Oxygen-Carrying Proteins: a Comparison of the Oxygenation Reaction in Hemocyanin and Hemerythrin with That in Hemoglobin

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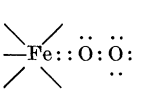
FOR the vast majority of organisms, oxygen in the atmosphere is one terminus of chemical metabolism. From the air oxygen becomes dissolved in water and then in blood, or enters directly into the blood, where the uptake is increased enormously when certain respiratory pigments are present. One hundred milliliters of sea water may hold about 0.5 ml of oxygen, whereas with oxygen-carrying pigments, 100 ml of blood may contain as much as 25 ml of oxygen.

These oxygen-carrying pigments are usually grouped into four classes: hemoglobins, chlorocruorins, hemocyanins, hemerythrins. Three of these contain iron at the center of the active site, but the fourth, hemocyanin, contains copper. Two of the iron-containing carriers, hemoglobin and chlorocruorin, have the metal centered in a porphyrin ring; however, the structure of the heme groups differs slightly in these two classes of protein. In the remaining two groups of pigment, pyrrole prosthetic groups do not occur, and it is likely that the metals are bound to a site whose configuration is determined by a constellation of amino acid side chains.

The uptake of oxygen is accompanied by a redistribution of electrons that produces changes in the colors of these pigments. With hemoglobin, these optical changes are relatively small shifts of certain spectroscopic absorption bands, but with hemocyanin and hemerythrin the color changes are profound. Neither of these last two proteins is colored in the nonoxygenated state; in contrast oxyhemocyanin is deep blue, oxyhemerythrin is dark violet-brown.

State of iron in hemoglobin and chlorocruorin. The nature of the electron distribution and the state of iron in hemoglobin has been established for some time, primarily from potentiometric (*1*) and magnetic (*2*) studies. In the nonoxygenated form, iron exists in the ferrous state; in oxyhemoglobin, the ferroheme group forms a complex with oxygen. The electronic structure of this complex may be written as follows (*3*).



The heme nature of the prosthetic group of chlorocruorin, as well as the general parallelism in behavior of this oxygen carrier and hemoglobin, warrants the belief that the oxygenation reaction is of essentially the same character in both classes of protein.

State of copper in hemocyanin. The situation with hemocyanin and hemerythrin is more confusing, however. Largely by analogy it has been assumed that each metal is in its lowest valence state in the deoxygenated protein, cuprous in hemocyanin (*4*), ferrous in hemerythrin (*5*), and that this valence state is retained in the oxygenated proteins. With hemocyanin, however, there are difficulties with this viewpoint. The blue color of the oxygenated pigment is very reminiscent of the colors of simple cupric complexes. In fact the spectra of cupric-protein complexes prepared *in vitro* (*6*) (Fig. 1) generally show absorption maxima, at the proper *p*H, close to 600 mμ, which is the region of the maximum in the visible spectrum for hemocyanin. For some time it was difficult to account for the additional peak near 350 mμ shown by oxy-

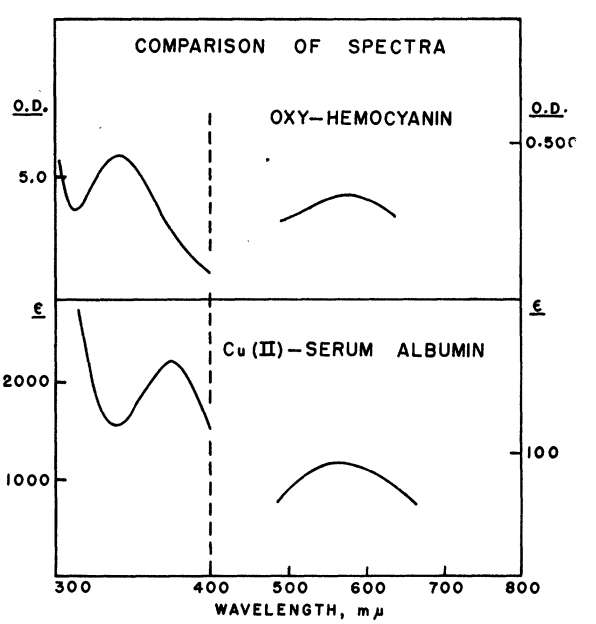


Fig. 1. Absorption spectra of oxyhemocyanin and of cupric-bovine albumin. *O.D.*, optical density; ε, molecular extinction coefficient.

hemocyanin, but it has now been observed (*7*) that a cupric ion on the sulfhydryl group of serum albumin has an absorption maximum at 375 mμ. Thus the optical properties of oxyhemocyanin parallel those of cupric proteins (Fig. 1) and suggest that the metal is in the divalent state.

