Physical Studies of Hemocyanins. IV. Oxygen-Linked Disassociation of *Loligo pealei* Hemocyanin*

H. A. DePhillips, K. W. Nickerson,† M. Johnson, and K. E. Van Holde

ABSTRACT: The 59S hemocyanin from the squid can be disassociated into 19S and 11S subunits in alkaline solution. We now find that such disassociation also occurs near pH 7, if the hemocyanin is partially oxygenated. The deoxyhemocyanin remains in the 59S form from pH 6 to 10, in buffers containing 0.01 M Mg^{2+}. Solutions equilibrated with air or pure oxygen exhibit disassociation at intermediate pH values. The disassociation is a function of the per cent oxygenation. At about 80% oxygenation only the subunits are observed; higher oxygenation leads to reassociation. That these are equilibrium phenomena is demonstrated by kinetic experiments, in which the same mixture of species is approached from different initial conditions. Sedimentation with ultraviolet scanning indicates that the disassociated subunits bind more oxygen than the 59S hemocyanin, at least at low oxygenation levels. The deoxy and oxy 59S species differ slightly in sedimentation coefficient and in the circular dichroism spectrum in the neighborhood of 250–280 nm.

Following a series of investigations of subunit structure and association equilibria in the hemocyanin from the squid, *Loligo pealei* (Van Holde and Cohen, 1964a,b; Cohen and Van Holde, 1964), we decided to investigate the oxygen binding of this protein. We originally planned to conduct comparative oxygen binding studies on the hemocyanin in various states of aggregation, for we had found that the 59S protein (mol wt 3.8×10^6) dissociated into particles one-fifth and one-tenth its weight in alka ine solution. These subunits have sedimentation coefficients of about 19 and 11 S, respectively. Earlier studies of oxygen binding by squid hemocyanin (see, for example, Redfield and Goodkind, 1929), while carefully conducted, had not taken the state of aggregation of the protein into account. In order to provide adequate controls, we carried out sedimentation velocity examinations of the solutions used for binding studies. These experiments yielded the surprising result that the state of aggregation at a given pH depended upon the degree of oxygenation of the hemocyanin. This development showed that the system was more complex than we had hitherto realized and that an investigation of the relationship between oxygenation and subunit aggregation was needed as a prelude to definitive binding studies. The results of such a study are detailed herein; preliminary reports have appeared elsewhere (DePhillips and Van Holde, 1968; Van Holde and DePhillips, 1968).

Experimental Section

Preparation of Solutions. The hemocyanin was obtained as described previously (Van Holde and Cohen, 1964a,b). In all experiments fresh material which had been stored in the cold for no more than 1 week was employed. Tris, phosphate, and bicarbonate buffers of ionic strength 0.1 were prepared according to the Biochemist's Handbook (Long, 1961). Hemocyanin concentrations were determined spectrophotometrically in bicarbonate buffer at pH 10.6, as described in the earlier publications in these series.

Deoxygenation of solutions was carried out in two ways. In the earlier experiments, and in all oxygen-binding experiments, solutions were evacuated as quickly as possible and flushed with argon; the process was repeated until absorption at 345 nm was negligible (see below). While effective, this procedure usually produced a small amount of denatured hemocyanin, and slightly increased the concentration. It was later found that directing a slow stream of water-saturated argon over the solution for a few hours produced effective deoxygenation with neither appreciable evaporation nor denaturation. This technique was used in about half of the experiments; no overall difference in results was observed. In a few experiments nitrogen was substituted for argon to test for any specific interaction with argon. No significant difference in behavior was found.

The apohemocyanin was prepared as described by Cohen and Van Holde (1964). Atomic absorption studies (Takesada and Hamaguchi, 1968) have shown this procedure to actually remove the copper atoms and not just make them unavailable for oxygenation.

Oxygen-Binding Studies. The measurement of oxygenation of the hemocyanin was based on absorbance of the 345-nm band, since this band disappears on deoxygenation (Cohen and Van Holde, 1964). The all-glass tonometers, with fused-quartz cuvets attached, resembled those used by Spoek *et al.* (1964). These tonometers have a side arm with two stopcocks, between which there is a small length of capillary. The volume of this capillary was determined by mercury weighing; the volume of the tonometer was determined from its weight when filled with water. A solution of hemocyanin was placed in the tonometer and deoxygenated by successive evacuation and argon flushing until the 345-nm band was essentially removed.

* From the Department of Chemistry, Trinity College, Hartford, Connecticut (H. A. D.), the Department of Chemistry, University of Cincinnati, Cincinnati, Ohio (K. W. N.), and the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon (M. J. and K. E. V. H.). *Received March 5, 1969.* This work was supported by U. S. Public Health Service Research Grants HE-12326-01 and HE-11671-02. K. W. N. wishes to acknowledge U. S. Public Health Service Predoctoral Fellowship 5-F1-GM-29,756-01.

† Present address: Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon.

