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Physical Studies of Hemocyanins. III. Circular Dichroism and Absorption Spectra*

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ABSTRACT: Studies of the circular dichroism of *Octopus vulgaris* and *Loligo pealei* hemocyanins reveal that the absorption band in the visible spectrum is not simple, but involves at least three components, centered at 440, 570, and about 700 mμ. This has been confirmed by measurement of the absorption spectrum in glycol–water glass at very low temperatures. The set of bands now observed in the hemocyanin spectrum corresponds closely to the structures of the spectra of a number of proteins containing cupric copper. On this basis, mechanisms for oxygen binding by hemocyanin are discussed.

Although it has been known for many years that the oxygen binding by hemocyanin depends upon the presence of copper in the protein, the mode of attachment of this copper, or even its oxidation state, remains uncertain. It appears that the metal in the deoxygenated protein is cuprous, for only by the use of cuprous salts can active hemocyanin be regenerated from the apohemocyanin (Kubowitz, 1938), and chemical studies reveal that all of the copper, when released from the deoxygenated protein, is cuprous (Klotz and Klotz, 1955; Felsenfeld, 1960). Similar experiments indicated that about one-half of the copper is cupric in the oxygenated protein. However, these results have been criticized because of the possibility of oxidation of the copper by released oxygen or its reduction by reducing groups on the protein (see, for example, Manwell, 1964.) Finally, oxygenated hemocyanin does not display the electron paramagnetic resonance (epr) signal which would normally be expected if cupric copper were present (Nakamura and Mason, 1960; Manwell, 1964; D. L. Lloyd and K. E. Van Holde, unpublished). Thus, it is held by some that the metal is in the cuprous form in both oxygenated and deoxygenated hemocyanin (for example, Malmstrom and Nielands, 1964). In contrast to this, the spectral changes induced by oxygenation have been cited. The deoxygenated protein is colorless, and exhibits a spectrum in the ultraviolet region very similar to that of other proteins. When oxygen is added, a rather strong absorption band appears in the visible region, yielding a deep blue color reminiscent of that of many cupric complexes. A very intense band also appears in the near-ultraviolet region. Because of its intensity (extinction coefficients of about 10^4 have been commonly reported), the latter band has been attributed by many to a charge-transfer transition. Those who hold that the copper remains cuprous upon oxygenation have suggested that the absorption in the visible spectrum also corresponds to charge-transfer bands. In this region a clear distinction cannot be made upon the basis of intensity, for the extinction coefficients are in the neighborhood of 500 l. (g-atom)$^{-1}$ cm^{-1}, which is somewhat high for cupric d–d transitions but low for typical charge-transfer bands.

Under these circumstances, any information which may lead to an understanding of the absorption spectrum is of potential value. Recent measurements of optical rotatory dispersion (ORD) (Foss, 1964; Cohen and Van Holde, 1964) have shown that both the band in the visible and that in the ultraviolet are optically active. However, the detailed analysis of these ORD spectra is complicated by the overlap of the Cotton effects, and the enormous contribution to the optical rotation from the peptide Cotton effects centered near 200 mμ. For this reason, it seemed that circular dichroism (CD) measurements, with inherently greater resolving power, might yield more information. Some preliminary measurements have been reported by Foss (1964). However, while the report of a negative

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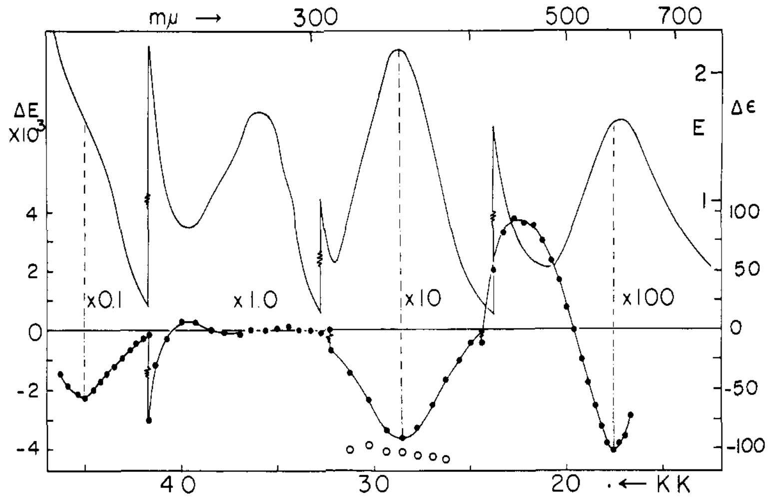