Nevertheless, the comparison of optical properties is not entirely convincing, for the intensity of absorption per gram atom of copper is 5 to 10 times greater in oxyhemocyanin than in the laboratory-prepared complexes. Evidently the copper in hemocyanin, even if cupric, must be in an unusual environment. For this reason we have looked for alternative methods of establishing the valence state.

Oxidation-reduction potentials have been measured by Conant, Chow, and Schoenbach (*8*), but as has been demonstrated by Rawlinson (*9*), the potentials drift over long periods of time, and it is likely that groups of the protein other than the metal are involved in these electrochemical reactions. Likewise an approach through magnetic measurements does not hold much promise, despite the substantial difference in the magnetic moments of cuprous and cupric ions (0 and 1.73 Bohr magnetons, respectively). For the copper content of hemocyanin is so small that a change in valence from +1 to +2 would change the mass susceptibility by only 0.03×10^{-6} per gram (*9*). Furthermore, the observed diamagnetism of the oxygenated protein (*9*) might be the result of the presence of cuprous ion combined with oxygen in which the two unpaired electrons have become paired, but it could also be explained in terms of copper in the cupric state with the free electrons of each pair of metal ions being coupled with the two unpaired electrons of a molecule of oxygen. Thus magnetic measurements lead to no decision about the state of the metal in hemocyanin. Finally, polarographic reduction of the metal ion, in which cuprous and cupric ions in complexes can usually be distinguished by the appearance of one or two reduction waves, respectively (*10*), is also of no avail with hemocyanin. In the deoxygenated form no wave corresponding to either form of copper can be detected (Fig. 2); in oxyhemocyanin only the oxygen wave is observed (*11*). Evidently the copper, whatever its valence, is held very strongly by the protein, or is sufficiently imbedded in the macromolecule that the electrons from the dropping mercury electrode are unable to reach the metal ion.

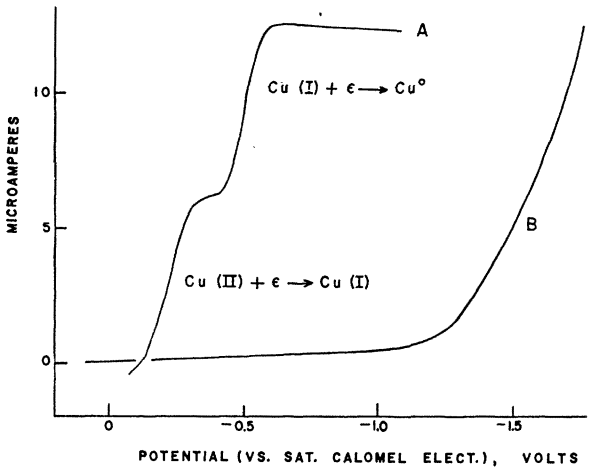


Fig. 2. (*A*) Polarogram of Cu(II) in aqueous ammonia solution; (*B*) of deoxygenated hemocyanin. The data for *A* were taken from Kolthoff and Lingane (*10*).

We have turned back, therefore, to the use of specific quantitative color reactions. Examination of a number of possibilities has finally led to the adoption of 2,2′-biquinoline in glacial acetic acid. The glacial acetic acid (*12*) serves as an agent for the release of protein-bound copper and as an excellent solvent, both for the biquinoline and for the residual protein. The biquinoline gives a strong pink color with cuprous ion (*13*), none with cupric ion.

Application of this reagent to hemocyanin in the blood of the whelk (*Busycon canaliculatum*) gives results such as those shown in Table 1. We can see

Table 1. Application of 2,2′-biquinoline in glacial acetic acid to hemocyanin in the blood of the whelk (*Busycon canaliculatum*).

