Limulus* hemocyanin is heterogeneous in subunit composition (Sullivan et al., 1976).

Osmotic pressure measurements indicate the monomer molecular weight to be 69 000 (Burk, 1939). Ultrafiltration indicates 68 100 (Cohn, 1925). Bonaventura and co-workers (Sullivan et al., 1976) found that the monomers were heterogeneous on $NaDodSO_4$[1] gel electrophoresis with molecular weights ranging from 57 000 to 75 000 and with a predominant species of approximately 70 000.

Svedberg & Heyroth (1929) determined the molecular weight of the 36S component as 2 million by the combination of sedimentation and diffusion measurements at pH 6.63. Bancroft et al. (1966) reported a molecular weight of 1.94×10^6 for the 36S component as observed by sedimentation

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; RMS, root mean square of the residuals.

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equilibrium. Unfortunately, this report was an abstract which did not present experimental details. Tanford and co-workers, using the Archibald procedure, reported a value of $(2.4 \pm 0.2) \times 10^6$ for the molecular weight of the 60S component (Nozaki et al., 1976).

It is not known whether the self-association proceeds as a random mixing of subunits, or if there are specific relationships between various subunits in the self-assembly process. An appropriate study of the subunit interactions could answer this question.

Materials and Methods

Adult female *Limulus polyphemus* were obtained from the Marine Biology Laboratory, Woods Hole, Mass. Before extraction of the serum, the *Limulus* were placed in a cold room for several hours to extend the clotting time. While still in the cold room an incision was made between the carapace and the abdomen on the dorsal surface and the serum was poured from the body cavity into a polyethylene beaker. The serum was immediately centrifuged to minimize clotting and remove large particles and cells (10 min at 15 000 rpm in a Sorval). The hemocyanin remains in the supernatant since it is an extracellular protein. If clots formed during storage at 4 °C, the solution was again centrifuged. Further purification of the hemocyanin is described in the text. Glass-distilled water was used throughout and all chemicals were reagent grade.

Sedimentation equilibrium measurements were made using the meniscus depletion method (Yphantis, 1964). Each experiment was performed at three different loading concentrations using a 12-mm external loading six channel cell (Ansevin et al., 1970). Some of the experiments used a pulsed argon-ion laser light source for the Rayleigh interferometer (Paul & Yphantis, 1972a,b). The interference patterns were automatically scanned using a Gaertner microcomparator interfaced to a PDP-8/L computer. The fringe displacements were determined by a procedure which involves finding the phase shift by a Fourier transform of the total fringe envelope at each radial position (deRosier et al., 1972). Appropriate water blanks were determined as described previously (Ansevin et al., 1970).

The resulting net fringe displacements as a function of radial position were then analyzed by two computer programs: BIOSPIN and NONLIN. BIOSPIN was used to calculate various molecular weight averages as a function of concentration (Roark & Yphantis, 1969; Roark, 1971). These molecular weight averages were used to postulate various association models (including nonideality) for each experiment and these models were then tested and refined using NONLIN (Johnson, 1973). NONLIN performs a simultaneous nonlinear least-squares fit of one or more channels of data at different loading concentrations and radial positions to a specific association and/or nonideality scheme.

This nonlinear least-squares fit of the concentration distribution directly determines monomer molecular weights, virial coefficients, and association constants. The molecular weight, M_i, of the ith component of an ideal system is related to its σ_i by

$$\frac{d \ln c_{r,i}}{d(r^2/2)} = \sigma_i = \frac{M_i(1 - \bar{v}\rho)\omega^2}{RT} \quad (1)$$

where $c_{r,i}$ is the concentration of the ith component at a radius r, R is the gas constant, T is the absolute temperature, $\bar{v}$ is the partial specific volume, ρ is the density, and ω is the angular velocity. The concentration distribution, $c_{r,i}$, of the ith species at a radius r is obtained by integration of eq 1:

$$c_{r,i} = c_{0,i} e^{\sigma_i(r^2/2 - r_0^2/2)} \quad (2)$$

where $c_{0,i}$ is the concentration of the ith component at a radius r_0. For a monomer–n-mer association, the total concentration at any radius, $c_{r,t}$, can be expressed in terms of the monomer concentration, $c_{r,1}$, and an association constant, K_n, by

$$c_{r,t} = c_{r,1} + K_n(c_{r,1})^n \quad (3)$$

The equivalent of eq 1 for a nonideal system is

$$\frac{d \ln c_i}{d(r^2/2)} = \sigma_{i,a} = \frac{\sigma_i}{1 + c\dfrac{\partial \ln y_i}{\partial c}} = \frac{\sigma_i}{1 + 2BM_ic + 3CM_ic^2 + \cdots} \quad (4)$$