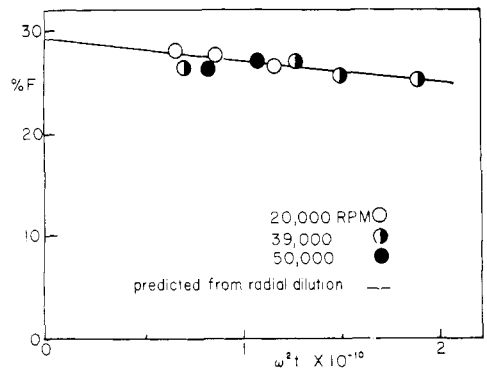

FIGURE 1: Variation of the apparent per cent 59S hemocyanin with $\omega^2 t$ during sedimentation velocity experimnets. The solution has been equilibrated with air at pH 7.7. Experiments at different rotor speeds show that there is no appreciable effect of rotor speed on the apparent composition of the mixture. The slope of the solid line is that predicted from the radial dilution effect.

One or more increments of air (or oxygen at 1 atm) were then introduced, one capillary volume at a time. The solution was gently shaken at 23° for 5 min or more and the absorbance at 345 nm was redetermined. The shakings and readings were repeated until no significant difference was obtained in 5 min. Usually, apparent equilibrium was obtained almost immediately. The process was then repeated successively, until a pressure of about 1 atm of O_2 was obtained. The oxygen pressure at each stage was calculated by the method described by Spoek *et al.* (1964).

All of the binding studies were carried out at protein concentrations between 3 and 4 mg per ml, a 1:25 dilution of the purified hemolymph.

Sedimentation Experiments. Spinco Model E analytical ultracentrifuges were used. One of these instruments was equipped with an ultraviolet-scanning optical system. Most of the studies of the distribution of hemocyanin between the 59S particles and subunits utilized phase-plate schlieren optics. Since a large number of areas under schlieren peaks had to be determined, a rapid but reasonably accurate method was sought. It was found that images of the patterns projected on the viewing screen of a Nikon comparator with 20× magnification could be accurately traced on tracing paper. The areas could then be quickly measured with a planimeter. The over-all precision and accuracy of this method (as compared with direct comparation and numerical integration) were about ±2%, quite sufficient for our purposes. The areas were corrected for radial dilution by extrapolation to an estimated starting time (see Figure 1). No attempt was made to correct for the Johnson–Ogston effect, since the sedimentation coefficients of the 59S particle and the 19S and 11S subunits differ appreciably, and the concentration dependence of S is small for these particles (Van Holde and Cohen, 1964a). Also, we have made no attempt, in the present study, to distinguish between 19S and 11S subunits, but rather have restricted the analysis to "fast" (59S) and "slow" (19S plus 11S) components because previous experiments had shown that the 19S and 11S mixtures were in rapid equilibrium, and usually not resolvable (Van Holde and Harrison, 1963).

All sedimentation studies were made at 20 ± 1°, and most at about 42,000 rpm. We were concerned that the pressure gradient in the ultracentrifuge might affect the position of equilibrium when more than one component was present. Therefore, a series of experiments were carried out with the same mixture at different rotor speeds. As can be seen in Figure 1, there appears to be no rotor speed dependence of the apparent composition of the mixture. This may be either because the equilibrium is pressure insensitive, or simply because equilibrium is attained slowly in these systems. In the latter case, the mixture represents that existing at 1 atm.

In experiments on solutions under oxygen, argon, or nitrogen, the ultracentrifuge cells were filled in a glove bag, after first being flushed repeatedly with the desired gas. They were then closed in the same environment.

Circular Dichroism. All experiments were performed in a Cary Model 60 optical rotatory dispersion apparatus with circular dichroism attachment. Cylindrical cells of length between 0.5 and 10 mm were employed; those containing solutions under argon or oxygen were filled in a glove bag, tightly stoppered, and covered with parafilm. The calibration of the circular dichroism apparatus was checked with a solution of epiandrosterone.

Results and Discussion

Binding Studies. We present first some of the results of our preliminary studies on oxygen binding by hemocyanin. It should be emphasized at the outset that these experiments must be regarded as possibly subject to small corrections. There are two reasons for this. In the first place, use of the optical density at 345 nm as a quantitative measure of oxygen binding involves the assumption that no significant changes in extinction coefficient and/or light scattering occurs as oxygenation progresses. More serious is the objection that these experiments cannot, in some cases at least, correspond to equilibrium conditions. As will be shown below, there are changes in subunit association accompanying oxygen binding, and these changes may require several hours under the conditions of concentration and pH used here. Since such changes in state of aggregation may in turn be accompanied by changes in binding, it is evident that the determination of equilibrium binding curves will be a laborious process. It is not believed that these complications have greatly modified the binding curves, since several hours were required to record each one. Nevertheless, we feel that these results should be treated with some reservation. At the same time, since the general features of the binding curves are important in what is to follow, we shall present these preliminary results here.

