Molecular Weights of Aggregation States of Busyccon Hemocyanin*

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Molecular weights of all hemocyanin aggregates which can be homogeneously isolated have been measured by sedimentation equilibrium. The larger aggregates, which are the ones present under physiological conditions, are, to a very close approximation, integral multiples of a 4.4 x $10^6$-dalton, 60 S species. Dissociation of the 60 S species at high pH gives heterogeneous samples in which the smallest species has a molecular weight of 300,000. The smallest subunit which can be produced in denaturing solvents also has a molecular weight of 300,000.

The existence of multiple aggregation states of hemocyanin has been recognized for many years. Work on these proteins has been the subject of a recent comprehensive review (1). Reproducibly occurring species of molluscan hemocyanins can be identified by their sedimentation coefficients. Predominant species occurring near neutral pH have sedimentation coefficients of 100 S and 60 S and these interconvert in response to changes in solution ligand concentrations, H*, divalent cations, and oxygen favoring the larger aggregate. Molecular weight studies by various methods, including light scattering and sedimentation/diffusion, some of which were among the earliest made of proteins, place the molecular mass of the 100 S aggregate at about $9 \times 10^6$ daltons and of the 60 S particle at about half of this. There can be little doubt that these numbers are approximately correct.

Electron microscope studies show that the 100 S particle is a right circular cylinder 940 to 380 Å high and 260 to 300 Å in diameter and the 60 S particle appears as a cylinder of the same diameter and half the height (1). The weight, geometry, and hydrodynamic properties of the 100 S particle are self-consistent. Thus, 100 S and 60 S aggregates have come to be regarded as dimer and monomer having respective molecular weights of 9 and $4.5 \times 10^6$, although the molecular weight of the 60 S species has not been measured precisely.

Larger aggregates of the same cylindrical form but longer on the cylinder axis by increments the height of the 60 S particle have been observed in electron microscope preparations, and boundaries sedimenting at 130 S, 150 S, and faster have been observed in the ultracentrifuge (1). It is reasonable to assume on hydrodynamic grounds that these observations correspond to the same molecular species. Molecular weights of these have not been measured. Under some conditions very long arrays of "stacked" cylinders can be produced.

Increasing pH above the physiological range leads to dissociation of the 60 S particle to species having sedimentation coefficients between 13 S and 19 S. Sophisticated electron microscopy correlated with sedimentation work indicates that the predominant dissociation products of the 60 S molecule correspond to tenths and twentieths of the 100 S molecule and that different conformational forms of each of these exist, which accounts for the range of sedimentation coefficients observed. The observation of 5- and 10-fold symmetry in the larger aggregate has contributed heavily to conclusions about the stoichiometry of 60 S subunits. (The established convention regards the 100 S as the unitary particle; thus the 60 S is the half, etc.) An elegant model of the subunit architecture of the molluscan hemocyanins has emerged from this work (2, 3).

Two problems remain. First, the molecular masses of the putative 19th and 20th molecules have been measured to be 730,000 and 365,000 daltons, respectively (2). Although these molecular masses seem too low for tenths and twentieths, the combined uncertainty in all of the measurements does not permit the conclusion that the data are inconsistent with the model inferred from electron microscope observations.

The second problem is that measurements of the molecular weight of the ultimate subunit produced in denaturing solvents exhibit considerable variability and do not fit in a convincing way into the proposed model. The molecular weight of the subunit has been reported as 220,000 by sedimentation/diffusion in Gdn·HCl (4) and 240,000 to 300,000 by gel filtration in Gdn·HCl, gel electrophoresis in SDS, and sedimentation equilibrium in Gdn·HCl (5, 6).