where c is the total solute concentration (Σc_i), the y_i are the activity coefficients of the ith species on the c scale, and the colligative virial coefficients of the system are denoted by B, $C, \cdots$. In addition to the $\sigma_{i,a}$, to the $c_{0,i}$, and to the communal $B, C, \cdots$, another parameter, δc, must be included for each channel used. This is an additive constant in the evaluation of the total concentration introduced because the interferometer does not measure absolute concentration. For the "meniscus depletion" technique this constant should be zero and is usually only a few micrometers of displacement on the photographic plate. However, it must be included as

$$c_{obsd} = c_{true} + \delta c \quad (5)$$

where the subscripts refer to the observed and true concentration (or fringe displacement). The corresponding concentration distribution for any association and/or nonideality scheme can be generated by an appropriate combination of eq 2–5. Implicit assumptions of this approach are that no volume change occurs on association ($\bar{v}$ is a constant) and that the activity coefficients of the monomer, γ_m, and any n-mer, γ_n, are related by eq 6:

$$n \ln \gamma_m = \ln \gamma_n \quad (6)$$

(see Roark & Yphantis, 1969).

The reported confidence limits were calculated by searching the variance space for an F-statistic corresponding to a 95% confidence probability (Ackers et al., 1975). This corresponds to approximately two standard deviations, but because of the correlation between successive data points these confidence limits are probably underestimates of the true value. In general such confidence limits will be asymmetric and are thus reported as a range of values, in parentheses, instead of a single value. These confidence limits reflect the precision of the fit of the experimental data to the model and do not necessarily indicate the accuracy of the determined parameters. The evaluation of the confidence region does not include possible effects of systematic errors in the data nor the use of an incorrect association and/or nonideality model.

"Goodness of fit" was determined by two commonly used criteria: First, the RMS must be approximately the same as the experimental noise level, i.e., a few micrometers of displacement on the photographic plate. Second, the residuals must appear to be random as a function of either concentration or radius. The latter criterion has been applied qualitatively rather than quantitatively.

One test for heterogeneity of an associating system is a determination of the apparent association constant for each of the three sets of data collected for each experiment. If the system is a homogeneous associating system the confidence regions of the apparent association constants will overlap

TABLE I: Sedimentation Equilibrium Measurements of the 6S Component[a]

Channel	Parameter	Value	Confidence region[b]	Units	RMS (μm fringe displacement)
A	σ	4.95	(4.88, 5.01)	cm^{-2}	5.1
B	σ	4.90	(4.85, 4.95)	cm^{-2}	5.4
C	σ	4.78	(4.73, 4.82)	cm^{-2}	4.7
All[c]	σ	4.86	(4.82, 4.91)	cm^{-2}	5.6
All[d]	σ	4.96[e]	(4.92, 5.00)	cm^{-2}	5.5
	K_2	0.0318[f]	(0.0011, 0.0694)	$L\ g^{-1}$	
	B	0.0003	(0.0001, 0.0005)	mol mL g^{-2}	

[a] Sodium bicarbonate buffer, 0.1 ionic strength, pH 10.6, 25.5 °C, and 24 019 rpm. In all cases the residuals appeared random. [b] Values in parentheses are the 95% confidence region described in the text. The initial (loading) concentrations were approximately 0.2 g/L for channel A, 0.8 g/L for channel B, and 2.0 g/L for channel C. [c] Evaluated assuming ideality and no association. [d] Evaluated assuming a nonideal monomer-dimer association. [e] Corresponding apparent molecular weight using $\bar{v}$ = 0.735 mL/g is 73 900 (73 300 to 74 400). [f] Corresponding free energy change of dimerization is −4.2 (−3.5 to −4.6) kcal/mol.

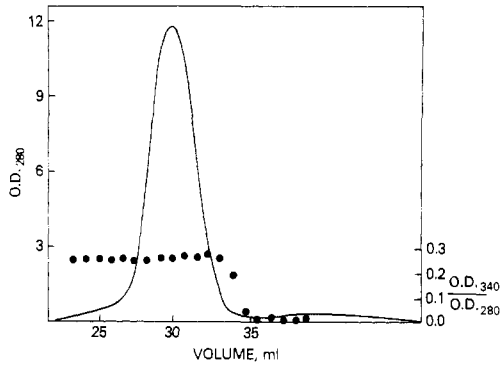

FIGURE 1: Gel filtration of *Limulus* hemocyanin. (●) The ratio of the optical densities at 340 and 280 nm. Column: Bio-Gel A-15m, 1 × 100 cm. Solvent: pH 7.5 Tris-HCl buffer, 0.1 ionic strength with 0.01 M $CaCl_2$ added. Load: 40 mg of *Limulus* serum protein in 1 mL. Flow rate: 2 mL/h.

(Johnson & Yphantis, in preparation; Yphantis & Johnson, 1971; Yphantis et al., 1972). If the model is not assuredly known, then such tests for heterogeneity can only be made by truncating all the data sets used so that they have a common concentration range. Thus in each fit the association constant, K_n, the monomer concentration, $c_{0,1}$, and the concentration offset, δc, were allowed to vary simultaneously, while σ was fixed at the value determined from the simultaneous fit of all of the channels.