FIGURE 1: The absorption spectrum (solid line) and circular dichroism spectrum (—●—) of *Octopus* hemocyanin at pH 6.6, ionic strength 0.1. The energy of the radiation is expressed in both kilokaisers (bottom scale) and wavelength (top scale). The absorption spectrum (E) is in units of milliliters (milligrams of protein)$^{-1}$ centimeters^{-1}, and the circular dichroism is expressed in terms of $\Delta E(=E_L - E_R)$ or $\Delta\epsilon(=\epsilon_L - \epsilon_R)$, where ϵ is in units of liters (gram-atoms of Cu)$^{-1}$ centimeters^{-1}. The scales apply to the 240–300-mμ region (X1); for other regions the numbers are to be divided by the factors given. A set of points (○) corresponding to $g'(=\Delta\epsilon/\epsilon)$ are given without scale.

dichroic band associated with the near ultraviolet band in consistent with the rotatory dispersion, the observation of weak positive dichroism in the visible seemed in contradiction to the ORD data. The circular dichroism studies reported in this paper reveal new detail in the spectra, which detail has been confirmed by absorption spectroscopy at very low temperatures.

Experimental Section

Materials. Hemocyanins from two molluscan species, *Octopus vulgaris* and the squid *Loligo pealei*, were employed. The *Octopus* hemocyanin was obtained from Dr. A. Ghiretti-Magaldi, of the Naples Zoological Station, and had been lyophilized in the presence of sucrose. This material, which was wholly soluble as received, was dialyzed exhaustively against the appropriate buffer before measurements were made. The *Loligo* hemocyanin was obtained from live squid at Woods Hole, Mass., and treated in the manner described previously (Van Holde and Cohen, 1964). The purified serum was quickly frozen, and kept at $-10°$ or lower until use. Examination of the thawed, diluted material in the ultracentrifuge revealed that at pH 6.7 the major component had a sedimentation coefficient of $s_{20,w} = 56 \times 10^{-13}$ sec. This is in good agreement with our previous results (Van Holde and Cohen, 1964), and the schlieren patterns indicated little if any disaggregation in freezing.

Phosphate or Tris buffers were used, and in all cases the ionic strength was 0.1. Protein concentrations were routinely determined by absorption measurements at 280 mμ. For the *Loligo* hemocyanin, the factor previously determined (Van Holde and Cohen, 1964) was used. Kjeldahl measurements of the *Octopus* hemocyanin led to a value for the extinction coefficient of E 1.70 ml mg^{-1} cm^{-1} at 280 mμ.

Circular Dichroism. The studies of *Octopus* hemocyanin were performed with a Roussel-Jouan Dichrograph, modified by the addition of an extra sensitivity scale. These experiments were performed at the Centre de Recherches sur les Macromolecules, Strasbourg, France. The experiments with *Loligo* hemocyanin utilized a circular dichroism apparatus constructed under the direction of Professors T. S. Piper and R. L. Belford of the University of Illinois. This instrument employs a special photomultiplier to extend the wavelength range to above 1000 mμ.

Absorption Spectroscopy. All absorption spectra were recorded on either Cary 14 or 15 spectrophotometers. For the low temperature measurements, a cryostat constructed by Dr. J. Brahms of the Centre de Recherches sur les Macromolecules was used. In these experiments the hemocyanin was dissolved in 30–40% (v/v) ethylene glycol–water mixtures. With care, these mixtures could be brought close to liquid nitrogen temperature for some time before cracking of the transparent glass occurred. Several spectra were obtained in this condition, although the glass was liable to crack upon the slightest disturbance.

