Copper and the Autoxidation of Hemoglobin†

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ABSTRACT: Copper is shown to catalyze the oxidation of oxyhemoglobin even at very low concentrations, while other metal ions have no significant effect even at much higher concentrations. The oxidation of deoxyhemoglobin by copper in the absence of oxygen indicates that this oxidation involves the transfer of an electron from Fe(II) to Cu(II). Oxygen is, however, required for the catalytic effect, which presumably requires that Cu(II) be regenerated from Cu(I). It is also shown that hemoglobin has a high Cu(II) affinity, suggesting that the oxidation involves copper bound to hemoglobin. Strong complexing agents are found to dramatically reduce the rate of autoxidation. The presence of significant concentrations of copper in both hemolyzed cells and purified hemoglobin preparations indicates that copper is actually responsible for a large part of what has been considered autoxidation. The copper in hemolyzed cells can be separated into two fractions. One fraction is associated with lower molecular weight proteins from which it cannot be removed by complexing agents. The second fraction chromatographs together with hemoglobin and can be removed with strong complexing agents. This second fraction is probably responsible for catalyzing the oxidation possibly in the same manner as added copper. The previously reported enhanced autoxidation of purified hemoglobin is shown to be caused by contamination with copper. It is, in fact, shown, that by eliminating both extraneous copper contamination and the oxidation due to copper present in hemolyzed cells, it is possible to prepare purified hemoglobin which has a slower rate of oxidation than hemolyzed cells.

Reversible oxygenation of hemoglobin occurs only with Fe(II) hemoglobin (Jaffé, 1964; Rifkind, 1972). Therefore, the effective transport of oxygen by hemoglobin requires that the autoxidation of ferrohemoglobin to ferrihemoglobin be minimized. Various studies indicate that the structure of hemoglobin with the heme located in a hydrophobic crevice plays an important role in stabilizing the Fe(II) state (Wang *et al.*, 1958; Rifkind, 1973). Hemoglobin is, nevertheless, oxidized at a significant rate (Brooks, 1931; Brown and Mebine, 1969; Rifkind, 1972).

In the erythrocyte, a low level of ferrihemoglobin is maintained by enzymatic reduction (Scott and McGraw, 1962; Jaffé, 1964). Purification of hemoglobin generally results in an enhanced rate of autoxidation, which is thought to be related to the removal of certain components of the erythrocyte (Rifkind, 1972).

A satisfactory method of maintaining purified hemoglobin in its functional reduced form has not been developed. Chemical reduction of the ferrihemoglobin formed as a result of autoxidation (Jaffé, 1964; Rifkind, 1972) is thought to irreversibly damage the hemoglobin (King and Winfield, 1963). We have recently found that the reaction of sulfhydryl reagents with the β-93 sulfhydryl groups inhibits the autoxidation (Rifkind, 1972). However, since this reaction also influences the oxygenation of hemoglobin (Brunori *et al.*, 1967), it also is not an appropriate method for most purposes.

The involvement of metal ions in the autoxidation of frog hemoglobin has been previously suggested (Salvati *et al.*, 1969). In this paper we report the effect of various metal ions and complexing agents on the autoxidation of mammalian hemoglobin. We are able to show that, while most metal ions have essentially no effect on the oxidation, Cu(II) has a catalytic effect even at the very low concentrations present in purified hemoglobin, and may, therefore, be responsible for a major portion of the autoxidation. The source and nature of the copper responsible for autoxidation are studied by correlating changes in the rates of autoxidation with changes in the relative concentration of copper to heme produced by modifying the purification procedure. These studies suggest methods for obtaining very stable purified hemoglobin. A mechanism is proposed for the catalytic oxidation of hemoglobin by copper.

Experimental Section

Hemoglobin. Horse blood was obtained from the Johns Hopkins Animal Center, the Beltsville Agricultural Research Center, and Bioquest. The hemolyzed cells were prepared from whole blood by centrifuging at 8000 rpm and then resuspending and recentrifuging several times in 0.9% NaCl. The washed cells were hemolyzed in two volumes of cold distilled water, and separated from the cell membranes by centrifugation at 35,000 rpm. The hemolyzed cells, when necessary, were further purified by gel filtration as previously described (Rifkind, 1972). Donkey hemoglobin was obtained already purified from Professor R. Lumry, University of Minnesota, Laboratory of Biophysical Chemistry.

Other Materials. Triethylenetetraamine was obtained from two sources: purified by redistillation over sodium from Strem Chemicals and technical grade from Aldrich Chemicals. Suprapur NaCl from E. Merck, Darmstadt, Germany, was used for some hemoglobin preparations. Radioactive ethylenediaminetetraacetic-2-^{14}C acid was obtained from New England Nuclear. All other chemicals were reagent grade.