In order to use the 345-nm band to measure the per cent oxygenation, a value for the limiting absorption at saturation is required. This may be obtained by extrapolation to $(1/P_{O_2}) = 0$, making use of the fact that the oxygenation becomes linear in oxygen pressure near saturation. A linear extrapolation to zero oxygen pressure is also needed because scattering from these solutions is not negligible. The fraction oxygenation is then calculated from

$$Y = \frac{A_p - A_0}{A_\infty - A_0} \tag{1}$$

where A_p, A_0, and A_∞ are the absorbancies at pressure P, zero

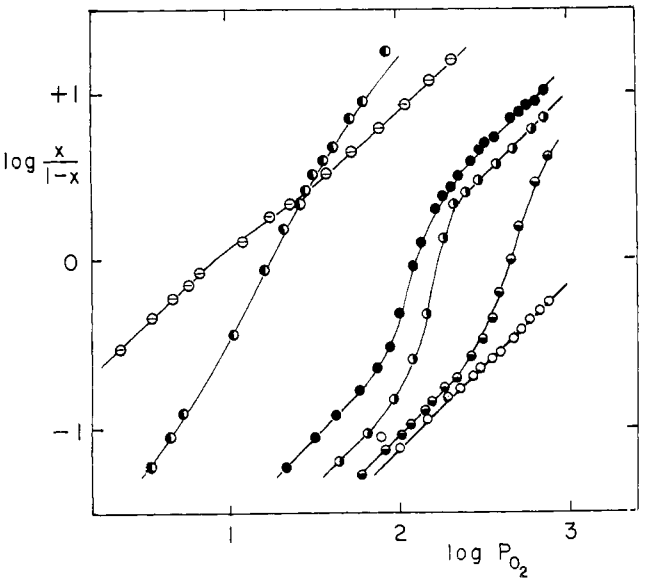

FIGURE 2: Hill plots of the oxygen binding by squid hemocyanin. The quantity x is the fraction oxygenation. Experiments at different pH values are indicated as follows: (○) 6.24,(◒) 6.98,(◑) 7.36, (●) 7.44, (◐) 8.44, and (⊖) 10.42. All except the last contain 0.01 M Mg^{2+}. Only data corresponding to $0.05 \leq x \leq 0.95$ are recorded.

TABLE I: Oxygen-Binding Experiments.

pH	Buffer[a]	$(A_{345,max}/A_{280})^b$	log K_1[c]	Max Slope
6.24	Phosphate	0.208[d]	−3.06	*e*
6.98	Phosphate	0.220	−2.98	3.3
7.36	Tris	0.280	−2.70	3.9
7.44	Tris	0.222	−2.55	3.5
8.44	Tris	0.212	−1.9[f]	1.9
10.42	Bicarbonate	0.226	−0.90	1.0

[a] All solutions except the last contained 0.01 M Mg^{2+}. [b] $A_{345,max}$ is the limiting absorbance (corrected for scattering) obtained by extrapolation to infinite O_2 pressure. [c] K_1 is the initial binding constant, deduced from the low-pressure form of the equation: $\log (x/(1-x)) = \log K_1 + \log P_{O_2}$. [d] Obtained by a rather long extrapolation. [e] The data for oxygenation at this pH extend only to about 50% oxygenation. It is not clear whether or not a steeper portion would be observed at higher P_{O_2}. The fact that the data when extrapolated linearly to infinite O_2 pressure yield a value of $(A_{345,max}/A_{280})$ in agreement with those at other pH values argues that the linear extrapolation is correct. If this is the case, there is no cooperativity at pH 6.24. [f] Estimated from the limiting form of the curve at low P_{O_2}.

oxygen pressure, and infinite oxygen pressure, respectively.

Hill plots from the data obtained at different pH values are shown in Figure 2. Two facts are immediately obvious. (1) The curves show the typical form for cooperative binding by molecules containing many subunits (Wyman, 1969). At low and high P_{O_2} the slope is equal to unity, but becomes much greater in the intermediate region. Data are summarized in Table I. Evidently the initial binding constant rises sharply between pH 7 and 8; also, the cooperativity, as judged from the maximum slope, reaches a maximum at about pH 7.4. Finally, it should be noted that at pH 10.4, where the molecules are disassociated completely to the 11S form (Van Holde and Cohen, 1964a), positive cooperativity vanishes. In fact, the Hill plot shows a portion with slope less than unity, suggesting either negative cooperativity or the presence of more than one type of binding site. (2) There is a very large Bohr effect. The magnitude of the Bohr effect is more clearly seen in Figure 3, where the oxygen binding by solutions in equilibrium with air is graphed *vs.* pH. Between pH 7.2 and 7.8 there is a very large and abrupt change in strength of oxygen binding. The very strong Bohr effect in squid hemocyanin has been noted in earlier studies (Redfield *et al.*, 1926; Redfield and Goodkind, 1929). This, together with the cooperativity in binding, results in a very effective mechanism for unloading oxygen to the tissues. It is of interest that the maximum slope in the Bohr effect curve lies close to the physiological pH of the blood, about pH 7.4 by our measurements, at a P_{O_2} corresponding to that of air.