Another test for heterogeneity of an associating system is to plot the weight average σ (or weight average molecular weight) for each channel as a function of concentration. If the associating system can be described as a single thermodynamic component, then the plots will superimpose. If heterogeneity is present, the apparent molecular weights will be a function not only of the local concentration but also of the radius and of initial loading concentrations. Consequently, the curves would not be expected to superimpose.

The partial specific volume of isoionic *Limulus* hemocyanin was taken to be the value (0.735 mL/g) measured by Svedberg & Heyroth (1929) at pH 6.63. This number is in close agreement with the calculated value of 0.730 mL/g estimated from the amino acid composition (Ghiretti-Magaldi et al., 1966; Roche & Jean, 1934) using the method of Cohn & Edsall (1943). The densities of the Tris buffers were taken from unpublished data of Dr. H. DePhillips (personal communication). All other densities were taken from the International Critical Tables.

Protein concentrations were determined from the interference patterns using the observed refractive index increment, 1.98 × 10^{-4} L/g for *Limulus* hemocyanin (Redfield, 1933).

$NaDodSO_4$ gel electrophoresis was performed as outlined by Shapiro et al. (1967).

Results

Hemocyanin is not the only protein present in *Limulus* serum and thus must be isolated. This hemocyanin has a sedimentation coefficient of approximately 60 S at pH 7.5 and it appears to be much larger than any other molecules in the serum. Accordingly we used Bio-Gel A-15m to provide effective gel filtration in the molecular weight range of several million. Before loading onto the column the serum was equilibrated with a known buffer: 0.1 M HCl, 0.01 M $CaCl_2$ titrated to pH 7.5 with solid Tris base. This equilibration was performed either by dialysis or, for small volumes, with a Sephadex G-25 column (2 × 15 cm). The Bio-Gel column (1 × 100 cm) was equilibrated and eluted with the pH 7.5 Tris buffer, at a flow rate of 2 mL/h. Figure 1 presents the effluent pattern of this column with a 40-mg load of protein. There are two major peaks in the pattern. The protein of the peak appearing after 35 mL of volume lacks the characteristic absorption bands of hemocyanin, has an $s_{20,w}$ of approximately 14 S and is thought to be the hemagglutinin reported by Marchalonis & Edelman (1968). The protein in the fractions eluting from 23 to 33 mL has the characteristic absorption spectrum of hemocyanin with a constant ratio of absorbance at 340 nm to that at 280 nm when in equilibrium with the atmosphere. The protein collected between 28 and 33 mL was pooled and used for later study. Equivalent fractionations have been obtained by chromatography on columns either of Sepharose 4B or of controlled pore glass beads, CPG-10-350 (Electronucleonics), that had been coated with polyethylene glycol (Hawk et al., 1972).

Neither sedimentation velocity experiments in the pH 7.5 Tris-$CaCl_2$ buffer nor $NaDodSO_4$ gel electrophoreses on the purified hemocyanin showed any detectable amount of any protein other than hemocyanin.

Purified *Limulus* hemocyanin has a sedimentation coefficient of approximately 6 S at pH 10.6 in 0.1 ionic strength sodium bicarbonate buffer. A sedimentation equilibrium ex-

TABLE II: Sedimentation Equilibrium Measurement of the 60S Component.[a]

Channel	Parameter	Value	Confidence region[b]	Units	RMS (μm fringe displacement)
A	σ	4.37	(4.33, 4.41)	cm^{-2}	2.4
B	σ	4.26	(4.22, 4.31)	cm^{-2}	4.7
C	σ	4.24	(4.21, 4.28)	cm^{-2}	5.2
All[c]	σ	4.26	(4.23, 4.30)	cm^{-2}	4.7
All[d]	σ	4.17[e]	(4.12, 4.21)	cm^{-2}	4.7
	K_2	0.154[f]	(0.103, 0.211)	$L\ g^{-1}$	
	B	1.23×10^{-5}	$(0.91, 1.55) \times 10^{-5}$	mol mL g^{-2}	

[a] Tris-HCl buffer, 0.05 ionic strength, pH 7.5, with 0.45 M KCl added, at 21.9 °C and 3400 rpm. In all cases the residuals appeared random. The initial (loading) concentrations were approximately 0.2 g/L for channel A, 0.8 g/L for channel B, and 2.0 g/L for channel C. [b] 95% confidence region as described in the text. [c] Evaluated assuming ideality and no association. [d] Evaluated assuming a nonideal monomer-dimer association. [e] Corresponding apparent molecular weight using $\bar{v}$ = 0.735 mL/g is 3 240 000 (3 200 000 to 3 270 000). [f] Corresponding free energy change of dimerization is −7.3 (−7.0 to −7.5) kcal/mol.

