Investigation of the Iron and Copper Complexes of Avian Conalbumins and Human Transferrins by Electron Paramagnetic Resonance*

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Received July 1, 1963

Electron paramagnetic resonance (EPR) spectra have been obtained on iron and copper protein complexes of human blood serum transferrin, human lactotransferrin, and conalbumins obtained from chicken, turkey, and Japanese quail egg whites. The EPR results indicate that Fe^{+3} and Cu^{+2} both bind to similar sites and that there is no magnetic interaction between the bound metal atoms. No difference between the sites binding the first and second moles of metal was detected by EPR. It is proposed that Cu^{+2} is bound to two tyrosyl oxygen atoms and two nitrogen atoms of the protein with square planar coordination, and that Fe^{+3} is octahedrally coordinated to three tyrosyl oxygen atoms, two nitrogen atoms, and one bicarbonate ion.

The specific iron-binding capacity of an egg white component was first recognized by Schade and Caroline (1944), and this component was identified as conalbumin by Alderton *et al.* (1946). These findings led to the identification of a similar protein in human plasma (Schade and Caroline, 1946) which is usually called "transferrin" but has also been named "siderophilin" and "β_1-metal-binding globulin." More recently lactotransferrin has been identified and isolated from human milk (Montreuil *et al.*, 1960) as well as a "red protein" from bovine milk (Groves, 1960; Gordon *et al.*, 1962).

These proteins all bind two atoms of Fe^{+3} per mole (Fraenkel-Conrat and Feeney, 1950; Schade *et al.*, 1949; Blanc and Isliker, 1961; Groves, 1960) to form a stable salmon-colored complex which is characterized by a broad visible absorption band with a maximum at about 470 mμ. Conalbumin (Fraenkel-Conrat and Feeney, 1950) and transferrin (Surgenor *et al.*, 1949) will bind two atoms of Cu^{+2} per mole with the formation of a less stable yellow complex. A weak Zn^{+2} complex can also be formed (Warner and Weber, 1953; Surgenor *et al.*, 1949). Azari and Feeney (1958, 1961) have shown the iron-protein complexes of conalbumin and transferrin to be stabilized to proteolysis, denaturation, and chemical treatment. The proteins contain no special prosthetic groups for binding metal ions, but, rather, it appears that the metal-binding capacity results from the fundamental properties and conformational arrangement of the amino acid residues in the protein molecule (Fraenkel-Conrat and Feeney, 1950). However, one molecule of HCO_3^- or $CO_3^=$ per metal ion bound is required for formation of the colored iron complexes (Schade *et al.*, 1949; Warner and Weber 1953). Because of their unique metal-binding properties these proteins have received considerable study. Also their complementary properties have made comparative studies useful in investigating the mode of metal binding.

* Part of this material was taken from a dissertation to be submitted by J. R. Clark (predoctoral fellow of the United States Public Health Service, GPM-16,261, 1962–1963) in partial fulfillment of the requirements for the Ph.D. degree in Agricultural Chemistry at the University of California, Davis. This research was supported in part by a research grant (AI-3484) from the National Institute of Allergic and Infectious Diseases of the National Institutes of Health, U. S. Public Health Service.

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Since no prosthetic groups have been found in the molecules (Fraenkel-Conrat, 1950), determination of the metal-binding site has been difficult. Chemical modification of metal-free conalbumin by reagents specific for amino, guanidyl, phenolic, imidazole, disulfide, hydroxyl, and carboxyl groups resulted in each case in a loss of iron-binding capacity (Fraenkel-Conrat and Feeney, 1950), making it difficult to ascertain which amino acid residues are involved in metal-binding. However, iron-conalbumin has recently been modified by iodination without loss of binding properties (Azari and Feeney, 1961) and both iron and iron-free conalbumins have been modified by mild amino group reagents and found to retain their metal binding properties.[1] Therefore, in the earlier studies, the loss of iron-binding capacity may have been due to denaturation rather than chemical modification of the proteins.