Autoxidation of Hemoglobin. The autoxidation was monitored by following the changes in the spectrum of hemoglobin (Rifkind, 1972). The spectrum at pH 7.0 gradually changed from that of oxyhemoglobin with peaks at 576 and 541 nm to that of the H_2O form of ferrihemoglobin with peaks at 630 and 500 nm (Figure 1). The proper changes in absorbance at 630, 576, 560, 541, and 500 nm (Jope, 1949) were considered

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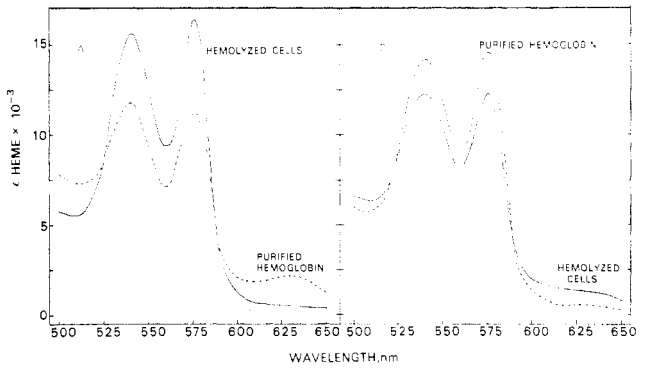

FIGURE 1: Comparison of the visible spectra of horse hemolyzed cells and horse hemoglobin purified by gel filtration after storage at pH 7.0 and 4° for extended periods of time. (A) Clean autoclaved glassware was used without additional precautions to eliminate trace metal ion contamination. After the hemoglobin sample was purified, a sample of hemolyzed cells and purified hemoglobin were diluted to a heme concentration of 6.7×10^{-5} M. At the time of dilution the spectra were indistinguishable. The diluted samples were stored for 29 days: (—) hemolyzed cells; (- - - - - - -) hemoglobin purified by gel filtration chromatography. (B) All glassware was acid washed, columns were washed with triethylenetetraamine, and hemoglobin was eluted with 10^{-4} M triethylenetetraamine. A sample of the hemolyzed cells was purified by gel filtration 22 days after hemolysis. The nondiluted hemolyzed cells and the purified hemoglobin with a heme concentration of 2.8×10^{-4} M was stored for an additional 77 days. The spectra of the purified hemoglobin was then compared with that of a sample of hemolyzed cells, then diluted to a heme concentration of 3.0×10^{-4} M: (—) hemolyzed cells; (- - - - -) purified hemoglobin.

a clear indication that the oxidation of the iron was actually being monitored. For relatively rapid rates of oxidation, the initial phase was followed at a single wavelength. The changes at other wavelengths were, however, checked after the initial rapid phase of the oxidation was completed.

Anaerobic Experiments Involving Deoxyhemoglobin and Carbonylhemoglobin. Hemoglobin samples were placed in a specially designed cuvet with a large bulb containing a side arm above the optical path (Keyes *et al.*, 1967). The copper solution was pipetted directly into the side arm. The cuvet was connected by standard tapered glass joints to a gas reservoir which could be connected to a vacuum line and/or a gas line.

Deoxyhemoglobin was prepared by repeated evacuation of the reservoir and cuvet, while gently rotating the hemoglobin solution in the bulb above the optical path, without permitting mixing of the hemoglobin and copper solutions. The proper deoxyhemoglobin spectrum indicated that all the oxygen had been removed. Carbonylhemoglobin was then formed, when desired, by filling the gas reservoir with carbon monoxide, and equilibrating this gas with the hemoglobin. Copper was added to the various anaerobic samples by tilting the apparatus to permit mixing of the copper solution in the side arm with the hemoglobin. As for oxyhemoglobin, oxidation was monitored by following the changes in the spectrum from that of deoxyhemoglobin or carbonylhemoglobin to that of ferrihemoglobin.

Binding of Ethylenediaminetetraacetic Acid (EDTA) and Copper to Hemoglobin. The binding was determined by equilibrium dialysis. The dialysis tubing was obtained from Union Carbide and cleaned by the procedure of Huang (1967). Glass tubes with stoppers or caps were used. The hemoglobin solutions adjusted to the desired pH were placed inside small dialysis bags. Various concentrations of EDTA or copper, together with buffer and/or other ions, were placed on the outside. The test tubes were sealed and placed on a rotating rack or in a shaker at 4°. A bubble was left inside the bag and outside the bag to facilitate mixing; 4–7 days were usually required to assure that equilibrium had been reached. At equilibrium the concentration of EDTA or copper was measured both inside and outside. The outside concentration was considered the free concentration and the difference between the inside concentration and outside concentration measured the amount bound.

The contribution of the Donnan effect to these concentrations was considered insignificant because of the close proximity to the isoionic pH of hemoglobin and the presence of a large excess of other ions.

