Physical Studies of Hemocyanins. II. A Comparison of the Hemocyanin and Apohemocyanin of *Loligo pealei**

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The apohemocyanin (copper-free protein) was prepared from the hemocyanin of the squid *Loligo pealei*, and physical properties of the two proteins were compared. The apohemocyanin exhibits the same species in sedimentation as does the hemocyanin, with the exception that small amounts of what probably corresponds to a dimer of the largest hemocyanin component are observed with the apo protein under some conditions. Dissociation of the two proteins is similar, except that the 58 S apohemocyanin component dissociates into subunits at a lower *p*H in solutions containing 0.01 M $MgCl_2$. The optical rotatory dispersion of the apohemocyanin and the deoxygenated hemocyanin differ from that of the oxygenated hemocyanin only in that the latter exhibits negative Cotton effects associated with the absorption bands of the oxygenated protein at 345 and 580 mμ. In the neighborhood of 233 mμ, the apohemocyanin and hemocyanin exhibit identical rotatory dispersion curves. It is concluded that the removal of neither oxygen nor copper produces measurable change in the conformation of the protein structure.

A good deal of evidence indicates that the copper in hemocyanins plays a role in oxygen binding somewhat analogous to that of the iron in heme proteins. There are, however, marked differences; apparently two copper atoms are required for the bonding of each oxygen molecule, and the copper appears to be attached directly to the protein, without the mediation of any porphyrin moiety.

Because of this, it is reasonable to inquire whether the removal of bound oxygen, or of the copper itself, will have an effect upon the tertiary or quaternary structure of the protein. Indeed, such effects have been reported; Klotz and Heiney (1957) observed a pronounced change in optical rotation at 436 mμ upon deoxygenation of *Busycon canaliculatum* hemocyanin. On the other hand, Van Bruggen *et al.* (1962a) could observe no difference in electron micrographs of the hemocyanin from *Helix pomatia* and the apohemocyanin (copper-free protein) prepared from it. However, electron microscopy might not reveal changes in the detailed conformation of the molecule, and the equilibria between the "native" molecule and its subunits might well be modified by removal of the copper.

With these problems in mind, we have continued the investigation of *Loligo pealei* hemocyanin described in the first paper in this series (Van Holde and Cohen, 1964) with a comparative study of the properties of the apohemocyanin. Special emphasis has been placed upon optical rotatory dispersion as the most sensitive method currently available for the study of protein conformation in solution.

This investigation looks toward another problem, the nature of the copper and oxygen binding. It was hoped that information about the optical activity (or lack of same) of the intense absorption bands of the oxygenated hemocyanin would provide a part of the evidence which will be necessary for the elucidation of these puzzles.

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EXPERIMENTAL

Materials.—The hemocyanin and buffer solutions were prepared as described previously (Van Holde and Cohen, 1964). Most experiments were performed with fresh samples; it was necessary to use material which had been frozen only for the optical rotatory dispersion measurements. Samples were checked by sedimentation at *p*H 10.6, as before. The apohemocyanin was prepared by a modification of the method described by Ghiretti (1956). Blood was dialyzed against a solution containing 0.01 M KCN, 0.01 M $MgCl_2$, and 0.1 ionic strength phosphate buffer (*p*H ≅ 6.6) until the absorption band at 345 mμ was absent in oxygen saturated solutions. Usually four changes of dialysis medium over a 24-hour period were required. To remove the KCN, the apohemocyanin was then dialyzed for 2 days against six changes of phosphate buffer containing 0.01 M $MgCl_2$. The entire process was carried out at about 5°.

Spectrophotometry and Spectropolarimetry.—Spectra were measured with a Zeiss spectrophotometer, using 1-cm cuvets. Concentrations were determined from absorption at 280 mμ, using the factors given in the previous paper. As will be shown, the far-ultraviolet spectrum of the apohemocyanin appears to be identical to that of the hemocyanin, justifying the use of the same factors.

