Biochemistry VOLUME 3, NUMBER 12

January 8, 1965

Physical Studies of Hemocyanins. I. Characterization and Subunit Structure of *Loligo pealei* Hemocyanin*

K. E. Van Holde and L. B. Cohen†

From the Marine Biological Laboratory, Woods Hole, Mass.; the Department of Chemistry and Chemical Engineering, University of Illinois, Urbana; and the Department of Zoology, Columbia University, New York, N. Y.

Received May 21, 1964

The hemocyanin from the squid *Loligo pealei* has been investigated by sedimentation and light scattering. In neutral or slightly acidic solutions the protein exists primarily in a form with M = 3,750,000 and $s^\circ_{20,w}$ = 58.7 S. In solutions more alkaline than *p*H 7 dissociation occurs, first into "$^1/_5$" molecules with $s_{20,w}$ = 19 S and M = 770,000, and, at high *p*H, into "$^1/_{10}$" molecules, with $s^\circ_{20,w}$ = 11.1 S and M = 385,000. The overall dissociation is accompanied by a decrease in the partial specific volume from 0.740 to 0.710 cc/g, but there is no pronounced change in tertiary structure. The presence of magnesium ion, in 0.01 M concentration, stabilizes the 58 S structure until *p*H 10, at which point dissociation into the 11 S component occurs.

Hemocyanins, which are respiratory pigments found in a wide variety of invertebrate species, were among the first proteins to be studied in detail by physical methods. The early researches of Svedberg and collaborators (see, for example, Svedberg and Pedersen, 1940) showed that many of these proteins were monodisperse substances of very high molecular weight, which could in many cases be dissociated into much smaller subunits. The current interest in subunit structure of proteins has been largely directed toward the study of relatively simple structures, most often proteins which are dimers, trimers, or tetramers. It would seem that this is an appropriate time to reopen the investigation of the complex subunit structure of the hemocyanins, making use of the many advances in physical techniques and their interpretation which have been made in the past 30 years. Considerable progress has already been made by electron microscopy (see, for example, Van Bruggen *et al.*, 1962a, 1962b, 1963), but it is necessary also to make use of techniques which will allow studies of structural changes in solution.

In remarkable contrast to the advanced state of our knowledge about the oxygen binding site in the iron containing heme proteins, very little is known about the mechanism of oxygen binding by the hemocyanins. The mode of attachment of the copper to the protein and even the oxidation state of the copper have been matters of controversy (see, for example, Klotz and Klotz, 1955, 1956; Williams, 1955; Lontie, 1958; Felsenfeld, 1960).

We have chosen the hemocyanin from the common Atlantic squid, *Loligo pealei*, for extensive investigation. A first aim is to elucidate the subunit structure of this protein, and to study the equilibria and kinetics of the dissociation and reassociation. A second objective is to make use of modern physical methods to attack the problem of the mechanism of oxygen binding, the copper-oxidation state, and the mode of binding of copper to the enzyme. The first paper in this series describes sedimentation and light-scattering studies of the hemocyanin and some of its dissociation products.

Experimental

Preparation of Hemocyanin.—Blood was taken from living squid by puncture of the post cava. About 1 cc, containing approximately 100 mg of hemocyanin, was obtained from each medium-sized (30-cm) animal. In each preparation, blood from several squid was pooled and centrifuged at low speed for a few minutes to remove particulate matter. Samples to be used within a few weeks were stored at about 5°. Longer periods at this temperature resulted in changes in the spectrum of the blood. For longer storage, the material was frozen and kept at −10°. Material which had been frozen was used in only a few of the experiments, specifically the determination of $\bar{v}$ at *p*H 10.6 and of the optical rotatory dispersion data. Samples to be used for experiments not requiring ultracentrifuge runs were checked by ultracentrifugation in *p*H 10.6 bicarbonate buffer. The observation of a single, symmetrical boundary with $s_{20} \cong$ 11 S (see Results) was used as a criterion of sample stability.

The hemocyanin appears to represent nearly all of the protein in the blood, as has been reported in other

* This research was supported in part by a grant (A3096) from the U. S. Public Health Service. One of the authors (LBC) received assistance in the form of a National Science Foundation Cooperative Fellowship. Preliminary results of this research have appeared as abstracts (Cohen and Van Holde, 1962, 1963).