The binding of EDTA was studied at a heme concentration of 1.4×10^{-4} M at pH 7.0 in 0.01 M NaCl and 0.1 M NaCl. The binding of copper was studied at a heme concentration of 1.5×10^{-4} M at pH 7.2 in 0.05 M Tris.

Exhaustive Dialysis of Hemoglobin. The hemoglobin sample was either placed in a collodion bag from a Schleicher Schuell apparatus or several small dialysis bags. Large volumes of water containing, when desired, NaCl and/or complexing agent were changed periodically. One of the small dialysis bags, or an aliquot from the collodion bag, was removed for analysis when desired. Dilution, which was a particularly serious problem using the Schleicher Schuell apparatus, was corrected for by measuring the hemoglobin concentration.

Determination of Metal Ion Concentrations. The total concentrations of metal ions were determined by aspirating directly into a Perkin-Elmer Model 306 atomic absorption spectrophotometer connected to a Honeywell Electronik 19 recorder. Standard metal solutions obtained from Hartman Leddon Co. were used for calibration purposes.

A calibration curve was determined by adding known concentrations of metal ions to the hemoglobin preparations in order to correct for any possible interferences.

The copper activity was determined with an Orion solid state Model 94-29 cupric ion electrode using a Radiometer Model 25 pH meter with scale expander.

The presence of heavy metal ions on glassware and in reagents was qualitatively determined by a color change observed in 0.001% dithizone dissolved in $CHCl_3$ (Thiers, 1957). Copper in various reagents and on glassware was quantitatively determined by the extraction of the copper complexed ammonium pyrrolidinedithiocarbamate (Mulford, 1966; Blomfield and MacMahon, 1969) into methyl isobutyl ketone. The copper in the organic solvent was determined on the Perkin-Elmer Model 306 atomic absorption spectrophotometer.

Determination of EDTA Concentration. Radioactive [^{14}C]-EDTA was used for the binding studies. A Beckman LS-250 liquid scintillation spectrophotometer was used to measure the carbon-14. Packard insta-gel emulsifier was used as a scintillation solvent. The aqueous solutions dissolved directly in this emulsifier when heated to 40°. Each sample was corrected for quenching by using radioactive EDTA as an internal standard. A broad window was used in order to minimize color quenching with the hemoglobin solutions.

Results and Discussion

The Effect of Complexing Agents on the Autoxidation of Hemoglobin. EDTA and triethylenetetraamine, which form very strong multidentate metal complexes (Sillén *et al.*, 1964), drastically inhibit the autoxidation of the relatively stable horse and donkey hemoglobins purified by gel filtration. In Figure 2 it is shown that similar concentrations of EDTA

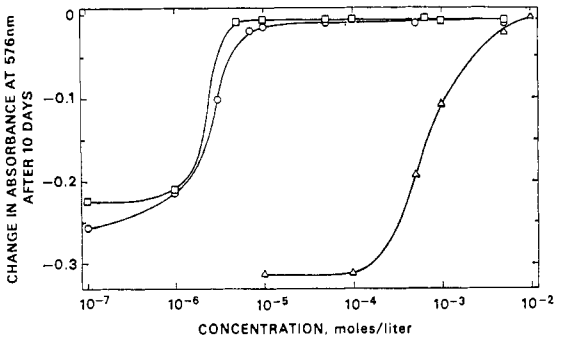

FIGURE 2: Concentration of various complexing agents necessary to prevent a decrease in absorbance at 576 nm for purified horse hemoglobin after storage for 10 days at pH 7.0 and 4°. The heme concentration was 6.3×10^{-5} M: (□) triethylenetetraamine; (○) EDTA; (△) ethylenediamine.

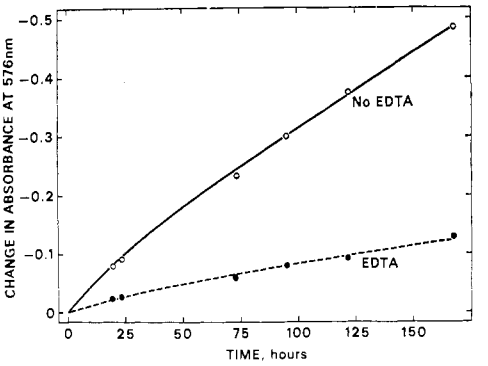

FIGURE 3: Change in absorbance at 576 nm as a function of time for horse hemolyzed cells at pH 7.0 and 24°. The heme concentration was 7.2×10^{-5} M and the initial absorbance at 576 nm in a 1-cm cell was 1.179: (○) nothing added; (●) 1×10^{-4} M EDTA.

and triethylenetetraamine are necessary to decrease the amount of oxidation taking place in 10 days. However, a much higher concentration of the much weaker complexing agent, ethylenediamine (Sillén *et al.*, 1964), is required to produce the same effect (Figure 2).