Optical rotatory dispersion measurements were carried out with two instruments, a Rudolph Instruments Engineering Corp. recording spectropolarimeter, and a Cary Model 60 recording spectropolarimeter. With the former instrument, measurements were made manually, alternating solution and blank measurements, to minimize the effects of zero drift. Readings were in many cases approached from two directions. With the Cary instrument, automatic recording was employed. In order to cover the entire wavelength range, from 700 to 225 mμ, a number of concentrations were used. Data employed for the final analysis always corresponded to a total rotation of at least 0.01°. To guard against artifacts, the concentrations were chosen so as to overlap data from the several runs. An additional check of the Rudolph instrument was performed

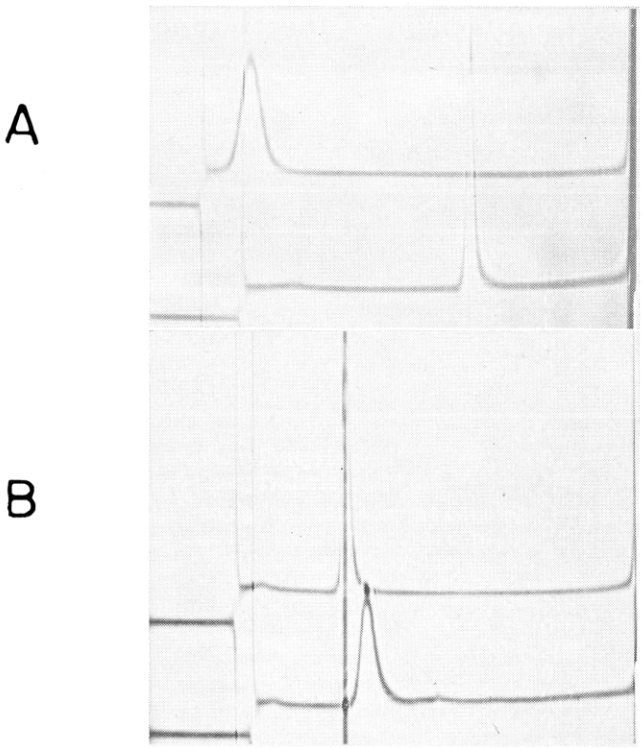

FIG. 1.—Sedimentation diagrams of *Loligo pealei* apohemocyanin. (A) Apohemocyanin at *p*H 7.5 in 0.1 ionic strength phosphate buffer (lower image) and at *p*H 9.0 in 0.1 ionic strength bicarbonate buffer (upper image). The photograph was taken 12 minutes after accelerating the rotor to 42,040 rpm. (B) Apohemocyanin at *p*H 4.6 in 0.1 M acetate buffer, containing 0.01 M $MgCl_2$ (lower image), and at *p*H 7.5 in 0.1 M phosphate buffer containing 0.01 M $MgCl_2$ (upper image). The photograph was taken 5 minutes after accelerating the rotor to 42,040 rpm.

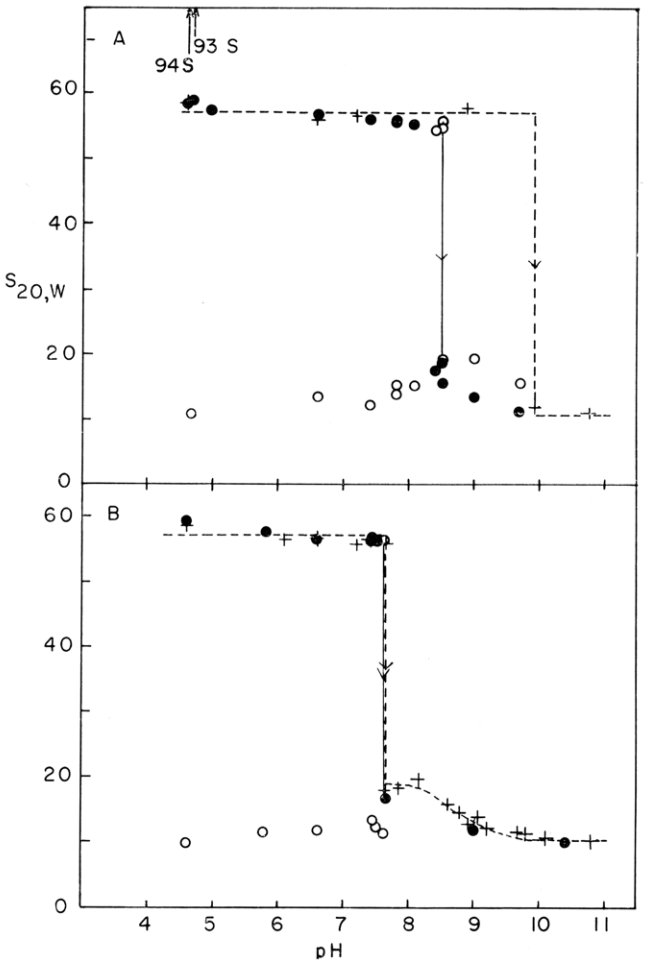

FIG. 2.—Sedimentation coefficient versus *p*H for *Loligo* apohemocyanin. The magnesium ion concentration in (A) was 0.01 M; in (B) it was about 0.0003 M. In each diagram the solid circles represent the major component, the open circles the minor component. The arrows labeled "93 S" and "94 S" indicate that small amounts of material with such sedimentation coefficients were present in two instances. The crosses (+) indicate the major component in the hemocyanin under comparable conditions, and the broken lines indicate the stability ranges of the hemocyanin. (See Van Holde and Cohen, 1964.)

by examining a saturated cupric sulfate solution in the range 600–700 mμ. No significant deviation from the baseline was observed.