TABLE III: Sedimentation Equilibrium Measurement of the 24S Component.[a]

Parameter	Channel	Value	Confidence region	Units
σ	All	4.323[b]	(4.270, 4.376)	cm^{-2}
B	All	1.7×10^{-5}	$(0.5, 2.9) \times 10^{-5}$	mol mL g^{-2}
K_2	All	0.23[c]	(0.16, 0.30)	$L\ g^{-1}$
$c_{0,1}$	A	1.72	(1.62, 1.82)	$\mu g/mL$
	B	1.51	(1.42, 1.60)	$\mu g/mL$
	C	1.62	(1.53, 1.72)	$\mu g/mL$
δC	A	−0.05	(−3.39, 3.34)	μm fringe displacement
	B	1.59	(−1.64, 4.88)	μm fringe displacement
	C	−1.98	(−5.36, 1.27)	μm fringe displacement

[a] Three channels of data were fit to a nonideal monomer-dimer association, eq 2-4, with an RMS deviation of 4.4 μm on the photographic plate. Conditions were: 0.05 ionic strength sodium aetate buffer, pH 5.0, 0.45 M KCl added, 25.8 °C, and 6800 rpm. The initial (loading) concentrations were approximately 0.2 g/L for channel A, 0.8 g/L for channel B, and 2.0 g/L for channel C. [b] Corresponding apparent molecular weight using $\bar{v}$ = 0.735 mL/g is 850 000 (840 000 to 860 000). [c] Corresponding free energy change of dimerization is −6.8 (−6.6, −7.0) kcal/mol.

periment in this buffer at 26.5 °C and at 24 019 rpm is summarized in Table I. When the individual channels are analyzed separately there is a 3.5% variation in σ and thus the corresponding molecular weight. When analyzed simultaneously, a good fit was obtained assuming an ideal nonassociating model.

Hemocyanin at pH 10.6 should exhibit significant Donnan nonideality (Roark & Yphantis, 1971) because of the charge on the protein at this elevated pH. Since the observed fringe displacements can be fit with reasonable precision to an ideal non-associating model there must be compensation for the effects of the expected nonideality. Two possible origins of this compensation are self-association and the heterogeneity observed by Bonaventura and co-workers (Sullivan et al., 1976). An analysis of these data as a nonideal monomer-dimer association is also presented in Table I. The σ value obtained from this nonideal self-associating analysis is 4.96 cm^{-2} corresponding to an apparent molecular weight (uncorrected for component definition, volume changes on titration, etc.) of 73 900 (73 300 to 74 500).

This hemocyanin exists in an associated state with a sedimentation coefficient of approximately 60 S and appears to be a single component at pH 7.5 in 0.05 ionic strength Tris-HCl buffer with 0.45 M KCl added. A sedimentation equilibrium experiment under these conditions can be successfully analyzed as a single ideal component, as shown in Table II. The channel with the lowest loading concentration yields a value of σ that is 3% higher than the values of σ from the other two channels when each channel is analyzed separately assuming a single ideal component. On joint analysis the combined data can be fit to the same model with some increase (0.44 μm) in the RMS of the residuals of the fit.

The 60S component is expected to show nonideality from both excluded volume and charge repulsion. Consequently, the experiment summarized in Table II was also analyzed assuming a nonideal monomer-dimer association. The value of σ determined for this model was 4.17 cm^{-2} corresponding to an uncorrected molecular weight of 3 270 000 (3 230 000 to 3 300 000).

Table III, Table IV, and Figure 2 summarize a sedimentation equilibrium determination of the molecular weight of the 24S component. This experiment was performed in an 0.5 ionic strength pH 5.0 acetate buffer (see Table III). The solid line in Figure 2 corresponds to a nonideal monomer-dimer equilibrium. The apparent uncorrected molecular weight so determined was 850 000 (840 000 to 860 000) and the free en-

TABLE IV: Heterogeneity of the 24-36S Association.[a]

Channel	K_2[b] ($L\ g^{-1}$)	Confidence region	RMS (μm fringe displacement)
A	0.213	(0.177, 0.250)	2.76
B	0.189	(0.163, 0.216)	2.49
C	0.118	(0.066, 0.171)	5.21
C[c]	0.119	(0.084, 0.155)	3.53

[a] This corresponds to the experiment in Table III. In this case the data were truncated to a common concentration range (0.0 to 1.0 g/L) and fit individually to an ideal monomer-dimer association while the monomer σ was fixed at 4.323. The initial (loading) concentrations were approximately 0.2 g/L for channel A, 0.8 g/L for channel B, and 2.0 g/L for channel C. [b] Apparent dimerization constant. [c] Calculated after removing an anomaly in the blank fringe pattern. The anomaly was replaced with a linear interpolation from outside the anomalous region.

ergy change of dimerization was −6.8 (−6.6 to −7.0) kcal/mol.[2]

Table IV and Figure 2 can be used to test whether the 24S-36S association is heterogeneous. The curves in this figure almost superimpose and the small differences present could have arisen either from heterogeneity or from small systematic errors in the data. The confidence regions shown in Table IV do not overlap, but the discrepancy is not large. Consequently this association appears to be slightly heterogeneous. The large RMS observed for channel C arises primarily from an anomaly on the baseline fringe pattern. When this anomaly was removed from the blank fringe displacements (by linear interpolation from outside the anomalous region), the association constant remained essentially unchanged, but the RMS decreased substantially.