The binding of [^{14}C]EDTA to purified hemoglobin was studied by equilibrium dialysis in order to determine whether the complexing agents are binding directly to the protein. No protein binding was observed in the concentration range required to completely inhibit the autoxidation. These binding results are consistent with the fact that the concentration of complexing agent required to inhibit autoxidation correlates with the stability of the metal complexes formed by a particular chelating agent (Figure 2), indicating that metal ions are being complexed. This conclusion is supported by the similarity between the effect of EDTA and triethylenetetraamine (Figure 2), which are structurally very different.

The Source of Metal Ions Involved in Autoxidation. The inhibition by complexing agents of the autoxidation of purified hemoglobin (Figures 1 and 2) can be attributed to metal ions present in the erythrocyte, and/or metal ion contaminants.

As shown in Figure 1A, purified hemoglobin is generally oxidized more readily than hemolyzed cells (Rifkind, 1972). Since metal ion impurities, to varying extents, are found in reagents, on glassware, on columns, and even in distilled water (Thiers, 1957), the possibility exists that at least some of this autoxidation is caused by metal ion contamination during the purification procedures. Therefore, our preparative procedures were modified to eliminate metal ion contamination. We used Suprapur NaCl, filtered deionized water with a resistance of 15 megohms, acid-washed glassware exhaustively rinsed with distilled water, and Sephadex columns washed with EDTA or triethylenetetraamine prior to application of the sample. This treatment very significantly stabilized the purified hemoglobin. The purified hemoglobin prepared in this manner is as stable with respect to oxidation as hemolyzed cells.

The rate of oxidation of purified hemoglobin can be further reduced by the addition of complexing agents to the eluent (Figure 1B). This procedure results in purified hemoglobin actually more stable than hemolyzed cells (Figure 1). A similar increase in the stability of hemoglobin is obtained by the addition of complexing agents to hemolyzed cells (Figure 3). Since every precaution necessary to eliminate metal ion contamination was performed when doing these experiments, some of the metal ions responsible for autoxidation seem to originate within the erythrocyte.

Thus, the purification of hemoglobin by gel filtration does not seem to remove anything which is necessary to maintain a stable reduced form of hemoglobin under our conditions. Hemoglobin is, therefore, surprisingly a very stable molecule. The structure of hemoglobin with the heme in a low dielectric pocket (Perutz, 1965; Rifkind, 1973) seems to stabilize the Fe(II) of oxyhemoglobin to the extent that a negligible amount of oxidation is observed (Figures 1–3) as long as metal ion oxidation catalysts are eliminated. The fact that the complexing agents do not reduce oxidized hemoglobin and do not seen to directly interact with the hemoglobin suggests that they can be used without altering the functional properties of hemoglobin.

The Specific Catalytic Oxidation of Hemoglobin by Copper. In order to determine which metal ion or metal ions could be responsible for the enhanced rate of autoxidation in the absence of complexing agents (Figures 2 and 3), we added various metal ions to the purified hemoglobin preparation so as to obtain a final concentration of $1.0–0.5 \times 10^{-4}$ metal and compared the rates of autoxidation. This concentration corresponds to 1–2 metal ions per heme. The metal ions used for these experiments were Ag(I), Mg(II), Mn(II), Fe(II), Co(II), Ni(II), Cu(II), Zn(II), Cd(II), Hg(II), Pb(II), Bi(III), Cr(III), Tl(III), Fe(III), Co(III) (hexacyano complex), Ce(III), and Ce(IV). Certain metal ions such as Hg(II), Cu(II), and Zn(II) have a tendency to precipitate the hemoglobin when more than one metal ion is added for every 2 hemes. In these cases, the experiments were repeated at lower metal ion concentrations in the range of $1–5 \times 10^{-5}$ M, which produced no precipitation.

Hg(II) and Ag(I) partially inhibit the autoxidation (Rifkind, 1972). This stabilization is related to the very high affinity of these metal ions for sulfur ligands, which results in a reaction with the β-93 sulfhydryl groups. This reaction has previously been shown to lower the rate of autoxidation (Rifkind, 1972).

With the exception of Cu(II), all of the other metal ions have a negligible effect on the rate of autoxidation. The insensitivity of autoxidation to most metal ions is demonstrated in Figure 4 by a comparison of the autoxidation of hemoglobin with and without the addition of several typical metal ions. Cu(II), however, has a very dramatic catalytic effect with significant increases in the rate of autoxidation observed even for 1–2 copper ions/1000 hemes (Figure 4). These results with horse hemoglobin differ from those of Salvati *et al.* (1969) using the relatively unstable frog hemoglobin. They find that the concentration of Cu(II) required is in the range of the heme concentration, and many other metal ions also significantly enhance the rate of autoxidation, although Cu(II) has the largest effect.