Sedimentation Experiments.—The conditions for sedimentation velocity experiments were essentially the same as in the earlier work. In correcting data to water at 20°, it was assumed that $\bar{v}$ for the apohemocyanin was the same as that of the hemocyanin.

RESULTS

Sedimentation Experiments.—In Figure 1A is shown the schlieren diagram of a freshly prepared solution of *Loligo pealei* apohemocyanin at *p*H 7.5, in 0.1 ionic strength phosphate buffer. In sedimentation coefficient and boundary sharpness, the diagram is virtually identical to those obtained with the hemocyanin under the same conditions. There appears in general to be slightly less of the slowly sedimenting component in our apohemocyanin preparations than in hemocyanin samples under the same conditions. This may be a consequence of the prolonged dialysis against solutions containing 0.01 M $MgCl_2$.

The *p*H-stability diagrams shown in Figure 2 are also similar to those observed with the hemocyanin. For comparison, the crosses show the sedimentation coefficient of the main component of the hemocyanin as a function of *p*H, and the broken lines show the course of dissociation of the hemocyanin. Very little difference can be observed between the sedimentation coefficient of the large apohemocyanin component and that of the corresponding hemocyanin, although with increasing *p*H the $s_{20,w}$ values for the apohemocyanin seem to decrease slightly. Although the apohemocyanin dissociates into the same smaller species as does the hemocyanin, in 0.01 M $MgCl_2$ the dissociation occurs at a lower *p*H (about 8.5 as compared to 10.0 for the hemocyanin). Examination of the apohemocyanin schlieren photographs at *p*H 4.6 shows a very small amount of a more rapidly sedimenting component with $s_{20,w} \cong$ 94 S. A photograph is shown in Figure 1B. The broadening of the 58 S boundary should also be noted; this would also be expected if there were a slight tendency to association of the 58 S material. Under identical conditions, the hemocyanin yields a considerably sharper boundary, and no evidence of a 94 S peak.

Spectra and Optical Rotatory Dispersion.—Typical hemocyanin and apohemocyanin spectra are shown in Figures 3A and 4. The optical density of the apohemocyanin in the visible and near-ultraviolet regions is probably a consequence of scattering. By subtracting this contribution from that of the hemocyanin, estimates can be made of the extinction coefficients at the maxima at 345 and 580 mμ. The values based on the copper content are of most interest for comparison with