Schade *et al.* (1949) and Warner and Weber (1953) determined the release of 2 and 3 protons, respectively, per Cu^{+2} and Fe^{+3} bound. Because of the comparative titration behavior of the iron and iron-free conalbumin as well as the electrophoretic properties of the respective proteins, Warner postulated a binding site involving 3 tyrosyls, HCO_3^-, and another group, probably a carboxyl anion (Warner and Weber, 1953; Warner, 1953). The involvement of the phenolic groups of the tyrosyl residues was further substantiated by spectrophotometric tiration data (Wishnia *et al.*, 1961). During the course of the present investigation an abstract of a study on transferrin and conalbumin has appeared in which it is concluded from equilibrium dialysis and EPR[2] results that guanidyl groups of arginine may be involved in the metal binding (Malmström *et al.*, 1963).

We have obtained the electron paramagnetic resonance (EPR) spectra of the iron and copper complexes of three avian conalbumins and two human transferrins. These spectra have been compared in an attempt to determine the mode of binding of the metal ions and the type of interaction, if any, between the active sites.

Experimental Procedures

Preparation of Proteins.—Chicken conalbumin was isolated from egg white by chromatography on CM-

[1] Clark, J. R., and Feeney, R. E., part of other studies to be published later.

[2] Abbreviations used in this work: EPR, electron paramagnetic resonance; CM-cellulose, carboxymethyl-cellulose; DEAE-cellulose, diethylaminoethyl-cellulose; hf, hyperfine.

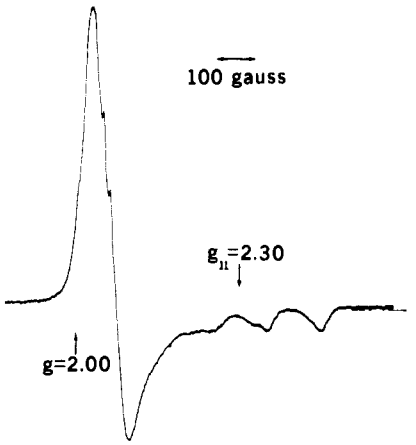

FIG. 1.—The EPR spectrum at liquid nitrogen temperature for a lyophilized sample of chicken conalbumin saturated with copper. In this spectrum and all the others the magnetic field, H_0, is increasing to the left.

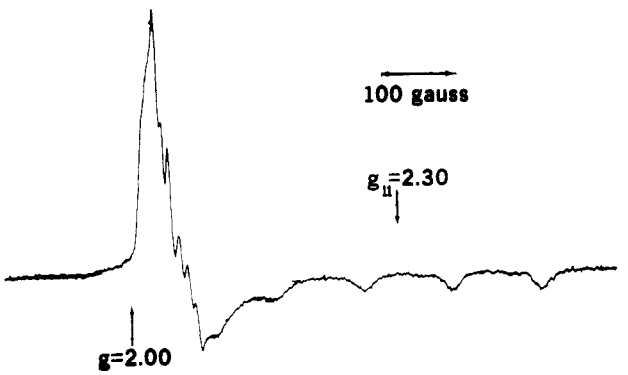

FIG. 2.—The EPR spectrum at liquid nitrogen temperature for an aqueous solution of copper-conalbumin. The extra-hyperfine splitting on the peak near $g = 2.00$ is evidence for coupling to nitrogen atoms.

cellulose according to the procedure of Rhodes *et al.* (1958) and crystallized as the iron complex by the method of Warner and Weber (1951). Iron-free conalbumin was obtained by titrating the iron complex to *p*H 4.7 in the presence of 0.01 M citrate and removing the iron-citrate complex by adsorption on Dowex-1 ion-exchange resin (Warner and Weber, 1951). Turkey and Japanese quail (*Coturnix coturnix japonica*) conalbumins were prepared from the respective blended egg whites by chromatography on CM-cellulose and DEAE-cellulose.[3] Turkey conalbumin was crystallized as the iron complex[3] and the iron was removed from both proteins as above (Warner and Weber, 1951).