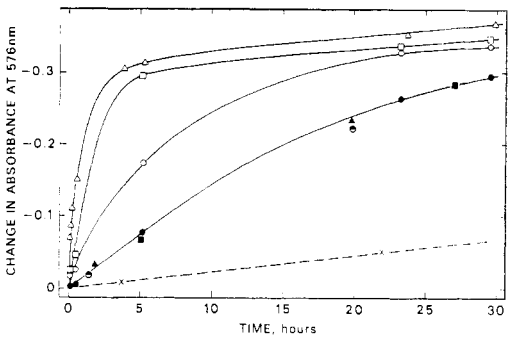

FIGURE 4: Change in absorbance at 576 nm as a function of time for purified horse hemoglobin at pH 7.0 and 24°. The heme concentration was 6.3×10^{-5} M and the initial absorbance at 576 nm in a 1-cm cell was 0.960: (●) nothing added; (×) 5×10^{-4} M EDTA; (○) 1×10^{-7} M Cu(II); (□) 3×10^{-7} M Cu(II); (△) 1×10^{-6} M Cu(II); (■) 1×10^{-4} M Cr(III); (▲) 5×10^{-5} M Cd(II); (◓) 5×10^{-5} M Ce(III).

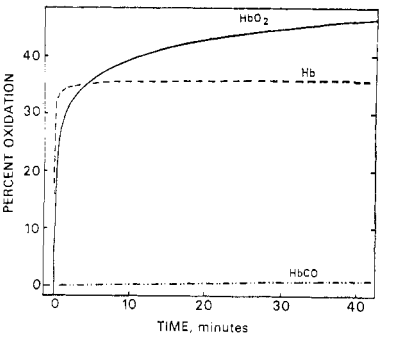

FIGURE 5: Per cent oxidation produced as a function of time for horse hemolyzed cells at pH 7.0 and 24° in 10^{-2} M Bis-Tris by the addition of 5×10^{-6} M Cu(II) to a heme concentration of 1.42×10^{-4} M. The per cent oxidation was determined by dividing the change in absorbance at a suitable wavelength by the change in absorbance at that wavelength for complete conversion to ferrihemoglobin; 630 nm was used for oxyhemoglobin, and 560 nm for deoxyhemoglobin and carbonylhemoglobin: (—) oxyhemoglobin at atmospheric oxygen pressure; (- - - -) deoxyhemoglobin in the absence of oxygen; (—·—·—) carbonylhemoglobin in the absence of oxygen.

A Mechanism for the Specific Catalytic Oxidation of Hemoglobin by Copper. Copper as well as other metal ions are frequently found to function as oxidation reduction catalysts (Grinstead, 1964). However, while copper will frequently function as a better catalyst than many other metal ions, the unique role of copper in our system deserves special attention. This specificity can perhaps be explained by a favorable potential and rate for the oxidation and reduction of copper between Cu(I) and Cu(II), and/or the manner in which copper binds to hemoglobin.

In the presence of oxygen, it is not evident whether the copper is directly involved in the oxidation of hemoglobin (eq 1), or whether it influences the structure of hemoglobin,

$$\text{Cu(II)}\cdots\text{Hb}^{2+}\text{–O}_2 \rightleftharpoons \text{Cu(I)}\cdots\text{Hb}^{3+} + \text{O}_2 \qquad (1)$$

thereby facilitating the oxidation of hemoglobin by oxygen (eq 2). The direct oxidation of hemoglobin by copper (eq 1)

$$\text{Cu(II)}\cdots\text{Hb}^{2+}\text{–O}_2 \rightleftharpoons \text{Cu(II)}\cdots\text{Hb}^{3+} + \text{O}_2\cdot^- \qquad (2)$$

was demonstrated by investigating the oxidation of deoxyhemoglobin by copper in the absence of oxygen (Figure 5). At a Cu/heme ratio of 0.35, all of the added copper is quantitatively used to very rapidly oxidize hemoglobin, and no further oxidation takes place even after much longer periods of time (Figure 5).

The effect of oxygen on the copper oxidation is indicated by comparing, at the same Cu(II) concentration, the results for deoxyhemoglobin in the absence of oxygen with those for oxyhemoglobin (HbO_2) at atmospheric oxygen pressures (Figure 5). The major distinction is that for HbO_2 the initial very rapid oxidation is followed by a slower oxidation which results in the total oxidation of more than 35% of the hemes. The initial rapid oxidation, as for deoxyhemoglobin, is probably due to the oxidation of hemoglobin by the Cu(II) added. As this Cu(II) is converted to Cu(I), additional oxidation is limited by the slower rate at which oxygen reoxidizes Cu(I) back to Cu(II). This step, which regenerates the Cu(II), is

$$4\text{Cu(I)} + \text{O}_2 + 4\text{H}^+ \rightleftharpoons 4\text{Cu(II)} + 2\text{H}_2\text{O} \qquad (3)$$

essential for the catalytic oxidation observed at low copper concentrations (Figure 4).