Disassociation Accompanying Oxygen Binding. In the course of the experiments described above, we found that disassociation of the 59S structures occurs upon oxygenation. The kind of situation encountered is shown in the schlieren photographs in Figure 4. In all cases the hemocyanin is in 0.1 M Tris buffer (pH 7.50) containing 0.01 M $MgCl_2$. The deoxygenated protein is almost entirely in the 59S form. The small amount of slow component may not be hemocyanin. We have found that at very low degrees of oxygenation scanner traces of the boundaries (at 345 nm) reveal oxygenation of the 59S material, but no absorbance in the region just ahead of the slow boundary. In contrast to the deoxygenated material, a sample equilibrated with air for 3 hr, which is about 70% saturated with oxygen, appears to be wholly disassociated into smaller subunits. Finally, samples under pure oxygen for 3–7 hr, which are about 95% oxygenated, contain about 40% of the 59S form. One of these samples had been oxygenated

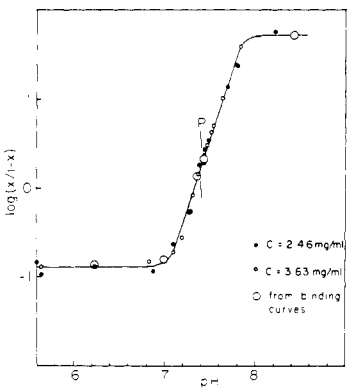

FIGURE 3: Oxygen binding (expressed as $\log x/(1-x)$) *vs.* pH for solutions equilibrated with air. Data from two concentrations are shown. The large open circles are taken from the corresponding points in Figure 2. All solutions contained 0.01 M $MgCl_2$. The point, P, represents the approximate pH of the hemolymph of *L. pealei*.

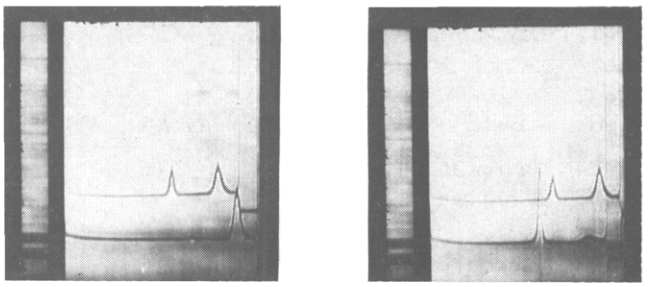

FIGURE 4: Sedimentation velocity studies of squid hemocyanin at pH 7.50, in a buffer containing 0.01 M Mg^{2+}, and equilibrated with different atmospheres. Right, lower pattern: under argon; upper pattern: under oxygen. Left, lower pattern: under air; upper pattern: under oxygen. In each case, sedimentation has proceeded for about 10 min at 42,000 rpm.

immediately after preparation; the other had first been allowed to disassociate in air.

It was our initial thought that this unusual behavior might be an artifact resulting from nonequilibrium situations. For example, disassociation might simply be slower in some circumstances than in others, or some processes might be irreversible. Since an exploration of the kinetics and reversibility of these phenomena seemed necessary to determine if indeed such was the case, the set of experiments described in Figure 5 was performed. A concentrated hemocyanin solution was diluted into pH 7.50 Tris buffer and equilibrated with air. The solution was quickly divided into three aliquots, one left in air, one placed under pure oxygen, the third deoxygenated by evacuation and argon flushing. Sedimentation velocity experiments were performed at the times indicated on the graph. As can be seen, each solution rather slowly approached a particular distribution of 59S and subunit species, in accord with our previous observations. Then, when solutions were transferred to new conditions, the disassociation and association processes reversed. Thus, a deoxygenated solution first became nearly entirely 59 S, but when subsequently placed in air, complete disassociation occurred. Solutions initially disassociated in air regained the characteristic amounts of 59S component when later deoxygenated or placed under pure O_2. In other words, the same equilibrium mixtures have been approached from two sides. The data show, however, that the rate of approach to equilibrium can be quite slow. It is not the oxygen-binding reaction which is rate limiting, for the time required for the oxygenation process is clearly much shorter than the periods of hours required for these association and disassociation processes. In spectrophotometric binding studies, attempts were made to rapidly equilibrate deoxygenated solutions with oxygen; following a few minutes of shaking the absorbance of the solution showed little or no subsequent change with time. Thus, the association and disassociation reactions follow, rather than accompany, the oxygenation and deoxygenation.

We have determined the distribution of 59 S and subunit species at various pH values, in solutions equilibrated with argon, air, and oxygen. Since kinetic experiments showed that the attainment of equilibrium could be slow, all data reported here were taken on solutions which had been equilibrated for at least 12 hr, at 22 ± 2°, with the appropriate atmosphere. The results are shown in Figure 6. First, it is clear that deoxygenated squid hemocyanin, in the presence of 0.01 M Mg^{2+}, remains almost entirely in the 59S form over the entire pH range studied. Solutions equilibrated with air or oxygen, however, exhibit disassociation in intermediate pH ranges. The pH for maximum disassociation is *not* the same for solutions equilibrated with O_2 and with air (Figure 6). This result was confusing, until it was realized that the parameter upon which the distribution of components depended was the *degree of oxygenation*, and that the apparent pH dependence resulted from the Bohr effect, the dependence of oxygenation upon pH. If the pH is below 8, a solution will be oxygenated to a very different extent in O_2 than in air; this can be seen in Figure 2. When the data from Figures 2 and 3 were used to