Lactotransferrin was isolated from human milk[4] by ammonium sulfate precipitation according to the procedure of Montreuil *et al.* (1960) and further chromatographed on DEAE-cellulose with 0.01 M Tris-HCl buffer, *p*H 8.6. Stepwise elution was carried out by adding 0.01 and 0.1 M NaCl successively to the buffer system. The iron was partially removed by Dowex-1 ion-exchange resin (Warner and Weber, 1951) after titration to *p*H 3.5 in the presence of citrate. Human plasma transferrin fraction IV 7, 2 (lot 47825) was obtained through the courtesy of Merck and Company and was further purified by chromatography of the iron complex on DEAE-cellulose. Stepwise elution was employed using 0.01 M Tris-HCl buffer, *p*H 8.5, plus 0.05, 0.075, 0.1, and 0.15 M NaCl. The 0.05–0.075 M NaCl fraction was used in this study. The iron was removed in the same manner as for the conalbumins.

[3] Clark, J. R., Osuga, D. T., and Feeney, R. E., in preparation.

[4] Human milk was obtained from the Mothers Milk Bank, Inc., San Francisco, California, and either refrigerated or frozen depending upon the time of storage before use.

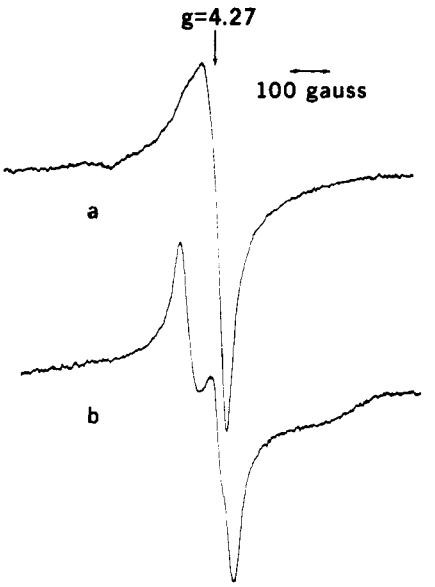

FIG. 3.—The portion of the EPR spectrum at liquid nitrogen temperature for Fe^{+3} centered at $g = 4.27$: (a) lyophilized human blood transferrin; (b) aqueous solution of iron-conalbumin.

Preparation of Samples for Analysis.—After removal of the iron, the conalbumin and transferrin solutions were dialyzed and lyophilized. The lyophilized proteins were weighed out and dissolved in 0.5 M $NaHCO_3$, 0.1 M sodium citrate buffer, *p*H 8.2. Sufficient metal ion to satisfy the metal-binding capacity of two atoms of metal per mole of protein (i.e., approximately 1.35 μg Fe^{+3}/mg protein) was added to the solution. $FeNH_4(SO_4)_2 \cdot 12\ H_2O$ was used as the Fe^{+3} source and $CuCl_2$ as the Cu^{+2} source. The metal-protein complexes were dialyzed extensively against 0.002 M NH_4Cl to remove excess buffer and metal ion and were then lyophilized. Concentrated aqueous solutions of iron and copper conalbumin were prepared from the lyophilized powders. Dry samples were packed into quartz tubes, 3 mm i.d., and solutions were placed in thin-wall capillary tubes for the EPR measurements.

EPR Measurements.—The EPR spectra were obtained at both room temperature and liquid nitrogen temperature utilizing an X-band spectrometer of our own construction in which a Varian 6-inch electromagnet and 100 kc sec^{-1} multi-purpose EPR cavity are employed. The first derivative of the absorption curve is plotted as a function of the applied magnetic field using an XY recorder. The g values near 2.0 were measured by comparison with the free radical standard diphenyl picryl hydrazyl. The g values for iron were obtained by comparison with the g value for iron in glass (Castner *et al.*, 1960). The magnetic field sweep was calibrated using an aqueous solution of peroxylamine disulfonate as a standard and the magnet was cycled to insure reproducibility of the sweep.