Metal	Nonoxygenated	Oxygenated
Cu(I)	$6.5 \times 10^{-4} M$	$2.9 \times 10^{-4} M$
Cu total	$7.5 (\pm 0.4) \times 10^{-4}$	

first that in the nonoxygenated protein, practically all of the copper is in the cuprous form. The deviation between the content of Cu(I) and total copper may be in part the result of the use of whole blood instead of crystalline hemocyanin and to the difficulty of removing the last traces of bound oxygen by a nonchemical method (*14*). Most striking and of greatest interest is the fact that oxygenation of hemocyanin converts approximately *one-half* of the copper to Cu(II), the remainder retaining the Cu(I) state. This observation has been confirmed many times with the dialyzed blood of *Busycon*, and comparable results have been obtained with the bloods of the lobster (*Homarus americanus*), squid (*Loligo*), and horseshoe crab (*Limulus polyphemus*).

Evidently, then, oxyhemocyanin contains copper in each of its two valence states, simultaneously This information allows one to harmonize the confusing physicochemical results, particularly the spectra, previously described, and to draw a picture of the active site of the protein (Fig 3), a picture that draws together all these data. Since the stoichiometry of O_2 to copper has been long known to be 1:2 (*15*), it is evident that one copper is cupric and one cuprous in the oxygen complex, as is illustrated in Fig. 3. The presence of some cupric ion would account for the blue color of hemocyanin. Furthermore the absorption peak near 340 mμ, close to that at 375 mμ of the cupric-sulfhydryl linkage in bovine serum albumin, suggests that the metal in hemocyanin is also

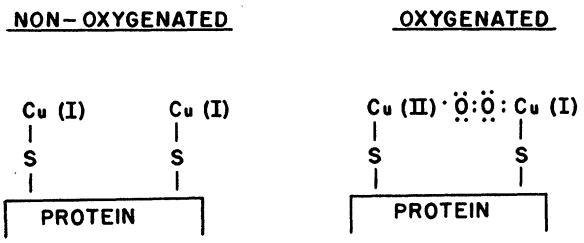


Fig. 3. State of copper in hemocyanin.

linked to the protein through a sulfur bridge. Of most significance are the very high extinction coefficients in oxyhemocyanin as contrasted to laboratory-prepared copper proteins, for enhancement of optical absorption is a definite characteristic of mixed valence states. One can point to familiar cases such as the ferroferricyanides; but perhaps the most pertinent example is that of McConnell and Davidson (*16*), who prepared chloro-bridge complexes containing Cu(I) and Cu(II) and found decided optical exaltation as compared with the simple cupric complex.

Thus the oxygenation reaction in hemocyanin, in contrast to that in hemoglobin, involves also a partial oxidation reaction. The O_2 molecule picks up 1 electron from one of the two cuprous ions and becomes a perhydroxyl ion, O_2^-. The perhydroxyl radical has been demonstrated in aqueous solutions (*17*), and compounds such as KO_2 have been recognized for some time (*18*). In hemocyanin, some stabilization of this radical may also arise from possible resonance of an electron between the two copper ions.

State of iron in hemerythrin. In view of the success of a specific color test with hemocyanin, it seemed worth while to attempt an analogous approach with hemerythrin. Marrian (*5*) reported some years ago that the iron liberated from hemerythrin with dilute hydrochloric acid "gave a distinct Prussian blue coloration" with potassium ferricyanide. It would seem reasonable to conclude from this observation that Fe(II) existed even in oxyhemerythrin. However, the Prussian blue reaction does not lend itself to quantitative work. Without a quantitative check, Marrian's observation might be merely the result of a trace amount of Fe(II), as has indeed turned out to be the case; consequently we turned to other color tests for the valence state of iron.

Table 2. Application of *o*-phenanthroline with dilute sulfuric acid to a solution of crystalline hemerythrin (from *Phascolosoma gouldii*) in sea water.

Metal	Nonoxygenated	Oxygenated
Fe (II)	$1.24 \times 10^{-4} M$	$0.245 \times 10^{-4} M$
Fe total	1.67×10^{-4}	1.70×10^{-4}

The most convenient quantitative procedure has been to use *o*-phenanthroline with dilute sulfuric acid. Ferrous ion forms a characteristic orange color with reasonable rapidity in this solution (*19*). Application of this reagent to a solution of crystalline hemerythrin (from *Phascolosoma gouldii*) in sea water gives results such as those shown in Table 2.