An additional difference between the results with deoxyhemoglobin and HbO_2 is the faster initial rate of oxidation for deoxyhemoglobin (Figure 5), *i.e.*, Cu(II) oxidizes deoxyhemoglobin faster than HbO_2. This difference can be due to the difference between the "deoxy" unliganded conformation of hemoglobin and the "oxy" liganded conformation of hemoglobin (Rifkind, 1973). However, it can also be explained by a dissociation of oxygen (eq 4) which is slower than the

$$\text{Cu(II)}\cdots\text{Hb}^{2+}\text{–O}_2 \rightleftharpoons \text{Cu(II)}\cdots\text{Hb}^{2+} + \text{O}_2 \qquad (4)$$

oxidation by Cu(II) (eq 5), if the dissociation (eq 4) is a

$$\text{Cu(II)}\cdots\text{Hb}^{2+} \rightleftharpoons \text{Cu(I)}\cdots\text{Hb}^{3-} \qquad (5)$$

necessary prerequisite for the oxidation (eq 5).

The significance of the dissociation of the sixth ligand (eq 4) is supported by the results on the effect of Cu(II) on carbonylhemoglobin (HbCO) (Figure 5). The same concentration of Cu(II) that rapidly oxidizes HbO_2 and deoxyhemoglobin has a negligible effect on carbonylhemoglobin (HbCO) which has the same liganded conformation as HbO_2. The major difference between HbCO and HbO_2 is the 1000-fold enhancement (Gibson and Roughton, 1955, 1957) of the rate for the dissociation of the sixth ligand for HbO_2 relative to HbCO (eq 4). The requirement for this step to precede the oxidation (eq 5) can, therefore, expain the dramatic difference between HbCO and HbO_2. Such a step has been postulated for the oxidation of hemoglobin by other oxidizing agents (Antonini *et al.*, 1965; Gorn, 1968) such as ferricyanide and even for autoxidation (Brown and Mebine, 1969). It is also consistent with the idea that the bound oxygen is not the oxidizing agent (Castro and Davis, 1969) and, in the presence of Cu(II), the metal ion oxidizes the hemoglobin.

The specific catalytic oxidation of hemoglobin by copper can now be partially attributed to a requirement for the proper oxidation potential. Thus, metal ions which cannot be readily reduced are not expected to affect the oxidation. However, among the metal ions investigated are those which are better oxidizing agents than copper (Latimer, 1952). Nevertheless, none of these other metal ions are able to oxidize hemoglobin even at stoichiometric concentrations, where a catalytic effect is not required.

It, therefore, seems reasonable that part of the copper specificity involves the binding of copper to a particular site on hemoglobin which enables it to participate in the oxidation process.

Copper has been reported to bind strongly to human hemoglobin on the basis of electron paramagnetic resonance studies (Bemski *et al.*, 1969; Nagel *et al.*, 1970). We have demonstrated the binding of copper to purified horse hemo-

globin by equilibrium dialysis. At pH 7.2 in 0.05 M Tris the apparent binding constant is 3×10^5 M^{-1} with a stoichiometry of 1 copper for every 2 hemes. The same stoichiometry was obtained for human hemoglobin (Nagel *et al.*, 1970).

A correlation between the binding and oxidation is indicated by the finding that the copper-induced oxidation, as well as the binding, involves only two of the four subunits. This behavior is shown in Figure 5 where the oxidation of HbO_2 levels off in the region of 50% and has also been reported by Nagel *et al.*, 1970.

These studies suggest that the copper specific catalytic oxidation of hemoglobin involves the binding of Cu(II) to a specific site on hemoglobin, which is suitable for the transfer of electrons between Cu(II) and Fe(II) (eq 1). The copper is then reoxidized by oxygen (eq 3), facilitating the oxidation of additional hemes.

The Copper Responsible for the Autoxidation of Hemoglobin. The specific catalytic oxidation of hemoglobin produced by Cu(II) indicates that the decreased rate of autoxidation produced by the addition of complexing agents to purified hemoglobin (Figure 2), as well as hemolyzed cells (Figure 3), is produced by complexing Cu(II).

We have measured the copper concentration in hemolyzed cells by atomic absorption spectroscopy. When the copper concentration is compared with the heme concentration determined by visible spectroscopy and/or the iron concentration, it is found that there are 1.7 copper atoms per 1000 hemes in hemolyzed cells (Table I).

The frequently observed enhanced rate of autoxidation for purified hemoglobin, when care is not taken to eliminate metal ion contamination (Figure 1A), is correlated with an increase in the relative concentrations of copper to that of hemoglobin (Table I). By atomic absorption spectroscopy, it was shown that copper is generally found on glassware prior to soaking in acid. It was also found that hemoglobin chelates additional copper in the chromatographic purification, unless the column is first washed with triethylenetetraamine or another strong complexing agent (Table I). Thus, a 440% increase in the relative concentration of copper to that of hemoglobin is observed when the column is not first washed with a complexing agent (Table I).