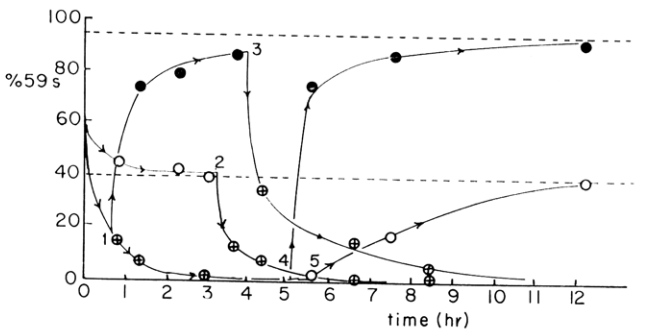

FIGURE 5: A set of experiments to show that the association equilibrium can be approached from either direction as the oxygen pressure is changed. Each point corresponds to a sedimentation velocity experiment. A solution (pH 7.50, 0.01 M Mg^{2+}) was prepared (in air) at zero time. The solution was divided into two parts, one in air, one placed under pure O_2. At point 1 part of the solution under air was deoxygenated and placed under argon. At times 2 and 3, the samples under oxygen and argon were opened to the atmosphere. At time 4, a part of the solution originally equilibrated with air was deoxygenated, and at time 5 another portion of this air-equilibrated solution was placed under pure O_2. The broken lines correspond to the results of other "equilibrium" (12 hr) experiments. Code: deoxygenated (●); sample in O_2 (○); sample in air (⊕).

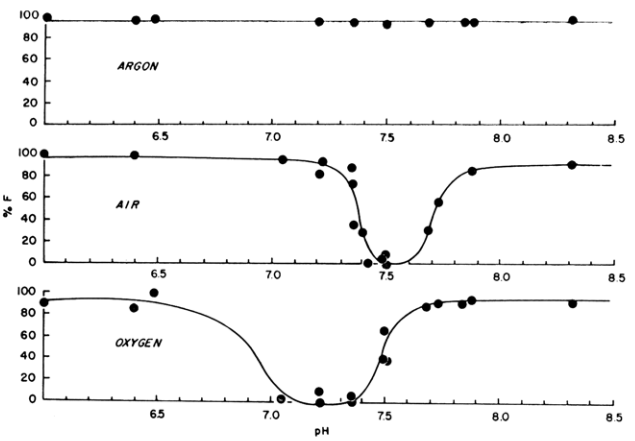

FIGURE 6: Per cent of 59S component in solutions equilibrated with argon, air, and pure O_2, shown as a function of pH. These data have been corrected for 5% of low molecular weight impurity. All solutions contained 0.01 M $MgCl_2$, and had been equilibrated for at least 12 hr with the appropriate atmosphere.

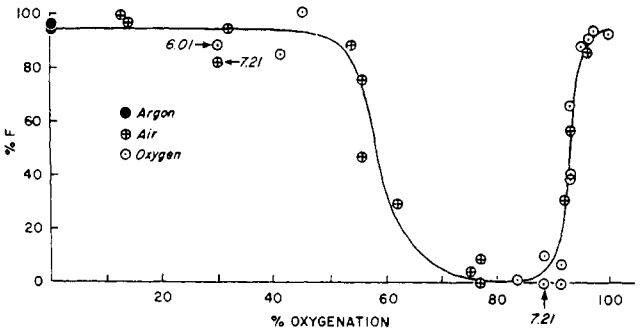

FIGURE 7: The same data as in Figure 6, graphed *vs.* per cent oxygenation rather than pH. Note that all of the points now fall close to a single curve. The numbers adjacent to three points are pH values; this is to point out that pH is *not* the factor determining the fraction of disassociation.

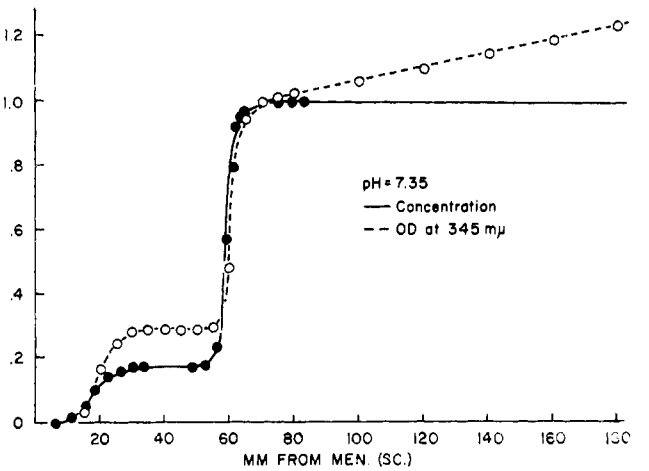

FIGURE 8: Comparison of integrated schlieren curve with scanner trace at 345 nm for a partially oxygenated solution. The distance axis of the scanner trace pattern has been transformed to that corresponding to a particular schlieren photograph. Both boundaries are present, but the comparison of the normalized heights shows that the slower component is more highly oxygenated.

calculate the per cent oxygenation corresponding to each of the points in Figure 6, the much simpler picture given in Figure 7 emerged. *Regardless of the pH, the fraction present as 59S component is determined by the per cent oxygenation.* Wholly deoxygenated or wholly oxygenated samples exist primarily in the 59S form, whereas at about 80% oxygenation, disassociation into subunits is complete. This result is obtained in solutions containing 0.01 M Mg^{2+}. In magnesium-free solutions, reassociation upon complete oxygenation is not observed (see Van Holde and Cohen, 1964a,b). This must mean that the 59S oxygenated form is distinct from the 59S deoxyhemocyanin, and that the former requires Mg^{2+} ions for stability. The fact that the pH for maximum disassociation in air-saturated solutions coincides with the physiological pH of squid hemolymph may be of significance. Although the degree of disassociation would be expected to be less in the 25-fold more concentrated hemolymph, the rate of disassociation should still be high, facilitating subunit interchange.