Limulus hemocyanin exhibits a sedimentation coefficient of approximately 36 S at pH 6.1 in 0.05 ionic strength phosphate with 0.45 M KCl added. This sedimentation coefficient increases with hemocyanin concentration, indicating the presence of a reversible association reaction (Johnson, 1973). Table V, Table VI, and Figure 3 summarize the results of a sedimentation equilibrium experiment under these conditions. The apparent point weight average molecular weight increases with concentration and the data can be fit to a monomer-dimer-tetramer association. The corresponding monomer (24 S) molecular weight (uncorrected) is 847 000 (837 000 to 861 000). The free energy change of dimerization obtained from this overall fit is −9.88 kcal/mol and the free energy change to form tetramers from monomers is −25.4 kcal/mol. The apparent weight average molecular weight is not a function of the observational concentration alone (Figure 3) since the three curves diverge significantly. The separate channels were fit individually to a simple ideal monomer-dimer association after first ensuring that each data set spanned the same range of concentration (from 0.0 to 0.7 g/L). In each of these fits we assumed the same value for σ_1 (2.511 cm^{-2}) that we obtained from the joint fit. Table VI shows that these individual fits require significantly and systematically different values of the apparent dimerization constants. Thus we must conclude that, under these conditions, the *Limulus* hemocyanin system is heterogeneous and cannot be described as a single thermodynamic component. Because of this apparent heterogeneity, interpretation of the apparent association constants (and their associated free energy changes) is difficult.

[2] All free energies of association are reported per mole of oligomer formed.

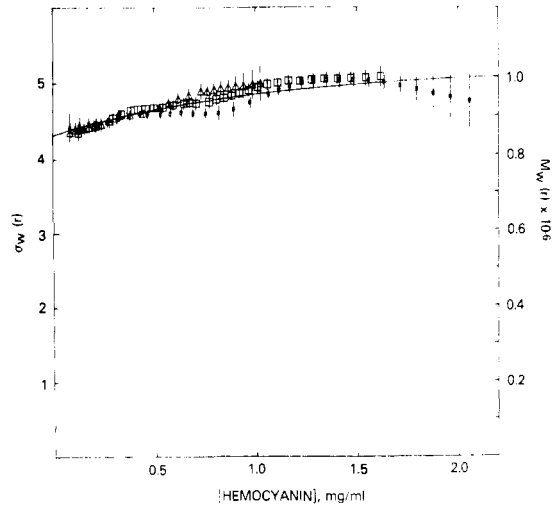

FIGURE 2: Weight average molecular weight and weight average σ as a function of hemocyanin concentration, $c(r)$, for the 24S component. This corresponds to the experiment summarized in Table III. The symbols, △, □, and ●, correspond to initial loading concentrations of 0.2, 0.8, and 2.0 g/L in channels A, B, and C. The solid line was calculated for the nonideal monomer-dimer association described in Table III.

Discussion

The apparent molecular weights observed are influenced by the charge on the solute of interest. Often the most striking effect is the Donnan nonideality occasioned by the requirement of bulk electroneutrality through the solution. In addition the infinite dilution values of the apparent molecular weights may also be significantly perturbed by electrostatic effects. Estimates of both effects may be obtained if the solute charge is known.

In order to estimate the charge we have calculated an approximate titration curve for *Limulus* hemocyanin from the amino acid composition, assigning reasonable typical values for the pKs of the side chains.[3] In general the charge expected from the titration curve does not appear to be fully expressed, probably because of counterion binding (Scatchard et al., 1946; Tanford, 1961). The effective charge for Donnan nonideality of proteins under similar charge densities appears to range from 30 to 60% of the titration charge (Szuchet & Yphantis, 1973, 1976; Szuchet, 1976). Accordingly we take the effective charge on *Limulus* hemocyanin to be 0.45 ± 0.15 times the titration charge under all our conditions.

Table VII lists the effective charge, Z_{eff}, per 74.9 kg of hemocyanin thus estimated for three of the experimental conditions, and the net effective charge per molecule. The corresponding contributions of the Donnan effect to the second virial coefficients (Roark & Yphantis, 1971) are listed, as B_D, in the same table. In addition we list B_E, the estimated contributions from the excluded volume of the assumed spherical molecules (Tanford, 1961). These excluded volume contributions vary only a little with reasonably possible values of assumed asymmetries for these molecules; for example, a tenfold ratio of length to diameter, for an assumed rod shape, increases these values 2.5-fold and, thus, increases the total range of the net second virial coefficient, B_T, only trivially (penultimate row of Table VII). The last row in Table VII

[3] We assumed 94 free carboxyl groups (including one C-terminal) with pK = 4.6, 46 histidyl groups with pK = 7.0, one α-NH_2 group with pK = 7.8, 22 tyrosyls with pK = 9.8, and 36 lysyl groups with pK = 10.2. The amounts quoted are per 74.9 kg of hemocyanin, as tabulated by Ghiretti-Magaldi et al. (1966). In addition we took the isoionic point to be the same as the observed isoelectric point of 6.4 (Redfield, 1933).