Results

In Figure 1 are shown the absorption spectrum and circular dichroic spectrum of *Octopus* hemocyanin after equilibration with air in phosphate buffer at pH 6.6. Very similar results were obtained in Tris buffer at pH 8.2; the only difference results from the some-

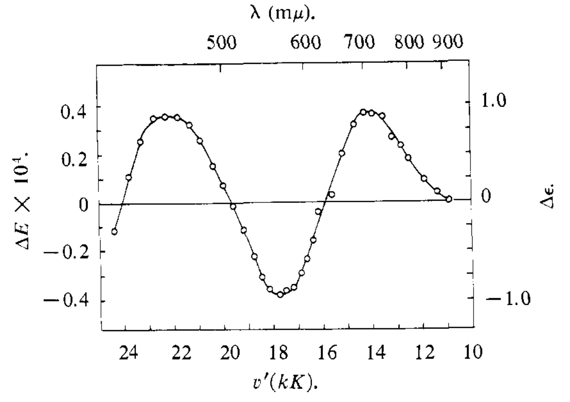

FIGURE 2: The CD spectrum of *L. pealei* hemocyanin (pH 6.6, ionic strength 0.1) at long wavelengths. Units same as in Figure 1.

what greater intensity of the copper bands at the higher pH. The data are expressed both in terms of protein concentration (milligrams per milliliter) and copper concentration (gram-atoms per liter). Since the intensities of both absorption and circular dichroism vary by several orders of magnitude over the entire wavelength range covered, the curves have been multiplied or divided by the factors indicated to yield roughly comparable results in the different regions of the spectrum. The absorption spectrum of the *Octopus* hemocyanin is very similar to that observed for other oxygenated hemocyanins. The circular dichroism can be described as follows.

(1) In the vicinity of the $n - \pi^*$ peptide absorption band (around 225 mμ), a fairly strong negative dichroic band is observed. This presumably originates in helical or other ordered structure of the polypeptide chains. If it is interpreted entirely in terms of α helix, according to the data given by Holzwarth and Doty (1965), about 19% helix is indicated. This is in the same range as the earlier estimate for the *Loligo* hemocyanin (about 13%) based upon optical rotatory dispersion data from the same spectral region. It should be pointed out that the instrument used in these measurements has poor spectral resolution below 240 mμ, and yields no reliable data below about 215 mμ. Thus, it was not possible to resolve the second dichroic band usually found for α-helical polypeptides at 207 mμ (Townend *et al.*, 1966). This prohibits an absolute distinction between α and β conformations. However, since the latter should yield a band at 217 mμ, instead of 222 mμ as observed, a large per cent of β structure is not likely. It should also be noted that use of the data of Townend *et al.* (1966) instead of that of Holzwarth and Doty (1965) leads to a value of 15% helix, if presumed to be all of the α form.

(2) The wavelength region corresponding to protein side-chain absorption (from about 240 to about 300 mμ) exhibits little or no optical activity. There may be a very weak positive dichroic band near 250 mμ, but this is at the border of detectibility.

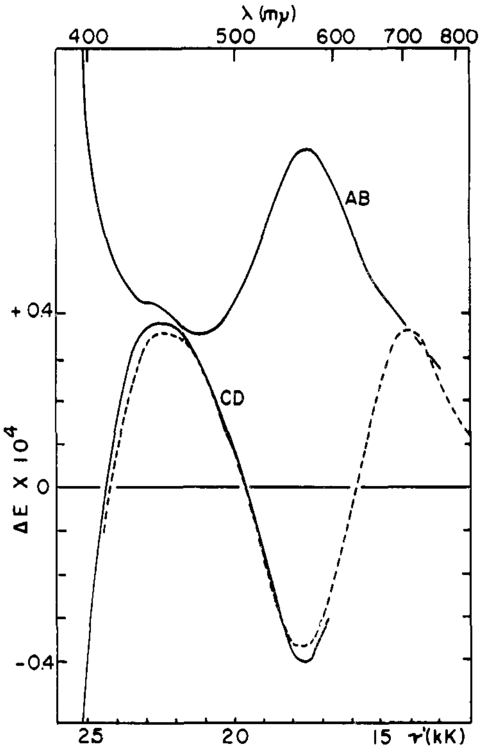

FIGURE 3: The absorption spectrum of *Octopus* hemocyanin in glycol–water glass at very low temperature (approximately −195°). The circular dichroic spectra of *Octopus* (-) and *Loligo* (--) hemocyanin are shown for comparison.