Comparative Oxygen Binding of 59S and 19S Hemocyanin. The fact that disassociation depends upon oxygenation suggests a corollary; there must be a difference in the oxygen affinities of these two species. This can be demonstrated very directly by use of the ultraviolet-scanning optics of the ultracentrifuge. We studied the hemocyanin under oxygenation conditions (pH 7.35 in air) which cause partial disassociation to occur. The schlieren optics were used to determine total protein concentration across the cell and the ultraviolet scanner was used to measure absorbance at 345 nm. The use of the schlieren method is justified since it has been shown (Van Holde and Cohen, 1964a,b) that the specific refractive index for 59S particles and subunits is the same.

In order to compare the results by the two techniques, alternate schlieren photographs and absorption scans were made. Comparation of the schlieren photographs followed by numerical integration gave the concentration as a function of distance. Interpolation of the scanner traces to the same times as the schlieren photographs gave comparable traces of absorbance. Results of one such experiment are shown in Figure 8. The curves have been normalized at a point just ahead of the 59S boundary to simplify comparison. It is obvious that the subunits bind oxygen more strongly than the 59S units since the ratio of absorbance to concentration is much larger for the slow boundary. In addition, there appears to be a pressure dependence of oxygenation, for the absorbance curve does not yield a plateau, but increases nearly linearly with radius below the fast boundary. The schlieren pattern, on the other hand, returns completely to the base line and exhibits no gradient in this region.

This interpretation is supported by a similar experiment at pH 7.70. At this pH, hemocyanin in equilibrium with air is nearly oxygen saturated. Therefore, we did not expect, and did not observe, an appreciable absorbance gradient in the plateau region. Furthermore, the relative oxygenation of the two components appeared to be approximately equal. A slight apparent deficiency in oxygenation in the slow boundary can be explained by the nonoxygenating contaminant referred to earlier. In summary, at moderate degrees of oxygenation, where some disassociation of the 59S component has occurred, more oxygen is bound to the 19S units; the more highly oxygenated subunits have preferentially disassociated. At high levels of oxygenation, where 59S particles have begun to reform, the difference in oxygenation between these and the subunits is not large. We have not attempted to make quantitative calculations of the binding by each component, since the observed absorbance depends upon both true absorbance and scattering. However, the sense of the conclusions reached above is not endangered. Since the 59S hemocyanin must scatter more than the subunits, the excess oxygen binding by the subunits must be, if anything, greater than Figure 8 would suggest.

Conformational Differences between Oxygenated and Deoxygenated Hemocyanins. The fact that oxygenation leads to disassociation of *L. pealei* hemocyanin strongly implies that there may be some conformational change accompanying the binding of oxygen. Further, the observation that progressive oxygen binding first promotes disassociation and then reassociation suggests that the oxygenated and deoxygenated 59S species must differ in some respect. This possibility was investigated by means of sedimentation velocity and circular dichroism studies.

Figure 9 shows the result of a comparative study of the sed-

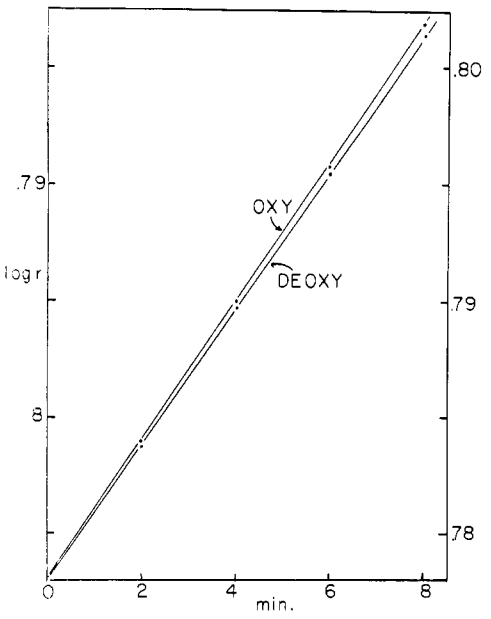

FIGURE 9: Comparison of the sedimentation velocities of oxygenated and deoxygenated "59S" hemocyanin at pH 8.2, 0.01 M $MgCl_2$. Right scale is log of distance from center of rotation for oxyhemocyanin, left is for deoxyhemocyanin.

imentation velocity of oxygenated and deoxygenated 59S hemocyanin. The experiment was designed to duplicate as closely as possible the conditions for study of the two samples. A solution was first deoxygenated, and an aliquot then removed and equilibrated with pure O_2. The pH was chosen so that the sample under O_2 was almost completely oxygenated. No concentration change occurred in the process of oxygenation. The solutions were then loaded into wedge and regular cells, one under argon, the other under oxygen. These cells