† Send reprint requests to Mr. Cohen at Columbia University.

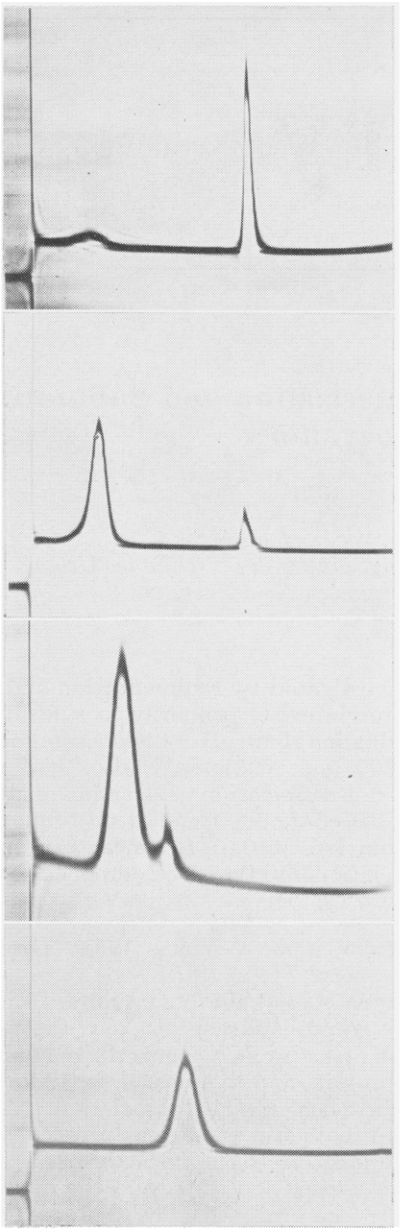

FIG. 1.—Sedimentation diagrams of *Loligo pealei* hemocyanin under various circumstances. (A) Blood diluted into 0.1 ionic strength phosphate buffer; *p*H 6.6. Sedimented for 12 minutes at 42,040 rpm. (B) Blood diluted into 0.1 ionic strength phosphate buffer; *p*H 7.2. The sedimentation experiment was started 10 hours after dilution. Sedimentation has proceeded for 12 minutes at 42,040 rpm. (C) Blood diluted into 0.1 ionic strength bicarbonate buffer; *p*H 8.9. Centrifuged for 12 minutes at 59,780 rpm. (D) Blood diluted into 0.1 ionic strength bicarbonate buffer; *p*H 10.7. Centrifuged for 56 minutes at 42,040 rpm.

cases (Ghiretti, 1962). Figure 1A shows a sedimentation pattern of blood diluted directly into 20 volumes of phosphate buffer (*p*H 6.6). The slowly sedimenting material, which represents only about 5% of the area under the schlieren diagram, appears to be identical with the dissociation products of the native hemocyanin which are observed in more alkaline solutions. Most of the experiments described herein were carried out with blood diluted directly into appropriate buffers.

Preparation of Solutions.—Buffer solutions were prepared according to standard recipes; in the various *p*H ranges the following were employed: acetic acid–sodium hydroxide (*p*H 4.5–5.5), potassium dihydrogen phosphate–sodium hydrogen phosphate (*p*H 5.5–7.6), Tris-hydrochloric acid (*p*H 7.6–9.0), and sodium bicarbonate–sodium hydroxide (*p*H 9.0–10.9). All buffers were 0.1 ionic strength, except in cases where 0.01 M $MgCl_2$ was added; in these the total ionic strength was 0.125.

Concentrations of the hemocyanin solutions were routinely determined from optical density measurements at 280 mμ. In order to calibrate this method, samples of the whole blood were dialyzed exhaustively against distilled deionized water, evaporated to dryness, and then dried to constant weight at 100–110°. Aliquots of the same dialyzed sample were diluted into phosphate buffer at *p*H 6.6 for optical density measurement. A value of $E_{1\,cm}^{1\%} = 15.8$ was obtained. Since the observed optical density is in part a consequence of scattering, and since the scattering depends upon the size of the particles present, the apparent extinction coefficient depends somewhat upon *p*H. The value given is appropriate when the 58 S material (see Results) predominates; if the 19 S or 11 S species were dominant, repeated experiments gave an average value of 14.5.