Stoichiometric ratios of copper, oxygen, and protein predict that the ultimate copper-binding subunit would have a molecular weight of 22,000 (2), and the minimum oxygen-binding subunit, one of 50,000. Brouwer and Kuiper (5) suggested the possibility that the minimum covalent subunit contains a number of independent conformational and functional domains. Electron microscopy (2) and limited proteolysis studies (2, 6, 7) indicate that the high pH dissociation products of the 60 S particle have a number of globular, 50,000-dalton subunits connected by short, nonglobular segments of polypeptide chain which are presumably labile to proteolysis. The number

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of functional subunits in the minimum polypeptide, if it is organized in domains, is uncertain. The data of Brouwer and Kuiper (5) suggest that it would be five to six; those of Siezen and van Bruggen (2) indicate that it would be seven to eight. A twentieth of the 100 S species should contain about nine.

These deductions assume, of course, that subunits are identical; interpretations of the electron micrographs of the cylindrical aggregates do not rule out the possibility that some material, not identical to the major type of subunit, is present in the form of a "collar" or "cap" on the ends (2, 3), and the presence of such material could be the reason why the major dissociation products of the larger aggregates have smaller than expected weights.

It seems appropriate to re-examine the molecular weights of the molluscan hemocyanin subunits using techniques sensitive to heterogeneity and nonideality and preparations in which precautions have been taken to prevent proteolysis. Molecular weights for the 60 S, 100 S, and 130 S species, high pH dissociation products, and the smallest coherent subunit are presented in this paper. They suggest an organization of the molecule somewhat different than that currently proposed.

MATERIALS AND METHODS

Hemocyanin Preparation—Busyccon canaliculatum were purchased from the Marine Biological Laboratory, Woods Hole, Mass., and kept in a salt water aquarium until used. Hemocyanin was collected directly from the heart cavity after excision of the heart. In puncturing the shell and opening the heart cavity, care was taken to avoid damaging the surrounding tissue and the animal was kept chilled during the collection procedure. The hemocyanin concentration in the serum is 20 to 50 g/liter and a single animal will yield up to 0.5 g of hemocyanin. The blood was chased from the Marine Biological Laboratory, Woods Hole, Mass., and kept in a salt water aquarium until used.

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RESULTS AND DISCUSSION

Fractionation of Hemocyanin Aggregates—Preliminary experiments with Busyccon hemocyanin revealed that subunit interactions of this protein conform to the pattern observed for other molluscan hemocyanins; aggregation and disaggregation may be readily effected by changes in solution ionic concentrations, but not by changes in protein concentration. This observation suggests that the various sized aggregates may be separated from one another (18) and Fig. 1 shows the results of such an experiment carried out using the technique of gel filtration. The sample applied to the column was resolved, incompletely, into zones reflecting the original composition. Sedimentation velocity analysis of the three zones showed that the leading edge of the compound peak, Fractions 89 and 90, contained only 100 S species, Fractions 110 to 111 contained 100% 100 S and 90% 60 S, and the trailing zone, centered at Fraction 152, contained material sedimenting at 15 S. The distributions observed in the fractions are stable as long as ligand concentrations are held constant, at least over a period of many days.

Partial Specific Volumes—The measured values for apparent partial specific volumes, \( \phi_{d, \mu} \), of native species agree well with those for Helix (2) and with the \( \phi = 0.727 \) ml/g calculated from the amino acid composition (6). The value in Gdn·HCl is lower by 0.028 ml/g than that for native species, a larger difference than is usual (10). Lee and Timasheff (10) have proposed a rationale by which absolute amounts of Gdn·HCl and water bound to the protein, and hence the preferential interaction parameter \( \xi = (c_1 g_1 + c_2 g_2) \), may be calculated from the amino acid composition. The published amino acid composition of Busyccon hemocyanin does not distinguish between free carboxyls and amides, so the proportions of these were estimated from the isionic point, which is about pH 5 in dilute salt solution. From this information we can calculate (19) that the net charge on the protein at pH 5 is rather small, so that the net negative charge contributed by carboxyl groups

\[ R. Roxby, unpublished results. \]
and denaturant solutions. The remainder of the difference is presumably produced upon transfer of the native protein to a-Gdn.HCl,-pH-7.0 - Gdn HCl