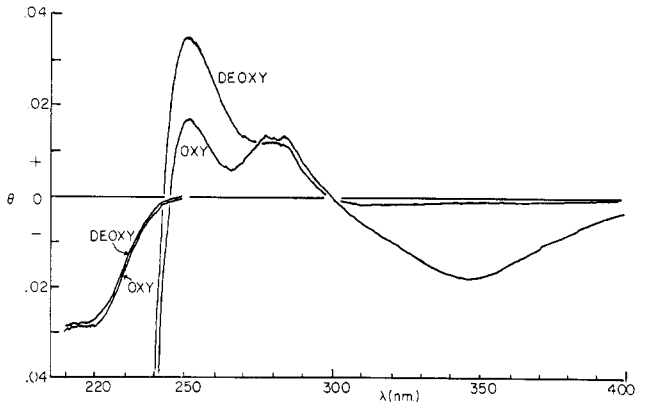

FIGURE 10: Comparison of the circular dichroism of oxygenated and deoxygenated 59S hemocyanin at pH 8.2, 0.01 M $MgCl_2$. The ellipticity in degrees for solutions containing approximately 1.9 mg/ml of protein in a 10-mm cell is given. The data below 240 nm were obtained with solutions of 1.9 mg/ml in a 0.5-mm cell. For these latter data, a twofold more sensitive scale was used; thus the ellipticity scale in the figure should be doubled for data taken below 240 nm. Note that the 345-nm band disappears upon deoxygenation.

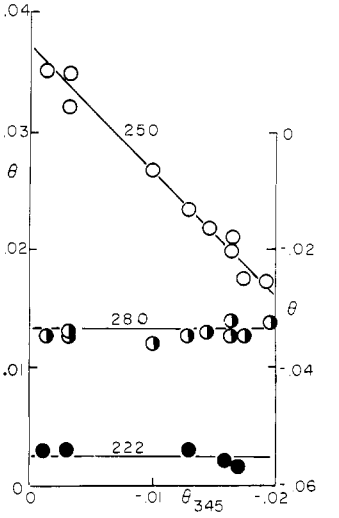

FIGURE 11: Data from a series of experiments which include those shown in Figure 10. The ellipticities at 250 and 280 nm (left scale) and 222 nm (right scale) are graphed *vs.* the ellipticity at 345 nm. The latter is used as a measure of oxygenation. Only the 250-nm band changes with oxygenation.

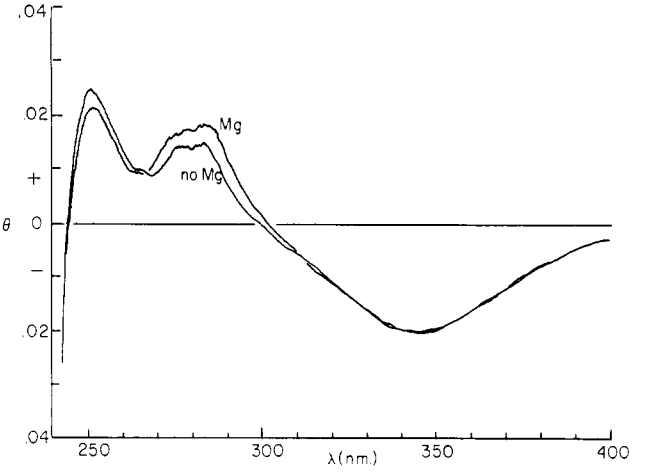

FIGURE 12: Comparison of the circular dichroic spectra of 59S and 19S hemocyanin solutions containing 4.3 mg/ml of hemocyanin in pH 8.2 Tris buffer. A 5-mm cell was used. In the presence of Mg^{2+}, the protein is in the 59S form, whereas without added Mg^{2+}, disassociation to 19S material results (see Van Holde and Cohen, 1964a). The difference in the curves, while not great, is reproducible and outside of experimental error.

were run simultaneously, so that the rotor speed and temperature were identical for the two solutions. It is evident from Figure 9 that the sedimentation coefficients differ. The oxygenated solution sediments 2.4% faster than the deoxygenated material. Since the increase in mass on oxygen binding is trivial (0.08%), the change in S must reflect a decrease in frictional coefficient upon oxygenation. Thus, there are really two 59S forms, one deoxygenated, the other oxygenated.

This conclusion is supported and amplified by circular dichroism studies. The results of these experiments are illustrated in Figures 10–12. These experiments supplement our earlier investigations (largely of octopus hemocyanin; Van

Holde, 1967), in which the main interest centered on the visible and near-ultraviolet bands. With the greater sensitivity of the apparatus now available, it was possible to investigate the relatively weak optical activity in the region between 240 and 300 nm. In this region, significant differences are observed, depending upon the oxygenation of the hemocyanin. In the first place, as shown in Figure 10, the oxygenated and deoxygenated proteins at a given pH show significantly different circular dichroism spectra. In both cases, two prominent bands are observed, one near 250 nm, the other near 280 nm. However, the 250-nm band is much more optically active in the deoxygenated sample, whereas the 280-nm band remains unchanged. These results are in general agreement with the observations of Takesada and Hamaguchi (1968) on the closely related hemocyanin from octopus. The unique behavior of the 250-nm band is emphasized in Figure 11, where the ellipticity at 250, 280, and 222 nm is graphed *vs.* per cent oxygenation, as measured from the 345-nm band. The 250-nm band decreases in ellipticity upon oxygenation, while the 280-nm band remains unchanged (see also Table II). It is also evident from this figure that the 222-nm minimum remains constant; there is apparently no gross change in secondary structure with oxygenation.