Sedimentation.—All experiments were carried out near 20°, using a Spinco Model E ultracentrifuge equipped with an RTIC unit and phase-plate schlieren optics. Filled-epoxy or Kel-F centerpieces were used. Sedimentation coefficients were corrected to $s_{20,w}$ values by the usual procedures. Where reliable literature values were not available, densities and relative viscosities of buffers were determined experimentally. Nearly all the sedimentation velocity experiments were performed at 42,040 rpm. Most of the sedimentation equilibrium experiments were carried out at 5785 rpm, using solutions columns 1–3 mm in height, in a double-sector cell. Photographic plates were measured with a two-way comparator.

Partial Specific Volume.—Solution density measurements were carried out in a pycnometer fashioned by replacing the neck of a 25-ml Erlenmeyer flask by a 1-mm (i.d.) capillary tube. The pycnometer was calibrated with distilled deionized water. All densities were measured at 25.00 ± 0.01°; weighings were always against an identically treated empty pycnometer, and were corrected for air buoyancy. The solutions to be used were dialyzed for several days against several changes of the appropriate buffer. Results are summarized in Table I, where the apparent specific volumes are given. Since the last figure in ϕ_v is uncertain, results were averaged to give $\bar{v} = 0.740$ cm³/g at *p*H 6.6 and $\bar{v} = 0.710$ cm³/g at *p*H 10.6.

TABLE I
PARTIAL SPECIFIC VOLUME

Buffer	Protein Concn (mg/ml)	ϕ_v
Phosphate, *p*H 6.5	10.1	0.73_6
	11.9	0.74_3
Bicarbonate, *p*H 10.6	8.9	0.70_6
	13.7	0.71_3

Light Scattering.—A Phoenix Precision Instrument Co. photometer was employed for the light scattering measurements. Light of wavelength 436 mμ was used, since the absorption of the hemocyanin is near a minimum in this region. Absorption corrections were calculated, and found to be negligible at the concentrations used. No appreciable fluorescence of the hemocyanin was found at this wavelength. Square or semi-octagonal scattering cells were employed. The dissymmetry of scattering was found to be too small for

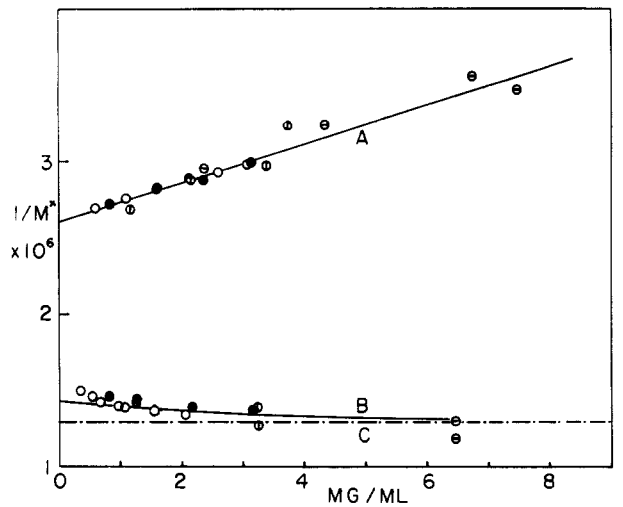

FIG. 2.—Apparent molecular weights for light scattering and sedimentation equilibrium for the 11 S component at *p*H 10.6 (A) and the 19 S component at *p*H 6.6 (B). The open and solid circles represent light scattering data, with solutions clarified by centrifugation and filtration, respectively. Other points are from sedimentation equilibrium, as follows: —⊙— $1/M_w^*$ versus $(c_a + c_b)/2$; —⊖— $1/M_z^*$ versus $(c_a + c_b)$. For the 11 S material $\bar{v} = 0.710$; for the 19 S material the upper sedimentation equilibrium points were obtained assuming $\bar{v} = 0.710$, the lower assuming $\bar{v} = 0.740$. The broken line (C) corresponds to a value of M = 770,000, twice the molecular weight of the 11 S component.

accurate measurement; hence only data obtained at 90° were used for computation. The small dissymmetry is in accord with the results of calculations based on reasonable dimensions of the particles (see Discussion) which would indicate that the greatest dimension of the largest molecule studied is only about 1/15 λ. The calibration of the scattering photometer was checked by a determination of the molecular weight of bovine serum albumin (Armour, 5 × recrystallized). A value of M_w = 69,500 was obtained, which is in good agreement with other light scattering results (see Dandliker, 1954). The residual refraction correction (R_w/R_c) (Brice *et al.*, 1950) was assumed to be 1.054; recently Tomimatsu and Palmer (1959, 1961) have suggested somewhat smaller values, dependent upon the type of cell employed. This would, on the average, decrease the molecular weights reported here by about 3%.