Turning to the oxygenated state first, we see that essentially all of the iron is in the ferric state (*20*). In contrast, in the deoxygenated protein, approximately two-thirds is ferrous, one-third ferric. Assuming that the stoichiometry (*21*) of O_2 to iron is 1 : 3, it is apparent that only 2 iron atoms change valence state in the oxygenation process. These results point to an active site with a configuration such as is shown in Fig. 4. Two of the irons in the nonoxygenated state, both ferrous, are evidently properly situated so that an O_2 molecule can fit in between them and pick up 1 electron from each, transforming the irons to Fe(III) and the oxygen to the peroxide ion, O_2^{--}. The third iron does not participate directly in the oxygenation reaction. Its function may be, therefore, to hold the other side chains together at a suitable distance apart.

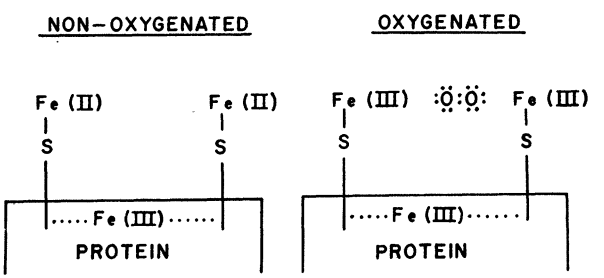


Fig. 4. State of iron in hemerythrin.

It is of interest that the intensity of absorption of hemerythrin is not greatly different from that of ferric-conalbumin (*22*). Thus the optical data conform with the specific color tests in indicating a single valence state, rather than a mixed Fe(II)–Fe(III) complex, in colored oxyhemerythrin.

As with hemocyanin, the attachment of the iron in hemerythrin is indicated as being through a sulfur atom. This assignment is based on the observation (*23*) that 1 mole of a mercurial is able to break up completely the oxygen-hemerythrin complex. Admittedly, mercury can combine with a great variety of protein side chains, but since its affinity for sulfur exceeds its affinity for any other ligand, it seems very likely that if only 1 mole of the metal is capable of affecting 1 mole of active site of the protein, the action comes about through the displacement of one of the iron atoms from a sulfur linkage to the protein. On the basis of the structure in Fig. 4, it seems reasonable that displacement from a sulfide linkage of either one of the two iron atoms holding the oxygen would destroy the stability of the complex and lead to the breakdown of oxyhemerythrin.

General conclusions. It thus seems clear that the nature of the oxygenation reaction is markedly different in hemocyanin and hemerythrin from that in hemoglobin. With this background the behavior of these proteins toward carbon monoxide becomes more

intelligible. The ability of carbon monoxide to combine with the iron of hemoglobin resides in its tendency to share a pair of its electrons just as oxygen does (*3*). To form stable complexes with hemocyanin or hemerythrin analogous to those with oxygen, the carbon monoxide would have to pick up 1 or 2 electrons, respectively; that is, it would have to act as an oxidizing agent, instead of as a reducing agent, which is its normal tendency. Thus a carbon monoxide complex with hemocyanin or hemerythrin would not be expected, in agreement with the most careful experimental observations (*9, 21, 24*).

From the viewpoint of the state of the combined oxygen, the three blood pigments may be arranged in a progressive series based on the stepwise change in the extent of reduction. Thus O_2 may be assigned to hemoglobin, O_2^- to hemocyanin, O_2^{--} to hemerythrin. Such a progression makes the observed heats of oxygenation much more understandable. These fall in the order −8 kcal for hemoglobin (*25*), −13 kcal for hemocyanin (*26*), and −18 kcal for hemerythrin (*27*), all being compared on a common basis of dissolved oxygen as a reference state. It would be particularly difficult to account for the wide difference between hemoglobin and hemerythrin on the basis of the old assumption that the mechanism of the interaction of oxygen with Fe(II) is the same in both cases. On the other hand, the trend in thermodynamic properties conforms well with the interpretations derived from spectroscopic investigations and specific color tests.

Thus the nature of the electronic changes responsible for the color and oxygen-carrying ability of the proteins in all major classes of blood have now been elucidated.

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In the course of experience of many generations of students, I have known far more to fail from lack of grit and perseverance than from the want of what is commonly called cleverness.—J. J. THOMSON.