Excess copper has been reported to have a deleterious effect on the erythrocyte (Shields *et al.*, 1960; Louria *et al.*, 1972; Deiss *et al.*, 1970). However, our results (Figure 3) suggest that the normal level of erythrocyte copper is responsible for at least some oxidation of hemoglobin. Additional studies were undertaken in order to demonstrate what fraction of the erythrocyte copper is involved in the oxidation, and the nature of this copper.

The extent to which the erythrocyte copper (Table I) is associated with other molecules was determined by a solid state electrode specific for Cu(II). A Cu(II) activity in the region of 10^{-15} mol/l. indicates that essentially all of this copper is tightly bound. An added complexing agent can effectively compete with the erythrocyte copper chelators only if is is capable of further lowering the Cu(II) activity. It is, therefore, not surprising that only the very strong complexing agents like EDTA and triethylenetetraamine, with copper association constants of 10^{16} and 10^{20}, respectively (Sillén and Martell, 1964), affect the autoxidation at relatively low concentrations (Figure 2).

A sample of hemolyzed cells was dialyzed against triethylenetetraamine in order to determine to what extent the erythrocyte copper can be complexed by this complexing agent which inhibits autoxidation (Figure 2). It was found (Table I) that only about 35% of the copper could be removed in this way and the remainder remained associated with the hemolyzed cells.

TABLE I: Effect of Preparative Procedure on the Removal of Copper from Hemoglobin.

Preparation[a]	[Copper][b]/[Heme][c] $\times 10^3$	% Change
Erythrocytes hemolyzed in two volumes of water	1.7 ± 0.1	
Hemolyzed cells exhaustively dialyzed against triethylenetetraamine	1.1 ± 0.1	−35
Chromatographed[e] on G-25 column not washed with complexing agent	7.5 ± 0.5	+440
Chromatographed[e] on G-25 column washed with triethylenetetraamine	1.5 ± 0.1	−12
Chromatographed[d,e] on G-75	1.1 ± 0.2	−35
Dialyzed against triethylenetetraamine and then chromatographed[d,e] on G-75	0.7 ± 0.2	−59
Chromatographed[d,f] on G-25 and then A-50; 10^{-4} M triethylenetetraamine in eluent	0.3 ± 0.1	−82

[a] In all cases acid-washed glassware was used. [b] Determined by atomic absorption. [c] Determined by visible absorption spectra and/or the Fe concentration determined by atomic absorption. [d] In these cases the columns were first washed with triethylenetetraamine. [e] These columns were eluted with 0.01 M NaCl (pH 7.0). The copper and heme concentrations are for the tube with the highest hemoglobin concentration. [f] The G-25 column was eluted with 0.05 M Tris (pH 8.0) and the A-50 column with 0.05 M Tris (pH 7.2). The A-50 hemoglobin peak was pooled and concentrated.

The separation of copper from hemoglobin was followed by various chromatographic techniques (Table I) in order to further characterize the fraction of copper removed by complexing agents. On G-25 Sephadex small molecules with a molecular weight lower than 5000 should be separated from hemoglobin, and everything else will be eluted together with hemoglobin. As expected G-25 Sephadex separates hemoglobin from metal ions like Mg(II) and K(I), as well as other low molecular weight substances such as glutathione, 2,3-DPG, and nucleotides (Rifkind, 1972). However, as indicated in Table I, essentially all of the copper is eluted together with the hemoglobin on G-25 Sephadex, when pH 7.0 0.01 M NaCl is used as an eluent. This finding indicates that all the copper is tightly bound to high molecular weight material.

G-75 Sephadex should separate from hemoglobin macromolecules of a molecular weight lower than 50,000. In Figure 6A it is demonstrated that, when hemolyzed cells are eluted with 0.01 M NaCl (pH 7.0) on G-75 Sephadex, the copper is separated into two overlapping fractions with the bulk of the copper in the second fraction trailing behind the hemoglobin in the region where lower molecular weight proteins should be eluted. This partial fractionation of the copper results in a hemoglobin preparation with a decreased concentration of copper relative to the heme concentration (Table I). The copper removed in this way is associated with proteins other

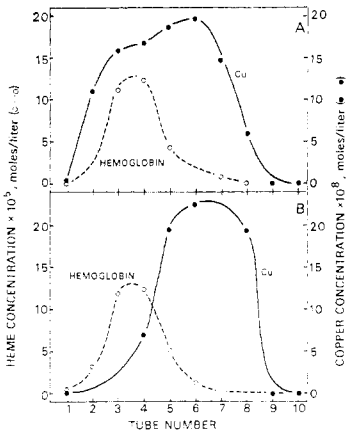

FIGURE 6: The effect of dialysis against triethylenetetraamine on the separation of copper from horse hemoglobin on G-75 Sephadex. The columns were washed with triethylenetetraamine, the samples were eluted with 0.01 M Suprapur NaCl at pH 7.0, and 3-ml fractions were collected in acid-washed test tubes. (A) A G-75 column, 2 cm × 40 cm, was loaded with 0.5 ml of hemolyzed cells: (●—●) copper concentration; (O- - - - - -O) heme concentration. (B) Hemolyzed cells were dialyzed against a liter of 10^{-4} M triethylenetetraamine for 4 days; 0.6 ml of these hemolyzed cells were loaded onto a G-75 column, 1.6 cm × 80 cm: (●—●) copper concentration; (O- - - - - -O) heme concentration.

than hemoglobin. This copper could not be involved in catalytic oxidation of hemoglobin involving Cu(II) bound to hemoglobin (see above).