Solutions for scattering were clarified in two ways: by filtration of individual solutions through millipore filters, or by centrifugation of a concentrated stock solution, which was then added to filtered solvent in the scattering cell. As can be seen in Figure 2, the two methods seemed to give results indistinguishable within experimental error.

The specific refractive index increment for the hemocyanin was determined with a Phoenix Precision Instrument Co. differential refractometer, which was calibrated with aqueous KCl solutions. Measurements on hemocyanin solutions were carried out at *p*H 6.65 (0.1 ionic strength phosphate buffer) and at 10.6 (0.1 ionic strength bicarbonate buffer). In each case, solutions were dialyzed against several changes of the appropriate buffer; the buffer used in the final dialysis was used for a reference.

Within experimental error the average value of dn/dc was 0.197 cm³/g at λ = 436 mμ for both *p*H values. We estimate the error in this number to be less than 1.5%.

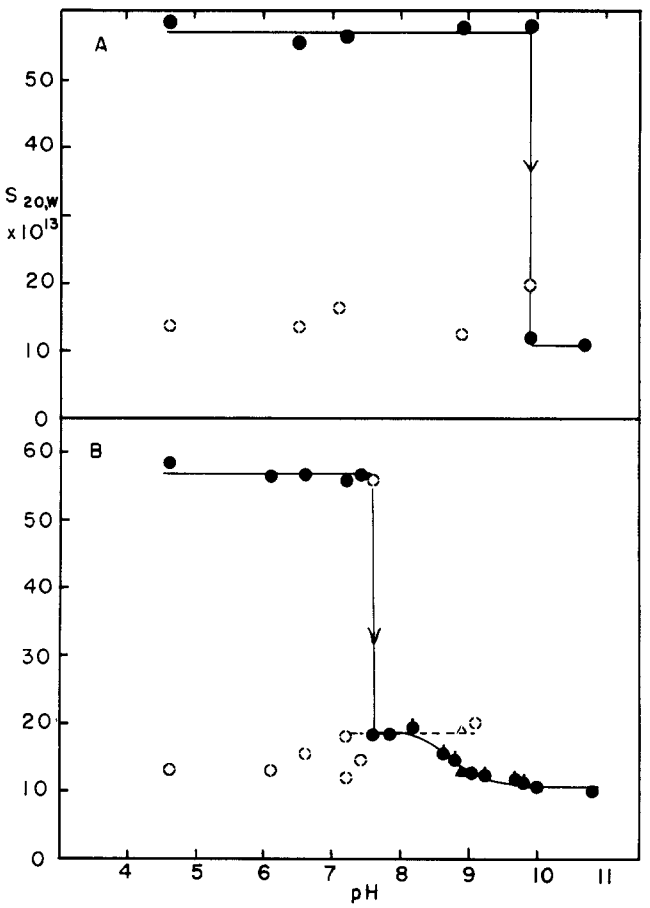

FIG. 3.—Diagrams of sedimentation coefficient versus *p*H in the presence (A) and absence (B) of 0.01 M $MgCl_2$ in the buffer. In each case the solid circles represent the sedimenting boundary present in greatest amount. All data were obtained at a total protein concentration of about 3.5 mg/ml, except for the points with flags, which represent a separate series of experiments in which concentrations were about 0.7 mg/ml. All experiments at 42,040 rpm, except for that indicated by triangles, which was at 59,780 rpm; most were run about 30 minutes after dilution into buffer.

RESULTS

The pH Dependence of the Hemocyanin Structure.—Figures 1 and 3B show how the sedimentation behavior of *Loligo pealei* hemocyanin depends upon *p*H. In neutral or slightly acidic solutions schlieren patterns resembling Figure 1A are observed; the principal component has a sedimentation coefficient of approximately 57 S at the concentration (approximately 3.5 mg/ml) used for the points on Figure 3. Extrapolation to infinite dilution yields a value of $s^0_{20,w}$ = 58.7 S (Fig. 4). The minor component under these conditions is a broad, slowly sedimenting boundary of average sedimentation coefficient about 15 S. Under some conditions it can be resolved into two peaks.