RESULTS

Copper Complexes.—The copper EPR spectra of chicken, turkey, and Japanese quail conalbumin and human plasma transferrin and lactotransferrin were all

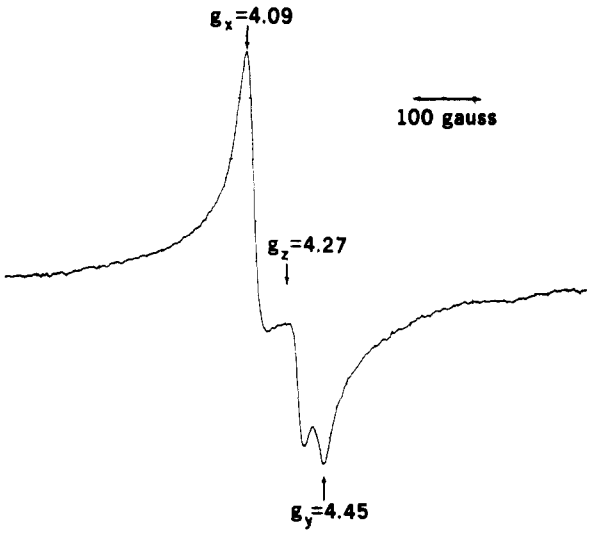

FIG. 4.—The portion of the EPR spectrum at liquid nitrogen temperature for Fe^{+3} centered at $g = 4.27$ for polycrystalline iron-conalbumin. The anisotropic nature of this spectrum is clearly evident and the three g values are shown.

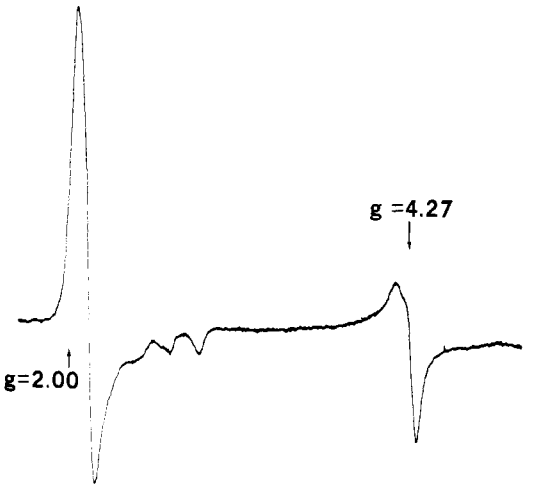

FIG. 5.—The EPR spectrum at liquid nitrogen temperature for conalbumin containing approximately equal amounts of both iron and copper. The abscissa is not linear in H_0.

essentially the same. A typical spectrum obtained at liquid nitrogen temperature is shown in Figure 1 for lyophilized chicken conalbumin saturated with copper. When conalbumin half saturated with respect to its binding capacity for copper was examined, an identical spectrum was obtained except that the amplitude of the signal was reduced correspondingly.

The copper spectrum is characterized by two peaks centered at g values designated as $g_\perp$ and $g_\parallel$ (Sands, 1955). The $g_\parallel$ peak is centered at a g value of 2.30 and is split into four components separated by 145 gauss due to the Cu nuclear hyperfine (hf) interaction. This spectrum is more clearly resolved in a concentrated solution of copper conalbumin at liquid nitrogen temperature (Fig. 2). The value of $g_\perp$ cannot be accurately determined from the spectrum. No copper interaction is observed on the $g_\perp$ peak, but an additional hf splitting of two or three lines is evident in Figure 1. These extra-hf lines are more clearly resolved in Figure 2 where six lines are observed. These extra-hf lines are due to coupling between the unpaired electron and the ligand nitrogen atoms.

Iron Complexes.—The EPR spectra were also essentially the same for all the lyophilized iron-protein complexes. The spectrum consists of a weak broad background spectrum extending from about $g = 1$ to $g = 10$ with a sharp, somewhat asymmetric peak at $g = 4.27$.[5] When only one mole of iron was present per mole of protein, the same spectrum was obtained with about half the amplitude. The portion of the iron spectrum centered at $g = 4.27$ for lyophilized human plasma transferrin is shown in Figure 3a. In aqueous solutions of iron conalbumin at liquid nitrogen temperature (Fig. 3b) the peak at $g = 4.27$ exhibits structure which is not apparent in the spectrum of the lyophilized complexes. A similar iron spectrum, but better resolved, was obtained at liquid nitrogen temperature on a polycrystalline sample of chicken conalbumin (Fig. 4).

Iron-Copper Complexes.—EPR spectra were obtained on metal-conalbumin complexes in which both copper and iron were bound. Mixtures were obtained by combining the metal ions in solution prior to saturation of the protein and by mechanically mixing the lyophilized powders of the metal-protein complexes prepared separately. In either case the lyophilized samples exhibit EPR spectra characteristic of each metal separately. This is clearly shown in the low-temperature spectrum of chicken conalbumin prepared by mixing the iron and copper ions prior to formation of the metal-protein complex (Fig. 5).