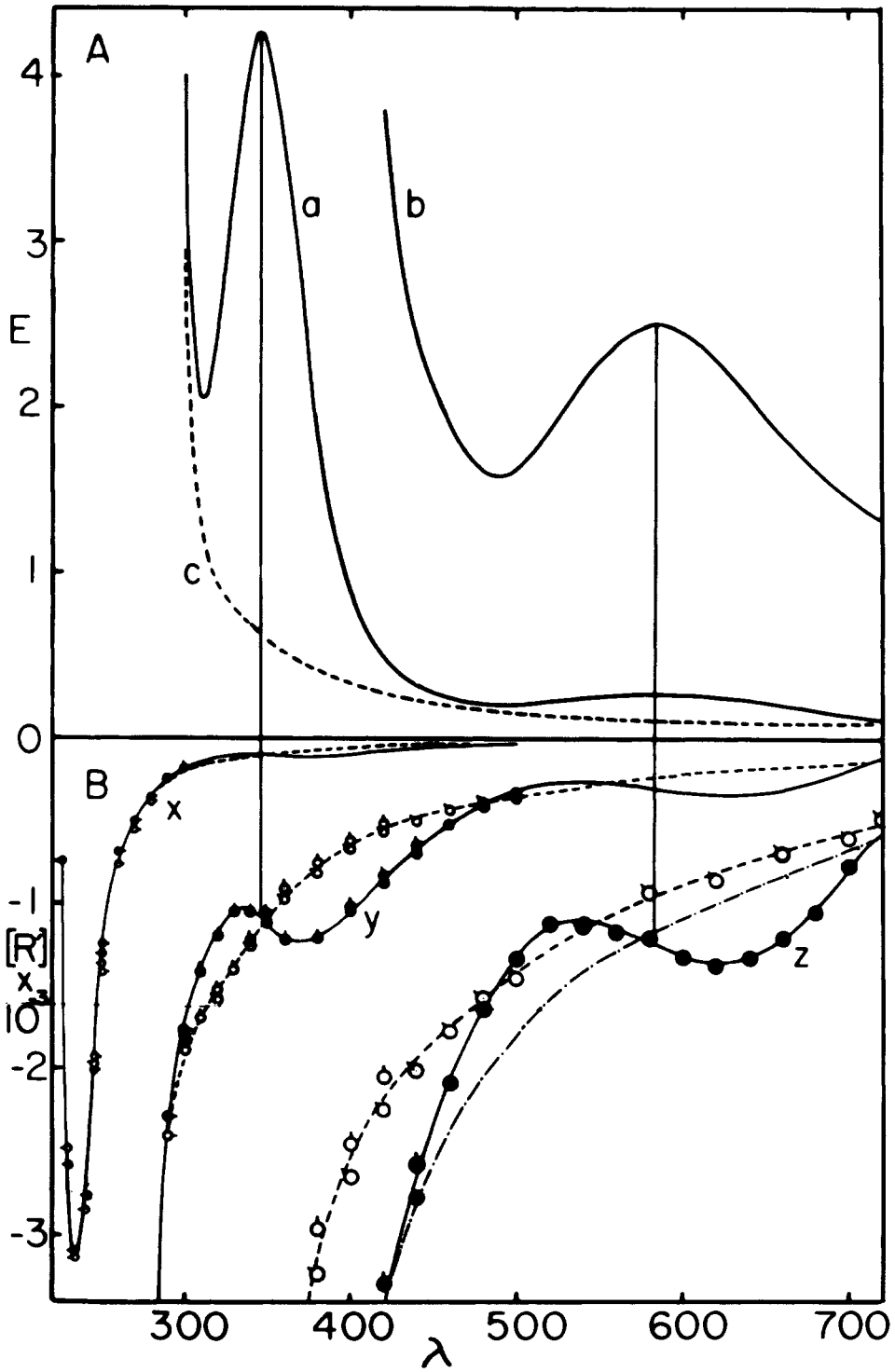

FIG. 3.—(A) Near-ultraviolet and visible spectra of *Loligo* hemocyanin at *p*H 8.2 (a and b) and apohemocyanin at *p*H 6.6 (c). Data are represented as $E^{1\%}_{1\,cm}$. Curve b was obtained with a solution ten times as concentrated as curves a or c, and the scale should be divided by ten. (B) Optical rotatory dispersion of *Loligo* hemocyanin, deoxygenated hemocyanin, and apohemocyanin. The mean residue rotation scale is given for curve x. For curve y, divide the scale by 10; for curve z divide the scale by 40. The identification of symbols is as follows: Hemocyanin, (→) 0.177 mg/ml, (←) 0.856 mg/ml, (◊) 3.72 mg/ml, (●) 34.9 mg/ml; apohemocyanin, (○) 0.211 mg/ml, (○) 1.12 mg/ml, (◊) 4.48 mg/ml; deoxygenated hemocyanin. (◡) 34.9 mg/ml. The solid line(——) indicates the hemocyanin data, the dashed line (- - -) the apohemocyanin or deoxyhemocyanin data, and the broken line (·····) is the apohemocyanin curve with the 345 mμ Cotton effect subtracted from it. All were measured at *p*H 8.2 in 0.1 ionic stength Tris buffer containing 0.01 M $MgCl_2$.

other copper complexes. Using a value of 0.26 wt % of copper (Prosser and Brown, 1961) the results obtained are ϵ = 8900 liters mole^{-1} cm^{-1} at 345 mμ and ϵ = 370 liters mole^{-1} cm^{-1} at 580 mμ. In the region below 300 mμ, shown separately in Figure 4, the spectra of the apohemocyanin and hemocyanin appear to be identical.