When the serum is diluted into buffers with *p*H >7.0, dissociation into more slowly sedimenting species occurs, as can be seen from Figure 3B. At *p*H 7.2 a slow dissociation is observed; the freshly prepared solution resembled Figure 1A. After 10 hours in the cold, the schlieren pattern shown in Figure 1B is found; most of the 58 S component has disappeared, and the predominant peak sediments with a velocity of 19 S. In more alkaline solutions the dissociation is complete and very rapid. In the *p*H range 7.8–10 the average sedimentation coefficient decreased with increasing *p*H. In this

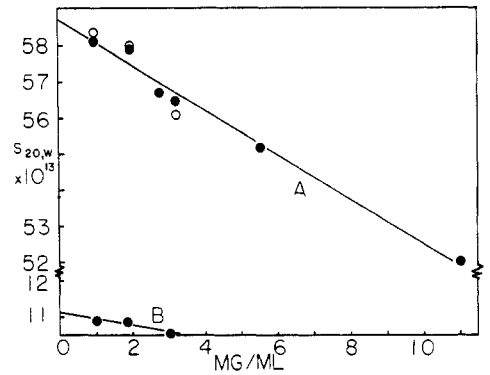

FIG. 4.—Sedimentation coefficient versus concentration for the 11 S and 58 S components. The open circles correspond to solutions which are 0.01 M in $MgCl_2$.

range broad boundaries, often partially resolved into two components, are observed (Fig. 1C). Above *p*H 10.0 only a single, symmetrical boundary (Fig. 1D) is found. The sedimentation coefficient of this species, upon extrapolation to infinite dilution, is found to be 11.1 S (Fig. 4).

As can be seen from a comparison of Figure 3A and B, the presence of magnesium ion has a marked influence on the stability of the 58 S material. While we will discuss the role of Mg^{2+} in the 58 S–19 S equilibrium in more detail in a subsequent paper, the salient facts are these: (a) The presence of 0.01 M $MgCl_2$ appears to stabilize the 58 S component, preventing dissociation up to *p*H 10, at which point the 11 S material is formed. (b) On the other hand, the magnesium ion does not seem to change appreciably the sedimentation coefficients of the components. This is perhaps best illustrated on the expanded scale of Figure 4, where the open circles correspond to solutions with 0.01 M $MgCl_2$, the filled circles to those without.

Characterization of the Components.—There is considerable evidence to indicate that the 58 S, 19 S, and 11 S components represent particularly stable subunit assemblies. Therefore these were chosen for detailed characterization. The principal method employed for molecular weight determination was light scattering, since preliminary experiments showed that there would be great difficulty in determining the "molecular weight" of a structure as large as the 58 S component by conventional sedimentation methods.

In Figure 2 are shown light scattering and sedimentation equilibrium data obtained for the 11 S and 19 S species. In incorporating the sedimentation equilibrium data, both apparent weight-average and apparent *z*-average molecular weight values have been included. These have been graphed versus concentration in the following way: Van Holde and Baldwin (1958) have shown that for nonideal homogeneous solutes the apparent average molecular weights from sedimentation equilibrium are given by

$$\frac{1}{M_w^*} = \frac{1}{M_w} + 2B\left(\frac{c_b + c_a}{2}\right) \qquad (1)$$

$$\frac{1}{M_z^*} = \frac{1}{M_z} + 2B(c_b + c_a) \qquad (2)$$

where c_a and c_b are the solute concentrations at the meniscus and bottom of the ultracentrifuge cell, respectively. The parameter B is the second virial coefficient, identical to that in the light scattering equation:

$$\frac{1}{M_w^*} = \frac{Hc}{\tau} = \frac{1}{M_w} + 2Bc \qquad (3)$$

Thus, the light scattering data are plotted versus c, the concentration of each scattering solution, the apparent weight-average molecular weight versus $(c_b + c_a)/2$, and the apparent *z*-average molecular weight versus $(c_b + c_a)$. The fact that the data from various methods of calculation fall near a single line argues for the homogeneity of the material. The rather large concentration dependence of the 11 S component is probably a charge effect; the isoelectric points of the hemocyanins are in the neighborhood of *p*H 4.5 (see Svedberg and Pedersen, 1940).