TABLE V: Sedimentation Equilibrium Measurements of the 36S Component.[a]

Parameter	Channel	Value	Confidence region	Units
σ	All	2.511[b]	(2.48, 2.55)	cm^{-2}
K_2	All	41.8[c]	(38.9, 44.9)	$L\ g^{-1}$
K_4	All	69.5[d]	(29.5, 106.2)	$L^3\ g^{-3}$
$c_{0,1}$	A	3.46	(3.29, 3.62)	$\mu g/mL$
	B	4.08	(3.89, 4.26)	$\mu g/mL$
	C	4.73	(4.51, 4.93)	$\mu g/mL$
δC	A	−11.33	(−14.01, −5.50)	μm fringe displacement
	B	−9.86	(−13.82, −4.45)	μm fringe displacement
	C	1.88	(−1.94, 8.27)	μm fringe displacement

[a] Conditions were 0.05 ionic strength phosphate buffer, pH 6.1, with 0.45 M KCl added at 26 °C and at 5200 rpm. The overall RMS was 5.16 μm fringe displacement. The initial (loading) concentrations were approximately 0.2 g/L for channel A, 0.8 g/L for channel B, and 2.0 g/L for channel C. [b] Corresponding apparent molecular weights are 847 000 (837 000 to 861 000) for the monomer (24S) under these conditions. The 36S component, i.e., the dimer, is the major macromolecular species with a molecular weight of 1.69 million (1.67 to 1.72 million). The 60S component is a tetramer of this monomer and has an apparent molecular weight of 3.39 million (3.35 to 3.44 million). [c] Corresponding dimerization free energy change is −9.92 (−9.88, −9.97) kcal/mol. [d] Corresponding tetramerization free energy change is −26.1 (−25.5, −26.3) kcal/mol.

TABLE VI: Heterogeneity of the 24S–36S–60S Association.[a]

Channel	K_2[b] $L\ g^{-1}$	Confidence region	RMS (μm fringe displacement)
A	92.6	(82.9, 102.2)	3.37
B	42.8	(37.4, 48.5)	3.81
C	23.8	(21.4, 26.4)	2.71

[a] This corresponds to the experiment in Table V. In this case the data were truncated to a common concentration range (0.0 to 0.7 g/L) and fit to an ideal monomer–dimer association with the monomer σ fixed at 2.511. The initial (loading) concentrations were approximately 0.2 g/L for channel A, 0.8 g/L for channel B, and 2.0 g/L for channel C. [b] Apparent dimerization constant.

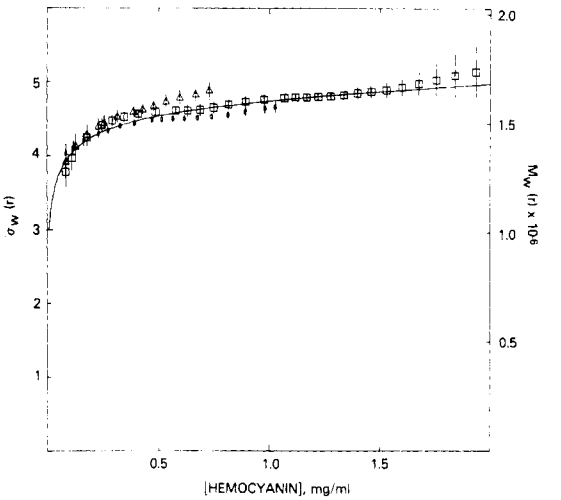

FIGURE 3: Weight average molecular weight and weight average σ as a function of hemocyanin concentration, $c(r)$, for the 36S component. This corresponds to the experiment described in Table V and the solid line is calculated for the monomer–dimer–tetramer association described there. The symbols, △, □, and ●, correspond to channels A, B, and C with initial loading concentrations of 0.2, 0.8, and 2.0 g/L, respectively.

presents the observed values of the second virial coefficient for these experimental conditions.

The estimated total second virial coefficient at pH 10.6 is quite similar to the value obtained when the experimental system is analyzed as a nonideal monomer–dimer association. Since the experimental observations can be analyzed almost as well (in terms of the RMS) by assuming only a single ideal component, it appears likely that the nonideality is compensated for either by some reversible dimerization and/or by some size heterogeneity, perhaps from some small irreversible aggregation or from the size heterogeneity reported by Bonaventura and co-workers (Sullivan et al., 1976). We cannot distinguish between these alternatives with the present data, but experiments bearing on these alternatives are under way.

Similarly, analysis of the 60S system at pH 7.5 as an associating system yields second virial coefficients close to those predicted. The small amount of apparent association observed for the 60S component (see Table II) could represent a real reversible assembly to a larger aggregate than the 60S component or it could reflect a small amount of irreversible aggregation or even molecular weight heterogeneity of the 60S component from random mixing of the different monomers reported by Bonaventura and co-workers (Sullivan et al., 1976).