TABLE II: Circular Dichroism Data.

	$\Delta\epsilon$ (in ml/g cm) at			
	222 nm	250 nm	280 nm	345 nm
Oxyhemocyanin	−16.6	+0.34	+0.25	+0.33
Deoxyhemocyanin	−16.6	+0.60	+0.24	0
Apohemocyanin	*a*	+0.43	+0.26	0

[a] Not measured. However, the earlier optical rotatory dispersion studies of Cohen and Van Holde (1964) showed no difference in this region between hemocyanin and apohemocyanin.

The behavior of the 250-nm circular dichroism band with oxygenation has at least two possible explanations. (1) There exists a previously undiscovered copper band at 250 nm which, like the 345-nm band, possesses negative circular dichroism and is developed upon oxygenation. This would then have to be superimposed upon another, positive circular dichroism band in the same region. An alternative version of this hypothesis would postulate a positive copper circular dichroism band in this neighborhood, which becomes less active on oxygenation. (2) The 250-nm band results from protein chromophores, the optical activity of which is modified by a limited conformational change upon oxygenation.

That the second hypothesis is correct is indicated by the fact that the same band exists in the apohemocyanin (see Table II). It is less intense than in the deoxygenated protein, but more so than in the oxy form. This fact is difficult to reconcile with the assumption of a negative copper circular dichroism band superimposed upon a positive protein band; were this the explanation, one would expect the deoxy- and apohemocyanin to show equal positive circular dichroism. If there were a positive copper circular dichroism band at 250 nm, it would have to change from positive to negative dichroism on oxygenation, to yield a circular dichroism spectrum for the oxygenated protein less positive than that of the apohemocyanin. This seems unlikely. A previous report (Urry and Eyring, 1964) of a 245-nm circular dichroism band due to charge transfer in a copper–histidine complex has recently been shown (Tsangaris *et al.*, 1969) to be due to the interaction of the copper with the free amino and carboxyl groups, not the imidazole ring; the band was *not* observed with copper bonded to histidine-containing peptides. Consequently, we believe that the circular dichroism changes at 250 nm indicate a limited conformational change accompanying oxygenation.

It should be emphasized that the fully oxygenated and fully deoxygenated samples illustrated in Figure 10 are both in the 59S form (see Figures 3 and 6). Thus, the conformational difference indicated by the difference in circular dichroism spectra corresponds to the difference in sedimentation coefficients noted in Figure 9. It is then of interest to inquire whether the oxygenated 59S and 19S forms are conformationally different. This may be investigated by taking advantage of the fact that at pH 8.2 oxygenated hemocyanin is in the 19S form in the absence of Mg^{2+}, but is in the 59S form in solutions containing 0.01 M Mg^{2+} (Van Holde and Cohen, 1964a). In Figure 12 are shown circular dichroism spectra at this pH in the presence and absence of Mg^{2+}; the observed difference, while reproducible, is very slight. We conclude that the conformational change results from oxygenation, and the tendency to disassociate in the absence of Mg^{2+} is a consequence of this change.

Discussion

The conclusions to be drawn from these experiments are as follows. Partial oxygenation produces a progressive change in the conformation, and when about 50% complete, leads to disassociation into subunits. When oxygenation becomes almost complete, the subunits will reassociate, *if* 0.01 M Mg^{2+} is present. Schematically, we may represent the results as follows

$$\text{59 S deoxy} \overset{O_2}{\rightleftharpoons} \text{59 S partially oxygenated} \overset{O_2}{\rightleftharpoons}$$
$$\text{19 + 11 S partially oxygenated} \overset{O_2}{\rightleftharpoons} \text{19 + 11 S oxy} \overset{Mg^{2+}}{\rightleftharpoons}$$
$$\text{59 S* oxy}$$

At the present time, we do not feel able to present a molecular explanation for these results, but the disassociation and reassociation with increasing oxygenation definitely appear to be determined by symmetry considerations and as such may be typical of other multiple subunit systems. The idea that oxygenation may result in disassociation is by no means new; such effects were observed some years ago with lamprey hemoglobin (Rumen, 1962; Briehl, 1963). However, we know of no case in which both disassociation and reassociation have been observed. It must be remembered that the 11S and 19S subunits, according to their known molecular weights and oxygen-binding stoichiometry, must contain about 8 and 16 binding sites, respectively. The progressive changes accompanying oxygenation of such subunits are apt to be complex. Recently,

Noble (1969) has emphasized that changes in intersubunit binding energy are to be expected when a multisubunit protein takes up or releases ligands. We may speculate that a molecule containing so many subunits as the 59S hemocyanin is unable to carry out the "symmetrical" conformation changes expected from the classical models of allosteric transitions. The resulting mixed conformational state may well be unstable (see Noble, 1969). It is possible to construct a model, based on an assumed distribution of oxygen molecules over sites, which fits the observed disassociation curves, but this model still involves *ad hoc* assumptions as to oxygenation levels at which disassociation and subsequent reassociation occur. Clearly, considerably more experimentation is required before the mechanism underlying this observed behavior will be evident.

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