There are negative Cotton effects associated with both the 345 mμ absorption band and the 580 mμ band. In Figure 3B are shown optical rotatory dispersion

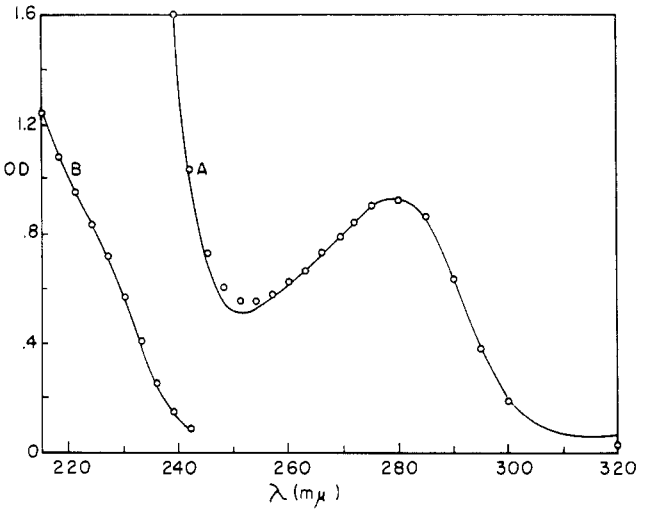

FIG. 4.—The ultraviolet spectra of *Loligo* hemocyanin and apohemocyanin in the wavelength region between 215 and 320 mμ. The line is drawn for the hemocyanin data; the points are for the apohemocyanin. Concentrations of solutions were: curve A, 0.59 mg/ml; curve B, 0.059 mg/ml. Both were in phosphate buffer, containing 0.01 M $MgCl_2$, at *p*H 7.5.

data (expressed as mean residue rotation $[R']$)[1] for the hemocyanin, the apohemocyanin, and the hemocyanin deoxygenated by the addition of 12 moles of sodium bisulfite per g-atom of copper. The points from the latter two experiments superimpose, giving a dispersion curve without Cotton effects in the visible or near-ultraviolet. These and other optical rotation measurements (data not given) show that the *deoxy*hemocyanin and the *apo*hemocyanin have identical optical rotatory dispersion curves, and presumably identical helix contents. The data for the hemocyanin and the apohemocyanin in the far-ultraviolet (left side, Fig. 3B) superimpose, indicating that these two proteins have identical helix contents. In addition, from the above identities we can conclude that the deoxyhemocyanin and the hemocyanin have the same helix content. Simmons *et al.* (1961) find that the depth of the minimum at 233 mμ for polypeptides varies from −1800 to −12,000 depending upon the helix content. Our value of $[R']$ = −3100 would correspond to about 13% helix.

From the foregoing results, we predict that in the near-ultraviolet and visible ranges the dispersion curves for the apohemocyanin and the hemocyanin are identical except for superposition of Cotton effects at 345 and 580 mμ on the rotation due to the protein. This means that the apohemocyanin and hemocyanin dispersion curves should intersect at the center of the Cotton effects at 345 and at 580 mμ. This is seen to be the case at 345 mμ. However the data around 580 mμ are more complex, and require a more extensive analysis. Evidently the negative limb of the 345 mμ Cotton effect extends well into the visible region with the result that the anomalous dispersion in the neighborhood of the 580 mμ absorption band also contains a contribution from the negative limb of the effect centered at 345 mμ. As a consequence, the rotatory dispersion curve of the apohemocyanin does not intersect the center of the 580 mμ Cotton effect. That this is the explanation can be confirmed in the following way. If an extrapolation of the 345 mμ Cotton effect into the visible is performed, the values so obtained can be subtracted from the rotatory dispersion curve of the apohemocyanin. This corrected curve should cross the observed hemocyanin curve at about 580 mμ so that the resulting difference between the two curves now reflects the true symmetry of the 580 mμ Cotton effect. The extrapolation has been carried out by assuming that the 345 mμ Cotton effect is described by the Kronig-Kramers equation (see, e.g., Djerassi, 1960):

$$[R'] = C_K \left\{ e^{-u^2} \int_0^u e^{y^2}\, dy - \Delta_K^\circ / 2(\lambda + \lambda_K^\circ) \right\} \quad (1a)$$

$$u = (\lambda - \lambda_K^\circ)/\Delta_K^\circ \quad (1b)$$

where C_K is a constant, λ_K° is the wavelength of the absorption band, and Δ_K° is a measure of the width of the circular dichroism band, which is assumed to be Gaussian. Equation (1) describes a typical Cotton-effect curve, with C_K, λ_K°, and Δ_K° as parameters. To obtain Δ_K°, equation (1a) may be differentiated and the result set equal to zero at the maximum and minimum. This yields