(3) The copper band in the near-ultraviolet region (centered at 347 mμ) corresponds to a fairly strong negative dichroic band. The value of $g' = \Delta\epsilon/\epsilon$ is about 1.5×10^{-3}. As seen by the constancy of g' values, this band appears to be simple in structure.

(4) In the visible region of the spectrum, the oxygenated hemocyanin exhibits not one, but two dichroic bands. These are of opposite sign and are centered at about 440 and 570 mμ. Moreover, the behavior of the circular dichroism at the upper wavelength limit of the instrument used for these measurements (600 mμ) suggests that the curve may again cross the axis at higher wavelengths, yielding a third band.

This suggestion is confirmed in the circular dichroic spectrum of *Loligo* hemocyanin shown in Figure 2. With an instrument capable of measurements into the near-infrared region, it is shown that there are in fact *three* dichroic components in the long-wavelength band. The two lower wavelength components are nearly identical in *Octopus* and *Loligo* hemocyanins. That these three components of the dichroic spectrum indeed correspond to separate absorption bands is shown by Figure 3, which depicts the absorption spectrum of *Octopus* hemocyanin at temperatures close to that of liquid nitrogen. A shoulder is clearly revealed at about 440 mμ, the central maximum is now at 570 mμ, and another shoulder appears to be present

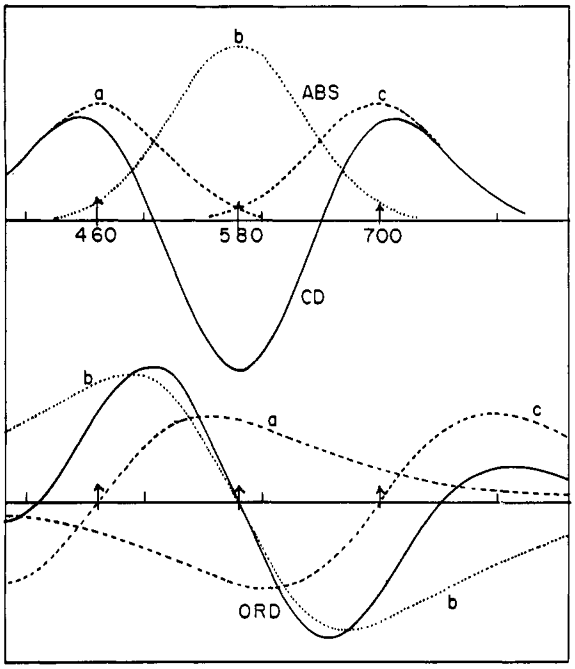

FIGURE 4: Theoretical circular dichroic spectra and ORD curves corresponding approximately to the optical activity of the hemocyanins in the visible region. The CD bands are all of the same half-width (67 mμ), and are centered at 460, 580, and 700 mμ. The central band is assumed to have 1.5 times the intensity of the flanking bands. The ORD curve strongly resembles a single negative Cotton effect centered at 580 mμ.

at about 700 mμ. Similar results have been reported by Mason in 1964,[1] who found in low temperature spectroscopy of frozen samples of *Cancer magister* hemocyanin bands at 420, 566, and 652 mμ. The demonstration of these components in both the absorption spectrum of the glass at low temperature and the circular dichroic spectrum argues strongly against their being artifactual.