From this data, the molecular weight of the 11 S material is found to be 385,000. The results for the 19 S material require further comment. This component dissociates rather easily into 11 S material in alkaline solution. However, if 58 S material is dissociated in slightly alkaline solution, and the solution is then dialyzed *in the cold* against *p*H 6.5 buffer containing no magnesium, reassociation will not occur as long as the subunits are kept at low temperature, and a relatively pure 19 S preparation can be obtained.[1]

The data for the 19 S component show a negative slope, with upward curvature at low concentration. This is apparently a consequence of partial dissociation into 11 S material upon dilution. In a later paper we shall show that this equilibrium is rapidly attained, at least in more alkaline solutions. Thus, the molecular weight estimated by extrapolation (709,000) is probably somewhat too low. At higher concentration the apparent molecular weight of the 19 S material approaches 770,000, corresponding to a dimer of the 11 S material.

In the case of the 58 S component, the molecular weight obtained by light scattering experiments had to be corrected for the presence of a small amount of slowly sedimenting material. Each "master" solution used for light scattering was examined in the ultracentrifuge, and the amount of slow component was measured. The amount found depended upon the method of clarification of the sample. For example, upon preparative centrifugation, 58 S material was selectively removed. Later experiments have shown that the slowly sedimenting material will, under these conditions, eventually reassociate to approach once more an equilibrium mixture; however, the process is extremely slow when the concentration of slow component is low. Hence, the nonequilibrium distribution of components may be taken as stable over the few hours required for these experiments. The slow material appears to be a mixture of 19 S and 11 S components; the proportions were judged from the average sedimentation coefficient of the slow peak. From these data, the molecular weights of the slow components, and the observed weight-average molecular weight, the molecular weight of the 58 S material could be calculated. The results are shown in Table II.

TABLE II
MOLECULAR WEIGHT OF 58 S HEMOCYANIN

Method of Clarification	Per Cent Slow	$\bar{s}_{slow}$	M_w(obs)	M_{58}
Centrifugation	19	19 S	3,220,000	3,810,000
Centrifugation	9	14 S	3,440,000	3,740,000
Filtration	4	12 S	3,550,000	3,690,000
Average				3,750,000

[1] The dissociation of the 58 S component can be made reversible, but different conditions are required. This will be discussed in a later paper.

TABLE III
COMPARISON OF SEDIMENTATION COEFFICIENTS OF WELL-DEFINED COMPONENTS OF DECAPOD HEMOCYANINS

	$s_{20,w}$ of Components (Svedbergs)			Reference
Loligo pealei	58.7[a]	19	11.1[a]	This work
Loligo vulgaris[b]	56.7	16.9	12.1	Eriksson-Quensel
Sepia officinalis	55.9	18.7	10.6	and Svedberg
Rossia owenii	56.2		10.9	(1936)
Ommatostrephes sloani pacificus	[c]	19.5[a]		Omura *et al.* (1961)

[a] Sedimentation coefficients extrapolated to $c = 0$. Others at finite concentration. [b] The value of 16.9 S represents an average of a sedimentation coefficient which varied considerably with *p*H. A "very heterogeneous" 36 S component was also sometimes observed. [c] The authors report that more rapidly sedimenting materials are observed at low *p*H. The schlieren diagrams illustrating them resemble Figure 1A.

The molecular weight obtained is about ten times that of the 11 S component, or five times that of the 19 S component.

DISCUSSION

The measurements of the molecular weights of the three hemocyanin components indicate that the 58 S "molecules" are decamers of the 11 S unit. This result makes a good deal of sense in view of the results of recent electron-microscope studies of hemocyanins of related species. The photographs published by Van Bruggen *et al.* (1962b) show the hemocyanin molecules from *Octopus vulgaris* and the squid *Sepia officinalis* to have the form of hollow right cylinders, about 140 A high by 300 A in diameter. The sedimentation coefficient of the high-molecular-weight *Sepia* component is given by Eriksson-Quensel and Svedberg (1936) as 55.9 S. The electron micrographs suggest a 10-fold symmetry (Van Bruggen *et al.*, 1963) in these cylindrical structures. Our data then suggest that the 58 S to 19 S dissociation involves a longitudinal split of the cylinder into five portions; these could again split to yield the 11 S material. The fact that the frictional ratio f/f_{min} for the 11 S material is greater than that for the 19 S component suggests that this second division is also longitudinal.