Under all three experimental conditions analyzed, the predicted and observed second virial coefficients are in substantial agreement suggesting that the ranges estimated for the effective charge on the hemocyanin molecules are reasonable. In the absence of direct estimates of the effective partial specific volume [the ψ' of Cassasa & Eisenberg (1961, 1964) that is directly obtainable by density measurements on dialysate and raffinate], we used these estimated ranges of the effective charge to infer correction factors for the charge effects at infinite dilution, following the procedure detailed by Szuchet & Yphantis (1973, 1976) and making the following assumptions: The volume change on titration alkaline to the isoelectric point was taken to be +16 mL/OH^- added and, on the acidic side, +11 mL/H^+ added; these are averages of the values observed by Rasper & Kauzmann (1962) for several proteins. The molal volumes of NaOH and Na_2CO_3 in aqueous solutions were both taken as −6.7 mL and the molal volumes of HCl and KOH required for the pH 5.0 and pH 7.5 experiments have been assumed to be 18 and 2.9 mL, respectively; these are the values summarized in Harned & Owen (1958). A value of 23.1 mL was used for the molal volume of $NaHCO_3$, as estimated from

TABLE VII: Calculated and Observed Values of Second Virial Coefficients.

	pH 10.6	pH 5	pH 7.5
Z_{eff}/74.9 kg	-37.8 ± 12.6	15.3 ± 5.1	-12.4 ± 4.1
Z_{eff}/molecule	-35.0 ± 11.7	170 ± 56.7	-551 ± 184
B_d, Donnan (mol mL g^{-2})	$7.4\ (0.8, 13.2) \times 10^{-4}$	$2.4\ (0.3, 4.3) \times 10^{-5}$	$1.6\ (0.2, 2.8) \times 10^{-5}$
B_E, excluded volume (mol mL g^{-2})	4.2×10^{-5}	3.5×10^{-6}	9×10^{-7}
B_T, total estimated (mol mL g^{-2})	$7.8\ (1.2, 13.6) \times 10^{-4}$	$2.8\ (0.7, 5.2) \times 10^{-5}$	$1.7\ (0.7, 2.9) \times 10^{-5}$
Obsd value of B (mol mL g^{-2})	$3(1, 5) \times 10^{-4}$	$1.7(0.5, 2.9) \times 10^{-5}$	$1.2(0.9, 1.6) \times 10^{-5}$

TABLE VIII: Parameters for Analysis of Infinite Dilution Apparent Molecular Weights.[a]

	6S monomer at pH 10.6		60S polymer at pH 7.5	24S polymer at pH 5
Z_{Titr}/74.9 kg	-83.9		-27.6	34.1
Z_{eff}/74.9 kg	-37.8 ± 12.6		-12.4 ± 4.1	15.3 ± 5.1
w_{salt}/74.9 kg	76.75 kg		75.95 kg	76.15 kg
v_{salt}/74.9 kg	54.32 L		55.08 L	56.04 L
	Only $NaHCO_3$	Only Na_2CO_3		
w_{Sc}/74.9 kg	75.16 kg	75.41 kg	75.49 kg	75.57 kg
v_{Sc}/74.9 kg	53.88 L	54.40 L	54.90 L	55.83 L
$\bar{v}_{Sc}$	0.7169 mL g^{-1}	0.7214 mL g^{-1}	0.7273 mL g^{-1}	0.7388 mL g^{-1}
$(1 - \bar{v}_p)$	0.2826	0.2781	0.2567	0.2453
M_{Sc} (g mol^{-1})	69 180	70 300	3.17×10^6	8.64×10^5
M_p (g mol^{-1})	68 940	69 820	3.14×10^6	8.56×10^5
	(70 730, 67 030)	(71 970, 66 270)	(3.19, 3.08)	(8.72, 8.34)
M_p (g mol^{-1})	69 380 (71 970, 66 270)			
M_{app} ($\bar{v}_2 = 0.735$)	73 910 (74 460, 73 320)		$3.27(3.30, 3.23) \times 10^6$	$8.50(8.60, 8.40) \times 10^5$
$\hat{n}$	[1]		45.3(48.1, 42.8)	12.3(13.2, 11.6)

[a] The ranges indicated for M_p and $\hat{n}$ are the propagated extremes calculated for the extremes of the assumed effective charge and for the ranges of the σ values (see Tables I–III) used. The ranges on M_{app} reflect only the range of the σ values.

density data in the International Critical tables. The value of 27.3 mL was used for the molal volume of HCl; this corresponds to the value observed in 0.5 M solutions (Harned & Owen, 1958).