The effect of dialyzing the hemolyzed cells against triethylenetetraamine before purification by gel filtration on G-75 Sephadex is shown in Table I and Figure 6. The relative concentration of copper in the purified hemoglobin is further decreased (Table I). Figure 6B shows that the dialysis against triethylenetetraamine removed the copper which chromatographs together with hemoglobin on G-75 without having much of an effect on the copper which chromatographs together with the lower molecular weight proteins. In fact, the appreciable concentration of copper remaining with the hemoglobin (Table I) seems to be due to the poor separation of the low molecular weight copper protein(s) from hemoglobin on G-75 Sephadex (Figure 6). This contention was supported by rechromatographing hemoglobin purified by gel filtration on A-50 Sephadex. The hemoglobin was eluted from the ion exchange resin with pH 7.2, 0.05 M Tris containing 10^{-4} M triethylenetetraamine. The use of triethylenetetraamine in the eluent should separate the complexable copper from hemoglobin and the A-50 column should improve the separation of the low molecular weight copper protein(s) from hemoglobin (Williams and Tsay, 1973). Consistent with these expectations, almost all of the copper is removed from hemoglobin by this procedure (Table I).

These studies imply that the erythrocyte copper responsible for the autoxidation (Figure 5) is chromatographed together with hemoglobin on G-75 Sephadex, and may be associated directly with hemoglobin. The mechanism for the oxidation of hemolyzed cells in Figure 3 is, therefore, possibly the same as that proposed for the addition of copper to hemoglobin (Figures 4 and 5).

Earlier studies also indicate two classes of erythrocyte copper. About 60% of the copper in human erythrocytes exchanges slowly with serum copper (Shields *et al.*, 1960; Bush *et al.*, 1956). This fraction is associated with erythrocuprein (Shields *et al.*, 1960) or superoxide dismutase (McCord and Fridovich, 1969). EDTA does not remove the copper from this metalloenzyme, with a molecular weight of 33,000, at neutral pH (Bannister *et al.*, 1971). Therefore, the copper fraction eluted after hemoglobin on G-75, which is not affected by prior dialysis against triethylenetetraamine (Figure 6), is most probably associated at least partially with superoxide dismutase.

The other fraction of copper exchanges rapidly with serum copper (Bush *et al.*, 1956), and can be removed by complexing agents even *in vivo* (Deiss *et al.*, 1970). This fraction of copper is most probably responsible for autoxidation, corresponding to the copper which chromatographs together with hemoglobin on G-75 Sephadex (Figure 6).

Superoxide dismutase catalyzes the dismutation of the highly reactive superoxide radicals into oxygen and hydrogen peroxide (McCord and Fridovich, 1969). This reaction prevents oxidative damage caused by the buildup of superoxide radicals formed by autoxidation of hemoglobin (Wever *et al.*, 1973) and other substances (McCord *et al.*, 1971). However, the role of the remainder of the erythrocyte copper is uncertain (Shields *et al.*, 1961, 1960).

A role for some of the erythrocyte copper in the proper development of the erythrocyte is suggested by studies indicating that copper-deficiency anemia results partially (Lee *et al.*, 1968) from a copper requirement for the proper transport of iron within the developing erythrocyte to the mitochondria (Goodman and Dallmon, 1969) where it is incorporated into protoporphyrin.

In the adult erythrocyte this copper may no longer be necessary but may still be retained in the erythrocyte because of the lack of a mechanism to actively transport the copper from the erythrocyte and the approximately equivalent copper concentration in the serum and the erythrocyte (Shields *et al.*, 1960). That the copper is not really required by the fully developed erythrocyte is indicated by studies which show a normal life span for normal cells placed in copper-deficient swine even though the red blood cells developed in this organism have a much shorter life span (Bush *et al.*, 1956).

Our studies suggest that this copper actually catalyzes the oxidation of hemoglobin in the fully developed erythrocyte. In fact, if hemoglobin by itself is actually as stable to autoxidation as our studies suggest, even the enzyme system (Jaffé, 1964), which is thought to be the major factor responsible for maintaining reduced hemoglobin in the erythrocyte, may only be required as an emergency protective mechanism if not for the presence of copper.

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