The question arises as to whether those components studied represent the only stable assemblies of the *Loligo* subunits. In the hemocyanin from *Busycon canaliculatum* and *Helix pomatia*, for example (Eriksson-Quensel and Svedberg, 1936), substantial amounts of a component with $s_{20} \cong 100$ S have been observed; in the latter case Van Bruggen *et al.* (1962a) have demonstrated that this is formed by the end-to-end association of two 60 S structures. We have seen no evidence for this component in the *Loligo* hemocyanin (however, see paper II of this series, Cohen and Van Holde, 1964). In preliminary experiments we have been unable to produce units smaller than the 11 S component; while 8 M urea produced a large decrease in $s_{20,w}$ (to about 3 S) the normal blue color of the protein had disappeared, and estimates of the molecular weight by the Archibald procedure indicate that no dissociation had occurred. Thus urea appears to unfold the hemocyanin, rather than to dissociate it. Similar behavior is seen at *p*H values above 11.

It might be thought that the observation of sedimentation coefficients between 19 S and 11 S in the *p*H range between 7.5 and 10 (and in the small amounts of slowly sedimenting material observed in more acidic solutions) argues for the existence of species between the 19 S and 11 S components. However, it seems more likely that these boundaries represent equilibrium, or near-equilibrium mixtures of the 19 S and 11 S components. The reasons are: (1) The boundaries are broad, and the average sedimentation coefficient varies continuously with *p*H over the range. According to Gilbert (1959) this is the behavior to be expected for an equilibrating monomer-dimer mixture. (2) As will be shown in a later paper, both light scattering and sedimentation velocity results in this range can be used to calculate the same equilibrium constant for dimerization. (3) If equilibration is rapid, but not instantaneous, the sedimentation diagram at high rotor speed should under certain circumstances show a small maximum corresponding to dimer, and a broad, more slowly sedimenting boundary (Belford and Belford, 1962). An illustration of such resolution is shown in Figure 1C.

Apparently, then, the principal stable association products of the 11 S hemocyanin are a dimer and a decamer. The existence of and role played by intermediates between the dimer and decamer will be discussed in a later publication.

Our results indicate a close similarity between the *Loligo* hemocyanin and those of other decapods; comparative data are given in Table III. Values for the frictional coefficients of the various *Loligo* hemocyanin components can be calculated from the sedimentation coefficient and molecular weight data. Results, expressed as f/f_{min}, where f_{min} is calculated for an unhydrated sphere of the same molecular weight, are: for the 58 S material, $f/f_{min} = 1.41$; for the 19 S material, $f/f_{min} = 1.5$–1.7 (depending on the value of $\bar{v}$ assumed); and for the 11 S material, $f/f_{min} = 1.88$. These values are relatively large, and suggest either a high asymmetry or a high hydration for the units. The same conclusion can be drawn from the intrinsic viscosities: for the 58 S material, $[\eta] = 5.6$ cm^3/g; and for the 11 S unit, $[\eta] = 11.4$ cm^3/g.

We have also carried out a preliminary investigation of the far-ultraviolet rotatory dispersion of the 58 S, 19 S, and 11 S materials. The depth of the 233 mμ minimum is (in units of mean residue rotation $[R']$) -3100 for the 58 S material, and -2850 for both the 19 S and 11 S components. This indicates (Simmons *et al.*, 1961) that the protein is relatively low in helix content, and that there is little difference between the native protein and subunits in this respect. In addition, each of the species here can be reversibly deoxygenated.

The decrease in $\bar{v}$ between *p*H 6.6 and 10.6 is surprisingly large. At the present time we do not know how this change is distributed between the 58 S → 19 S dissociation and the 19 S → 11 S step.[2] However, it seems likely that it is a consequence of dissociation, for there is no other obvious mechanism whereby a simple change in *p*H would cause such an effect. Changes in electrostriction would be expected to lead to an *increase* in volume in going from *p*H 6.6 to *p*H 10.6, since the number of charged groups on the protein molecule decreases in this change. Such an increase has been observed by Charlwood (1957) for bovine serum albu-