It was felt desirable to have available accurate values for the extinction coefficients of the hemocyanin bands. To obtain such numbers unambiguously, it is necessary to: (1) assure that the hemocyanin is completely oxygenated under the conditions of measurement, and (2) correct for the relatively large scattering produced by these large molecules. The spectra of *Octopus* hemocyanin were measured at various pH values, both at atmospheric oxygen tension and after saturation with pure oxygen, under an oxygen atmosphere. The results showed that while at pH 6.6 the protein is not quite saturated at atmospheric pressure, it is so at pH 8.2. Consequently, only spectra measured at the latter pH were used for these calculations. Corrections for scattering were made in the following manner. The sample, after measurement of the spectrum, was deoxygenated by the addition of a small amount of sodium sulfite, and the spectrum was rerecorded. The scattering spectra, in the visible and near-ultraviolet regions, were found to be linear in the inverse fourth power of the wavelength, as expected, but scattering was nonlinear in concentration at the higher concentrations. The scattering, when subtracted from the observed absorption spectrum, yields spectra such as that shown in Figure 1. The results have been expressed in terms of the copper molarity in the solutions; for this purpose, a value of 0.25% Cu in the hemocyanin was used (Ghiretti, 1962). The extinction coefficients found for the *Octopus* hemocyanin at pH 8.2 are: 8900 l. (g-atom)$^{-1}$ cm^{-1} at 347 mμ, and 500 l. (g-atom)$^{-1}$ cm^{-1} at 580 mμ. The corresponding values for the *Loligo* hemocyanin at pH 8.2 are, as reported previously (Cohen and Van Holde, 1964): 8900 at 345 mμ and 370 at 580 mμ. The weaker components at about 440 and 700 mμ, revealed in the low temperature spectral measurements, are not sufficiently well resolved to allow a meaningful estimate of extinction coefficients. It can only be said that they are much less intense than the 570-mμ band.

Discussion

These data, together with the results of Mason in 1964[1] show that the absorption of the copper chromophores of the hemocyanins (both molluscan and crustacean) is not so simple as had been hitherto assumed. Evidently, in the range between 300 and 800 mμ there are at least four transitions. While it remains possible that still more components are hidden in this region, exploration as high as 1200 mμ has revealed no additional bands at longer wavelength. The fact that these four components are seen in circular dichroism at room temperature, as well as in two types of low temperature–absorption measurements demonstrates that they are not artifacts, nor present only at low temperature.

Because of the considerable overlap of these absorption bands, it is not possible to make quantitative estimates of either the oscillator strengths or rotational strengths of the individual bands in the visible region. Furthermore, the locations of the individual absorption maxima are, for the same reason, subject to some uncertainty. However, the coincidence of apparent absorption and dichroic maxima for the 440- and 570-mμ bands in the molluscan hemocyanins give some confidence to their locations.

At first glance, it may seem surprising that this complexity of structure was not noted in the earlier ORD studies (Foss, 1964; Cohen and Van Holde, 1964). However, as is shown in Figure 4, the kind of combination of dichroic bands which could give rise to the circular dichroism spectrum of the type observed produces an ORD curve which is very difficult to distinguish from a single negative Cotton effect, especially if observed superimposed upon a very large background

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[1] The work of H. S. Mason was discussed following a paper given by Manwell (1964).

TABLE I: Properties of Copper Proteins.

Material	Cu/ Mole	Cu^{2+}/ Mole (max)	Epr?	Spectrum							
				Band I		Band II		Band III		Band IV	
				λ	ϵ	λ	ϵ	λ	ϵ	λ	ϵ
Hemocyanin	>2[a]	0	−	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
Oxyhemocyanin	>2[a]	?	−	347	8900	440	<500	570	500	700	<500
Ceruloplasmin[b]	8	4	+	332	500	459	140	610	1300	794	260
Laccase (fungal)[c]	4	2	+	330	1000	*d*	*d*	610	1000	650	$\ll 1000$
Plastocyanin[e]	2	2	+	. . .	. . .	460	590	597	4900	770	1700
R. vernicifera[f] blue protein	1	1	+	. . .	. . .	450	970	608	4030	850	700
Tyrosinase (*Neurospora*)[g]	1	0	−	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .

[a] The hemocyanins are very high molecular weight proteins, and, depending upon the state of aggregation, contain many pairs of copper atoms. [b] Blumberg *et al.* (1963). Extinction coefficients have been recalculated on a total copper basis for comparison. [c] Mosbach (1963). [d] While no maxima have been identified in this region, the absorption is not zero. [e] Omura (1962). [f] Omura (1961). [g] Fling *et al.* (1963).

resulting from lower lying Cotton effects (see Figure 3, Cohen and Van Holde, 1964). This provides an exceptionally good example of the superior resolving power of CD over ORD in studies of this kind.

The Oxidation State of the Copper in Hemocyanin

Recognition of the complexity of the spectra of the hemocyanins allows a more meaningful comparison of hemocyanin with other copper proteins than has been hitherto possible. In Table I are compared spectroscopic and other data for a selected group of such proteins. Those chosen include ceruloplasmin and fungal laccase (proteins which have both cupric and cuprous copper) plastocyanin, (a protein containing two cupric ions per molecule), *Rhus vernicifera* blue protein (which has a single cupric copper), and *Neurospora* tyrosinase (in which the single copper is cuprous).

It is indeed remarkable that most of these proteins (with the exception of the cuprous tyrosinase and the deoxygenated hemocyanin) exhibit absorption bands at about 450, 600, and 750 mμ. Furthermore, three of these (oxyhemocyanin, ceruloplasmin, and laccase) also exhibit bands at about 340 mμ. There is enormous variation in the extinction coefficients of the various bands, even if the values are calculated on the basis of the cupric copper content. However, the pattern of absorption bands in the visible region is similar throughout; the central band (near 600 mμ) is invariably much stronger than the two flanking bands.

We are faced then, with the necessity to explain the similarity and unique properties of a diverse group of copper proteins. The visible spectra are characterized by absorption intensities remarkably greater than those of most low molecular weight complexes. Two general kinds of explanations have been proposed.

A. Charge Transfer. This can, and frequently does, lead to exceedingly intense bands. However, there are a number of reasons for rejecting charge transfer as a general explanation for the intensities. Proposals involving cuprous ions as donors must obviously fail for plastocyanin and the *R. vernicifera* blue protein, which contain only cupric copper. Ligand $\rightarrow$ Cu^{2+}-transfer bands in the visible are observed with complexes like $CuBr_4^{2-}$ and $CuCl_4^{2-}$, but are exceedingly sensitive in wavelength to the nature of the donor ligand. It is hard to see how such a uniformity of bands could exist in such a variety of proteins were this the general explanation.

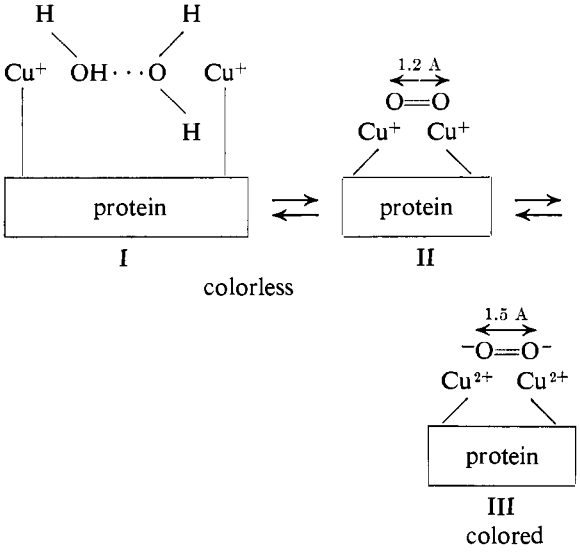
B. d–d Transitions. In most cupric complexes of high symmetry, the d–d transitions are strongly forbidden, and hence very weak. However, departures from cubic or square-planar symmetry can lead to much greater absorption. For example, tetrahedral Cu^{2+} complexes show quite strong absorption bands, but these lie in the neighborhood of 1000 mμ (Williams, 1964). Recently, Blumberg (1966) has proposed a type of distorted rhombic coordination site which can explain both the wavelengths and intensities of the three cupric d–d transitions in ceruloplasmin. This demonstrates that such visible spectra as have been discussed here can be explained in terms of d–d transitions, without specifically invoking charge transfer.