With these several assumptions and the observed partial specific volume for the neutral protein we calculated (and present in Table VIII) the following parameters per 74.9 kg of isoionic protein: the weight and volume of the protein salt obtained by adding base or acid to the protein as per the estimated titration curve; the values w_{Sc}, the weight, and v_{Sc}, the volume of the Scatchard et al. (1946) components[4] corresponding to 74.9 kg of isoionic hemocyanin; the values of $\bar{v}_{Sc}$, the partial specific volume of the Scatchard component (as the ratio of v_{Sc}/w_{Sc}) and the corresponding buoyancy factor $(1 - \bar{v}_{Sc}\rho)$. The observed values of σ (see Tables I–III) then yield M_{Sc} the molecular weights of the Scatchard components on the assumption that there is no preferential binding of any solvent components by the hemocyanin. These Scatchard molecular weights are then multiplied by the ratio (74.9 kg/the weight of the Scatchard component per 74.9 kg of isoionic protein) to obtain M_p, the number of grams of neutral, isoionic protein per mole of species under consideration. This procedure accounts for the change of electrostriction on titration of the protein, for the counterions associated with the protein, and for the appropriate component definitions needed to interpret the experiments. However, it specifically neglects any volume changes on association and any preferential binding of solvent components to the hemocyanin. The estimated degree of polymerization, $\hat{n}$, of the protein can now be obtained as the ratio of the values of M_p for the polymer to M_p for the monomer.

Unfortunately we are aware of no adequate theory that is available for solvents with significant amounts of more than one electrolyte. Accordingly we present two calculations for the pH 10.6 system, where there are comparable amounts of two electrolytes: in one calculation we assume the electrolyte to be all $NaHCO_3$, in the other all Na_2CO_3. The values calculated for M_p in the two cases differ only moderately. Accordingly, we take our best estimate of M_p in this system to be 69 400 (66 300 to 72 000), the average of the two calculated values of M_p, and include the extremes of the individual ranges as the ranges of the average. Similarly in order to be able to apply extant theory, we assume for the purposes of a charge correction that the electrolyte at pH 5.0 and at pH 7.5 is all KCl, and thus neglect the small contributions of the buffer components to these corrections. In all these calculations the buoyancy factors were estimated using the actual value of the solvent density.

Table VIII also lists the apparent uncorrected molecular weights estimated using the partial specific volume of the isoionic hemocyanin. The charge correction is negligible at pH 5.0 (and of even less importance at pH 6.1), significant at pH 7.5 and sizable at pH 10.6, where it amounts to over 6% of the molecular weight of the monomer. Our best estimates for the size of the monomer are in good agreement with values obtained in the early determinations. The molecular weights of the larger components are consistent with the interpretation of the electron microscopic data that the 60S aggregated state is a dimer of the 36S aggregated state which, in turn, is a dimer of the 24S component. We were unable to find buffer condi-

[4] For a negatively charged protein with charge $-Z$ in the presence of a simple electrolyte (with a positive ion, $B^{+\nu_+}$, of valence ν_+ and a negative ion, $X^{-\nu_-}$, of valence ν_-), this Scatchard component is $P^{-(Z)}B^{\nu_+}_{Z/(\nu_+ + \nu_-)}X^{\nu_-}_{Z/(\nu_+ + \nu_-)}$. This component can be formed from the usual macromolecular salt, $P^{-(Z)}B^{\nu_+}_{Z/\nu_+}$, by removal of $Z/(\nu_+ + \nu_-)$ moles of the salt $B_{\nu_-}{}^{+\nu_+}X_{\nu_+}{}^{-\nu_-}$. The usual macromolecular salt is generated, in turn, by titration of the neutral macromolecule P.

tions where the 16S component existed in significant quantities once the hemocyanin was purified. One possible explanation is that the 16S component reported in the literature actually may be the 14S hemagglutinin (Marchalonis & Edelman, 1968). By analogy with other arthropod hemocyanins and from the electron micrographs it is likely that *Limulus* hemocyanin can exist as a 16S component. The ratio of the molecular weights of the 24S and 6S components is 12.3 (+0.9, −0.7). Consequently, the assumption that the 16S component is one-half the size of the 24S component would require the 16S component to be a hexamer of the 6S component.

The heterogeneity of the 24S–36S–60S association (see Figure 3) probably does not arise from the impurity in the hemocyanin preparation, since the same preparation of hemocyanin was used for the 24S–36S association (see Figure 2) which showed much less heterogeneity. It should be pointed out that the effects of heterogeneity in association constant are very difficult to observe with relatively small degrees of association (as in Figure 2 and Table IV) but more readily visible with more extensive association (as in Figure 3 and Table VI) (Yphantis & Johnson, 1971). Some of the monomer species found by Sullivan et al. (1976) occur as approximately 4% of the preparation. This corresponds to an average of one of these monomers per 36S complex. Thus the 24S dodecamers differ in composition among themselves. This compositional difference may well be the source of the observed heterogeneity in association behaviour of these 24S units. Recent experiments on the reassembly of large aggregates from the purified monomer fractions indicate that several of the fractions are capable of substituting for each other (Schutter et al., 1976). This observation lends credence to the hypothesis that each of the dodecamer molecules consists of a mixture of different types of subunits.

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