[2] In this connection, it should be noted that Omura *et al.* (1961) found $\bar{v} = 0.724$ for their 19.5 S hemocyanin from *Ommatostrephes sloani pacificus*.

min. Three possibilities come to mind: (1) that there is a "waste space" in the 58 S (or 19 S) structure; (2) that hydration is increased by breaking hydrophobic bonds (Némethy and Scherega, 1962); or (3) that upon dissociation a number of charged groups, which are buried in the aggregated structure, come into contact with the solvent. The latter explanation has been advanced by Lauffer (1962) to explain the decrease in specific volume which occurs upon depolymerization of tobacco mosaic virus protein. The apparent increase in f/f_{min} with dissociation, which would be a consequence of increased hydration, would also be consistent with either this idea or the hydrophobic-bond hypothesis. A consequence of this finding is that the fairly common practice of assuming the partial specific volume of the subunit of a protein to be the same as that of the undissociated material is hazardous. Had only sedimentation measurements been employed, and this assumption been made, we would have concluded that there were only nine subunits. Furthermore, to "calculate" the partial specific volume from amino acid composition would also be invalid in this case.

The effect of magnesium ion in stabilizing the 58 S material is similar to that which has been observed for *Helix pomatia* hemocyanin (Brosteaux, 1937). A more detailed study of this effect will be discussed in a later paper.

Acknowledgments

Much of this work was carried out at the Marine Biological Laboratory, Woods Hole, Mass. The authors wish to express their appreciation for the facilities and animals which were placed at their disposal. Some of the sedimentation data were obtained by Mr. Stephen Harrison and Miss Mary Jane Tunis; Mr. R. Hyde assisted in a number of the experiments.

References

Belford, G. G., and Belford, R. L. (1962), *J. Chem. Phys. 37*, 1926.

Brice, B. A., Halwer, M., and Speiser, R. (1950), *J. Opt. Soc. Am. 40*, 768.

Brosteaux, J. (1937), *Naturwiss. 25*, 249.

Charlwood, P. A. (1957), *J. Am. Chem. Soc. 79*, 776.

Cohen, L. B., and Van Holde, K. E. (1962), *Biol. Bull. 123*, 480.

Cohen, L. B., and Van Holde, K. E. (1963), *Biol. Bull. 125*, 375

Cohen, L. B., and Van Holde, K. E. (1964), *Biochemistry 3*, 1809 (this issue; following paper).

Dandliker, W. B. (1954), *J. Am. Chem. Soc. 76*, 6036.

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

Felsenfeld, G. (1960), *Arch. Biochem. Biophys. 87*, 247.

Ghiretti, F. (1962), *in* Oxygenases, Hayaishi, O., ed., New York, Academic.

Gilbert, G. A. (1959), *Proc. Roy. Soc. (London), Ser. A: 250*, 377.

Klotz, I. M., and Klotz, T. A. (1955), *Science 121*, 477.

Klotz, I. M., and Klotz, T. A. (1956), *Biol. Bull. 111*, 306.

Lauffer, M. A. (1962), *in* The Molecular Basis of Neoplasia, Austin, Univ. of Texas Press, pp. 181–206.

Lontie, R. (1958), *Clin. Chim. Acta 3*, 68.

Némethy, G., and Scheraga, H. A. (1962), *J. Phys. Chem. 66*, 1773.

Omura, T., Fujita, T., Yamada, F., and Yamamoto, S. (1961), *J. Biochem. (Tokyo) 50*, 400.

Simmons, N. S., Szent-Györgyi, A. G., Wetlaufer, D. G., and Blout, E. R. (1961), *J. Am. Chem. Soc. 83*, 4766.

Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, Oxford, U. K., Oxford Univ. Press.

Tomimatsu, Y., and Palmer, K. J. (1959), *J. Polymer Sci. 35*, 549.

Tomimatsu, Y., and Palmer, K. J. (1961), *J. Polymer Sci. 54*, S21.

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

Van Bruggen, E. F. J., Wiebenga, E. H., and Gruber, M. (1962a), *J. Mol. Biol. 4*, 1.

Van Bruggen, E. F. J., Wiebenga, E. H., and Gruber, M. (1962b), *J. Mol. Biol. 4*, 8.

Van Holde, K. E., and Baldwin, R. E. (1958), *J. Phys. Chem. 62*, 734.

Williams, R. J. P. (1955), *Science 122*, 558.