Extinction coefficients and specific refractive index increments of *Busovon* hemocyanin

<table>
<thead>
<tr>
<th>Aggregate</th>
<th>(\epsilon_2)</th>
<th>(\rho^n)</th>
<th>(\alpha_3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkylated polypeptide chains in Gdn-HCl</td>
<td>276.0</td>
<td>1.383^-a-</td>
<td></td>
</tr>
<tr>
<td>Unreduced polypeptide chains in Gdn-HCl</td>
<td>276.0</td>
<td>1.389^-a-</td>
<td>0.746^-a-</td>
</tr>
<tr>
<td>Native hemocyanin^-b-</td>
<td>1.44</td>
<td>0.87</td>
<td>0.727 + 0.004</td>
</tr>
<tr>
<td>60 S</td>
<td>1.55</td>
<td>254.9</td>
<td>0.728 + 0.005</td>
</tr>
<tr>
<td>100 S</td>
<td>1.65</td>
<td>0.17</td>
<td>0.699 + 0.005</td>
</tr>
<tr>
<td>130 S</td>
<td>1.76</td>
<td>1.35</td>
<td></td>
</tr>
<tr>
<td>Specific refractive index (\Delta n/\Delta c)</td>
<td>4.08</td>
<td>0.186</td>
<td></td>
</tr>
</tbody>
</table>

-a Corrected for light scattering by the method of Leach and Scheraga (11).
-b See "Materials and Methods."
-c Calculated from the formula \(J = \Delta n/\Delta c\), where \(\Delta n\), the light path length is 1.2 cm and \(\Delta c\), taken to be 546 nm, was isolated with a Wratten No. 77a filter.

![Fig. 1. Elution pattern of hemocyanin. A sample originally containing 40% 100 S, 45% 60 S, and 15% 15 S was applied to a column (90 X 2.5 cm) containing 4% agarose (A-15m. Bio-Rad) equilibrated with Tris buffer, pH 8.0. Fraction size was 1.8 ml.](http://www.jbc.org/)

approximately balances the positive charges contributed by histidine, lysine, arginine, and \(a\)-amino groups, and thus that about 65% of the total glutamic acid + glutamine and aspartic acid + asparagine reported are present as free carboxyl groups. Alloting these to glutamic acid and aspartic acid proportionately, the calculated value for hemocyanin is \(\xi_2 = 0.15\ g/g\) of Gdn·HCl/g of protein. This is similar to the largest values found by Lee and Timasheff (10) (for chymotrypsinogen, \(\xi_2 = 0.15\ g/g\); for \(\alpha\)-chymotrypsin, \(\xi_2 = 0.17\ g/g\) and accounts for most of the difference in \(\Delta n/\Delta c\) between dilute salt and denaturant solutions. The remainder of the difference is presumably produced upon transfer of the native protein to denaturant, *i.e.* represents the difference between \(\rho^n\) and \(\alpha_3\) in the notation of Lee and Timasheff (10). In order to account for the remainder of the difference, assuming the contribution of preferential interactions is as calculated above, this volume change would have to be 0.011 ml/g, again a relatively large but not unprecedented value. Thus, the experimental result appears to be reasonable.

**Molecular Weights of Native Protein Species**—The result of Fig. 1 indicates that, in principle, pure samples of any of the larger hemocyanin aggregates can be prepared in homogeneous form and that, since they will neither associate or dissociate in response to concentration changes, molecular weight measurements by sedimentation equilibrium should be straightforward, and this technique will, in addition, provide a stringent test for re-equilibration of the fractionated samples.