DISCUSSION

Copper Complexes.—The EPR spectra of copper-conalbumin and copper-transferrin are characteristic of Cu^{+2} in an environment which has axial symmetry (Sands, 1955). The coordination appears to be square planar based on the similarity to the EPR spectra for known copper complexes of this symmetry. The extra-hf splitting of the $g_\perp$ peak (Fig. 2) is direct evidence for coordination of the copper to at least two equivalent nitrogen atoms. Equal coupling to two nitrogen atoms gives five lines with intensity ratios of 1:2:3:2:1. In Figure 2 six lines can be counted. However, the lack of complete resolution makes determination of both the number and intensity ratios of these components difficult. Also, it has been found for frozen solutions of copper chelates that another peak occurs near $g_\perp$ (Neiman and Kivelson, 1961) which can exhibit extra-hf lines and which, if it overlaps the $g_\perp$ peak, can lead to additional lines. This is believed to be the explanation for the appearance of six lines in Figure 2. The possibility that this spectrum may consist of seven or nine extra-hf lines due to coupling to three or four nitrogen atoms, respectively, must also be considered. However, on the basis of comparisons with spectra for copper chelates of known coordination to two, three, and four nitrogen atoms (Wiersema, 1963) it is concluded that the extra-hf spectrum more closely resembles that for two nitrogen ligands than for three or four. In addition, Warner and co-workers have concluded from titration and electrophoretic experiments (Warner, 1953; Warner and Weber, 1953; Wishnia *et al.*, 1961) that at least two of the atoms bonded to copper are phenolic oxygens of the tyrosine residues in the protein. Based on these titration and electrophoretic results and the EPR findings it is proposed that the copper is bonded to two nitrogen atoms

[5] A g value of 4.3 for iron was obtained from an EPR measurement by Dr. Helmut Beinert, Enzyme Institute, University of Wisconsin on a sample of "red protein" of bovine milk supplied by Dr. Merton L. Groves, Eastern Regional Research Laboratory, USDA (private communication from Dr. M. L. Groves and Dr. H. Beinert).

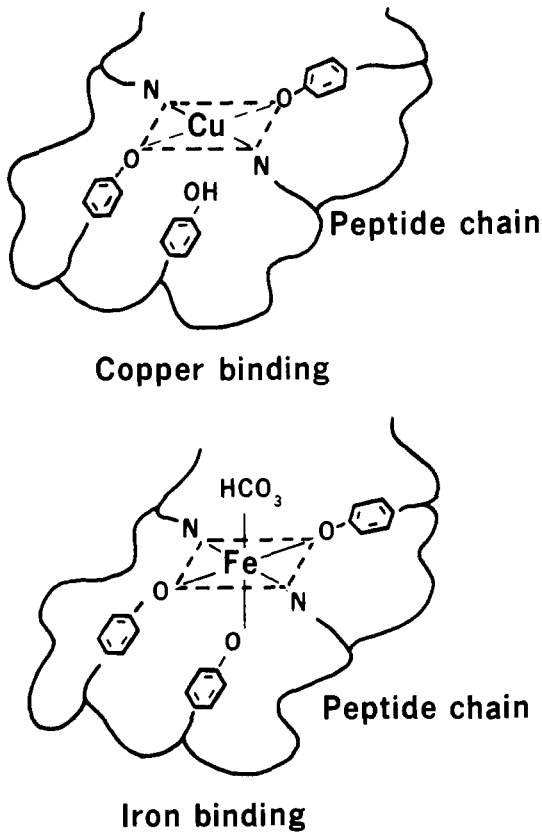

FIG. 6.—A schematic diagram indicating the possible binding of copper and iron in either conalbumin or transferrin showing the similarity of the sites for the two metals and their symmetry as inferred from the EPR results.

and two phenolic oxygen atoms in a square planar arrangement. Malmström *et al.* (1963) have proposed the guanidyl groups of arginine as the nitrogen ligands. However, the EPR results cannot rule out the alternative possibility of the imidazole groups of histidine or the ϵ-amino groups of lysine. A schematic diagram of a plausible coordination of copper in transferrin and conalbumin is given in Figure 6. The possible involvement of HCO_3^- in the copper binding is discussed below.