$$e^{(u')^2} = 2u' \int_0^{u'} e^{y^2}\, dy \quad (2)$$

where $u' = (\lambda' - \lambda_K^\circ)/\Delta_K^\circ$, λ' being the value of λ for the maximum or minimum in the dispersion curve. The (small) derivative of $\Delta_K^\circ/2(\lambda + \lambda_K^\circ)$ has been neglected in deriving (2). Graphical solution of (2) yields $u' = \pm 0.91$, which allows calculation of Δ_K° from λ' and λ_K°. The constant C_K can then be fixed from the value of the 345 mμ Cotton effect contribution at one wavelength. Then, this contribution can be calculated at any other wavelength from equation (1). In this way, the broken line in Figure 3B has been calculated. It does intersect the dispersion curve for the oxygenated hemocyanin at about 580 mμ. Thus the failure of the apohemocyanin curve to intersect the hemocyanin curve at 580 mμ is due to the effect of the superposition of a Cotton effect centered at 345 mμ upon the one centered at 580 mμ.

DISCUSSION

By the criterion of sedimentation velocity, the hemocyanin and apohemocyanin seem virtually identical. Thus both show 58 S, 19 S, and 11 S components, and no clearly demonstrable difference in sedimentation coefficient between corresponding materials can be seen. Of course, the loss of the mass of the copper would not in itself be detectable, but it might have been expected that removal of the copper could give rise to configurational changes, or changes in the mode of dissociation. If conformational changes occur, they must be very subtle, for the rotatory dispersion of the hemocyanin and apohemocyanin are virtually identical in the 233 mμ region, and differ at longer wavelengths only in the addition of Cotton effects associated with the 345 mμ and 580 mμ bands.

While this paper was being written, some results of studies by Foss (1964) of the rotatory dispersion of *Cancer magister* hemocyanin appeared. In this work the oxygenated and deoxygenated hemocyanin were studied over the wavelength region from 300 mμ to slightly above 600 mμ. The limited range of Foss' data and the failure to resolve clearly the 580 mμ

[1] The mean residue rotation $[R']$ is defined as

$$[R'] = \frac{3}{n^2 + 2} \cdot \frac{\bar{M}}{100} [\alpha]$$

where n is the refractive index, $\bar{M}$ is the mean amino acid residue weight (taken as 115), and $[\alpha]$ is the specific rotation. Refractive indices at the various wavelengths were taken from the International Critical Tables.

Cotton effect mean that the possibility of structural change in the protein was not excluded. However the evidence does suggest, as Foss has stated, that in this case as well the principal difference between the rotatory dispersion of oxygenated and deoxygenated hemocyanin arises from the optical activity of the near-ultraviolet and visible absorption bands.

Klotz and Heiney (1957) observed that the levorotation of *Busycon canaliculatum* hemocyanin at 436 mμ decreased markedly upon removal of the oxygen. They have suggested that this indicates that "the protein fabric is affected in a very drastic fashion." While we observe a similar change at this wavelength upon either deoxygenation or removal of the copper, we would interpret this change as a consequence of removal of the optically active 345 mμ and 580 mμ bands. Furthermore, the virtual identity of both the optical rotatory dispersion and the absorption spectrum of hemocyanin and apohemocyanin at short wavelengths would support this interpretation. It should be emphasized, of course, that the hemocyanin studied by Klotz and Heiney was from a different source.

The fact that the sedimentation coefficient of the 58 S apohemocyanin decreases slightly with *p*H in the range 4.6–8.5 may suggest a progressive "loosening" of the structure which is not observed with the hemocyanin. Similarly, in 0.01 M $MgCl_2$ the 58 S hemocyanin is stable to *p*H = 10.0, whereas the 58 S apohemocyanin dissociates at *p*H = 8.5. A similar effect with *Helix pomatia* hemocyanin has been described by Lontie (1958). Such an effect does not necessarily mean that the copper is directly involved in the binding together of subunits; a change in charge accompanying the loss of copper might account for the difference.

Finally, the observation of small amounts of a 94 S component is of considerable interest. In a number of other species, a component with $s_{20,w} \cong 100$ S forms the larger part of the hemocyanin under some conditions. In the case of *Helix pomatia*, it has been demonstrated by electron microscopy (Van Bruggen *et al.*, 1962a) that the 100 S hemocyanin corresponds to an end-to-end dimer of a 60 S component. The latter greatly resembles the most common structure observed in the serum of squid and octopi (Van Bruggen *et al.*, 1962b). Thus it seems likely that the apohemocyanin from the squid *Loligo pealei* can be induced to form a similar dimer at low *p*H values.

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