**100 S Aggregate**—Hemocyanin prepared by gel filtration chromatography in Tris buffer, pH 7.8, containing 0.01 M Mg\(^{2+}\) and found to contain only a single, 100 S boundary in a sedimentation velocity experiment, was used in sedimentation equilibrium experiments at different rotor speeds and initial protein concentrations. Plots of log \(f\) versus \(r^2\) were linear to a very close approximation in all cases. The data from an experiment carried out under optimal conditions of speed and concentration are shown in Fig. 2, A to C. In these plots the difference between the observed concentration at each radial position and the concentration calculated at the same point from the linear least squares lines fitted through the log \(f\) versus \(r^2\) data is plotted as a function of log concentration. This means of representing the data is chosen for its sensitivity in revealing the presence of inhomogeneity; if the 100 S samples had contained as much as 1% of the monomer or trimer (see below), a systematic deviation of the points from the horizontal line would have been detectable. Thus, within that limit, no detectable association or dissociation of the 100 S molecule has taken place within the period of several days required to conduct the experiment. The greater scatter of the points at high concentrations reflects the decreased precision in determining fringe positions in a region of large concentration gradient. Furthermore, while it is conceivable that conditions in a single experiment might be such that slight heterogeneity could be masked by nonideality to give the appearance of a homogeneous sample, it is very unlikely that this could be so in three samples at different initial concentrations, and we have never detected any evidence of such a complication over a range of rotor speeds and concentrations.

In the inner and center channels, the fringe positions were measurable to the bottom of the solution column. The molecular weight of the 100 S aggregate appears to be \(8.8 \pm 0.1\) x 10^6.

**60 S Aggregate**—This material was obtained in the gel filtration experiment described in Fig. 1. Since all fractions contain some 100 S material, a relatively high concentration fraction was chosen (0.8 mg/ml) and the experiment was carried out at a high rotor speed, conditions which permit estimation of the molecular weight of the smaller component (13). A linear log \(f\) versus \(r^2\) plot is obtained in the region of the cell in which fringe displacements could be measured and the molecular weight calculated was 4.35 x 10^6 (Fig. 2D).

**130 S Aggregate**—The sample for this measurement was obtained by twice fractionating a protein sample at pH 7.6, with 0.1 M Mg\(^{2+}\) present, on agarose A 15m. The initial sample contained 75% 100 S and 25% 130 S. The leading fraction obtained in the first fractionation contained some 150 S, which
Molecular Weights of Hemocyanin Aggregates

A) 100 S, \( M = 8.71 \times 10^6 \)

C) 100 S, \( M = 8.90 \times 10^6 \)

E) 100 S, \( M = 8.77 \times 10^6 \)

D) 60 S, \( M = 4.35 \times 10^6 \)

F) 130 S, \( M = 12.8 \times 10^6 \)

FIG. 2. Hemocyanin at sedimentation equilibrium. Data are presented as \( S_f \), deviation of the measured fringe displacement from a linear least squares line fitted to the \( \log f \) versus \( r^2 \) data, as a function of \( \log f \). The ordinate scale is the same on each plot and is indicated on part c. Points represented by solid circles were used to calculate molecular weight. A, B, and C are from inner, middle, and outer channels of an experiment on 100 S hemocyanins carried out at 2200 rpm; D and E, respectively, are from measurements on 60 S at 3200 rpm and 130 S at 1600 rpm.

was probably present in the original sample but at concentrations too small to be detectable in the sedimentation velocity experiment. Concentration and rechromatography of fractions containing only 150 S and 130 S did not yield a fraction containing only 130 S, so the same strategy was applied that was used to determine the 60 S molecular weight in the presence of 100 S. Again (Fig. 2E), a linear dependence of \( \log f \) on \( r^2 \) was found in the region of the cell in which fringes were resolved. The molecular weight is \( 12.8 \times 10^6 \).