Blumberg has also predicted the rotational strengths of the three bands in the visible spectrum. These agree in sign with the observations for oxyhemocyanin.

The band at about 340 mμ remains to be considered. It is exceedingly strong in the hemocyanins, but is absent entirely in the *R. vernicifera* blue protein and plastocyanin. These differ in two respects from the other copper proteins listed. They do not appear to bind oxygen under any circumstances, and contain no cuprous copper. Thus, it might be argued that the 340-mμ band corresponds either to ($Cu^+ \rightarrow Cu^{2+}$) charge transfer, or to charge transfer between copper and oxygen.

In the context of this analysis, we must include oxyhemocyanin among the copper proteins in which the copper is at least partially in the Cu(II) form. Unless the similarities of the spectra shown in Table I are to be attributed to coincidence, the common proposal that the copper in oxyhemocyanin is entirely cuprous must be rejected. Either the "half-cupric" hypothesis of Klotz and Klotz (1955) or the scheme recently presented by Frieden *et al.* (1965a) would seem consistent with these data. There remains to be explained the absence of an electron paramagnetic resonance signal in oxyhemocyanin. However, it has been suggested that either electron pairing with the oxygen or between cupric ions (Frieden *et al.*, 1965a) may occur. In this connection, it is of interest that the intensely colored complex between copper, oxalyldihydrazide, and acetaldehyde also does not exhibit an epr signal (see the comments by Frieden following the paper by Frieden *et al.*, 1965b). This complex also exhibits an absorption band at 380 mμ (Frieden *et al.*, 1965a).

A tentative mechanism for O_2 binding by hemocyanins can be presented. Following Frieden *et al.* (1965a,b), it is assumed that in the deoxygenated protein, one coordination site on each cuprous ion is occupied by a water molecule. When these are replaced by an oxygen atom, distortion of the coordination is required to hold the smaller ligand. Electron transfer to the O_2 is facilitated by the longer bond length in the O_2^{2-} ion. Schematically



The figure does not attempt to depict the number or arrangement of protein–copper bonds, nor the geometry of bonding. It is assumed that other intensely blue copper proteins involve similarly distorted sites for cupric ions (though not, in general, utilizing oxygen as a ligand).

It should be noted that it is specifically suggested that the strain in the copper binding sites is developed only upon oxygenation. A further implication of this is that irreversible oxidation of the copper, or disruption of the site may lead to a cupric protein which does *not* exhibit the intense absorption spectrum of oxyhemocyanin. This is in accord with observations that H_2O_2-treated hemocyanin and acid-denatured hemocyanin contain cupric copper but are not highly colored (Nakamura and Mason, 1960).

Comment on the Copper Ligands

The circular dichroism spectra of the hemocyanins do not, unfortunately, allow a certain assignment of the ligands which bind the copper. However, it has been suggested by a number of workers (see especially Lontie, 1958) that histidine residues are involved. In this connection, it is of interest to compare the CD spectra obtained for the molluscan hemocyanins with those of some peptide–Cu(II) complexes recently investigated by Bryce and Gurd (1966). In particular, the CD spectrum of the acetylglycylglycylhistidine (ML_2^{-2} form) seems very similar to those given here. In the spectral range between 400 and 600 mμ, this complex shows one maximum (490 mμ, $\Delta\epsilon$ +1.10) and one minimum (590 mμ, $\Delta\epsilon$ −1.36). While this correspondence is far from conclusive, it should be noted that none of the nonhistidine-containing complexes show CD spectra even remotely resembling those found for the hemocyanins. This may be taken as one more piece of circumstantial evidence implicating histidyl residues as ligands in hemocyanin.

Acknowledgments

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