Dissociation Products of the 60 S Species—At higher pH hemocyanin dissociates to products having sedimentation coefficients between 13 and 19 S. The dissociation is progressive with increasing pH (Fig. 3). Sedimentation coefficients at constant pH exhibit only the small, negative dependence on protein concentration which would be expected of nonassociating solutes. Examination of samples at high pH by sedimentation equilibrium always reveals them to be heterogeneous to some extent. In Fig. 4, results of two typical experiments are presented as plots of apparent weight average molecular weight \( M_{w, \text{app}} \) against concentration. Below pH 9 the samples are grossly heterogeneous. Protracted dialysis at pH 9 or above yields a less extensively heterogeneous sample in which the minimum molecular weight approaches 300,000. Plots of \( M_{w, \text{app}} \) obtained from the same sample at different speeds or different loading concentrations do not overlap, indicating that, like the larger aggregates, these smaller subunits do not associate reversibly. Attempts to analyze these plots as the sum of two independent components have not been successful. We conclude that the dissociation of hemocyanin at high pH yields mixtures of several components of which the smallest has a molecular weight near 300,000. Attempts to measure the molecular weight of the smallest component were not as successful as for 60 S and 130 S components because samples could not be as extensively enriched in the smallest component.

Molecular Weight of Minimum Covalent Subunit—The molecular weight of reduced and alkylated hemocyanin in 6 M Gdn·HCl was also measured by sedimentation equilibrium. The apparent liability of dissociated hemocyanin to degradation was minimized by rapid processing of samples in the cold. Proteolysis inhibitors, phenylmethylsulfonyl fluoride, iodoa-
from the calculations of reciprocal moments. In the main part of the figure reciprocal molecular weights moments \(1/M_\alpha\), \(1/M_\beta\), and \(1/M_\gamma\) are plotted as a function of concentration. The slopes of these plots have the expected relationship to one another and the direction of the deviation is toward lower molecular weights. Extrapolation gives \(M = 300,000\). The value of the virial coefficient, \(B\), is \(7.7 \times 10^{-4}\) mol cm\(^2\)/g\(^2\), in good agreement with the statistical value for the flexible polymer in a good solvent (20).

### CONCLUSIONS

These measurements affirm, with a high degree of precision, that the larger hemocyanin aggregates are a series of incremental polymers of the 60 S particle, which has a molecular weight of \(4.4 \times 10^6\). The molecular weight of the smallest subunit obtainable in denaturing solvents is close to 300,000. No fractionation was involved in the preparation of the reduced and alkylated subunits in Gdn-HCl; therefore, any nondialyzable component of the native hemocyanin should be present in the final sample. The data are complicated by nonideality, but consistent with the presence of a single component. Thus it appears that the subunits of the 60 S aggregate are all of about the same size. There is 1 copper atom/25,000 g of Busycon hemocyanin. Assuming that Busycon, like other hemocyanins, contains 2 copper atoms/oxygen binding site, the minimum polypeptide chain would contain six functional units, presumably in individual domains (3, 6).

The respective molecular weights suggest that the 60 S particle contains about 15 subunits. The combined uncertainties in the partial specific volume and sedimentation equilibrium data do not permit a precise statement about the number of subunits, but the presence of 5-fold symmetry in the 60 S and larger aggregates, which is seen in the electron microscope preparations (20), makes the number 15 a more attractive hypothesis than other numbers within the experimental error.

Studies of the 60 S dissociation products at high pH agree with this model. These preparations exhibit varying degrees of heterogeneity with the smallest component having a molecular weight near 300,000. These dissociation products all bind oxygen, thus, the minimum functional subunit appears to be the same size as the subunit obtained in denaturing solvents. These measurements differ from observations of Helix hemocyanin (2) in several respects. First, we do not find discrete stages in the dissociation process at high pH; disruption of the 60 S particle gives broadly heterogeneous samples which only approach homogeneity upon maximum dissociation, after prolonged exposure to high pH. Second, our results, taken as a whole, suggest that dissociation of the 60 S particle could only proceed through aggregates which are integral multiples of a 300,000-dalton subunit. A "\(1/15\)" subunit, molecular weight 900,000, could exist, but a "\(1/5\)" subunit could not. Helix and Busycon hemocyanins could differ in structure and in the way they dissociate, but the electron microscope studies reveal no substantial differences (3).

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### REFERENCES

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