Iron Complexes.—The EPR spectra of the lyophilized iron-conalbumin and iron-transferrin samples are characteristic of the spectrum of Fe^{+3} observed in different glasses (Castner *et al.*, 1960) and in nucleic acid samples (Shulman and Walsh, 1963). The spin Hamiltonian for iron may be written:

$$\mathcal{H} = \beta[g_z S_z H_z + g_y S_y H_y + g_z S_z H_z] + D[S_z^2 - {}^1/_3 S(S+1)] + E(S_x^2 - S_y^2)$$

The first line is the Zeeman interaction with the applied magnetic field. The second line describes the zero-field splitting due to local electric fields at the Fe^{+3} site.

The peak at $g = 4.27$ is characteristic of a highly asymmetric electric field with $E > D$ and $h\nu$ (Castner *et al.*, 1960; Shulman and Walsh, 1963). For $D = 0$ a single isotropic line will result. If D is not equal to zero an asymmetric line is obtained characterized by three unequal g values (Castner *et al.*, 1960): $g_x = 30/7 - (120D/49E)$, $g_z = 30/7$, $g_y = 30/7 + (120D/49E)$. The latter case applies to the line at $g = 4.27$ for iron-conalbumin and iron-transferrin. The asymmetry is most clearly evident in the spectrum of polycrystalline iron-conalbumin at liquid nitrogen temperature (Fig. 4). The line shape is characteristic of three unequal g values (Kneubühl, 1960). The g values are found to be $g_x = 4.09$, $g_z = 4.27$, and $g_y = 4.45$ with $D/E = 0.0735$. Castner *et al.* (1960) show that one will get $S_x^2 - S_y^2$ coupling with $E > D$ for octahedral coordination when the symmetry is orthorhombic.[6] Four of the ligand atoms are located at the corners of a rectangle with the Fe atom at the center, and two other groups are coordinated on the perpendicular axis through the iron. All the ligand atoms are equidistant from the iron. This protein ligand arrangement is quite similar to that proposed above for the copper in conalbumin and transferrin and is consistent with the evidence that the copper and iron bind to the same site in these proteins (Fraenkel-Conrat and Feeney, 1950; Warner and Weber, 1953; Surgenor *et al.*, 1949). Titration and electrophoretic data suggest coordination of the iron to three phenolic groups plus HCO_3^- (Warner and Weber, 1953; Wishnia *et al.*, 1961). By analogy with the analysis of the copper complex, the other two ligand atoms are probably nitrogen, although there is no direct evidence for nitrogen binding in the spectrum of the iron complex. The proposed coordination of iron is shown in Figure 6.

Interaction of Binding Sites.—The similarity of the EPR results for the metal-protein complexes of conalbumin and transferrin either containing both iron and copper simultaneously or one or two moles of either iron or copper separately shows that there is no magnetic interaction between the metal ions in these proteins which can be detected by EPR.

This conclusion is further supported by the results obtained at liquid nitrogen temperature in which a narrowing of the copper and iron spectra is observed, indicating that the line width is governed by spin-lattice rather than spin-spin interactions. These results may be interpreted to mean that the ions are at least 10 A apart. Thus, the apparent effect of the binding of the first iron atom on that of the second as indicated by their different association constants (Warner and Weber, 1953; Davis *et al.*, 1962) is not believed to be due to any direct interaction between the two iron atoms. The similarities of the EPR spectra for proteins containing one or two moles of bound metal implies that the two sites are essentially the same. The most plausible explanation for these differences in binding constants is the hypothesis of Warner and Weber (1953) that a change in the protein configuration has occurred upon binding of the first iron atom.

General Considerations of Evidence for Structural Formulas of Complexes.—A recent study of the optical rotatory dispersion properties of conalbumin and transferrin (Vallee and Ulmer, 1962) has shown that the formation of the iron-protein complex results in a negative Cotton effect centered at the absorption maximum of 470 mμ, but that no such effect is observed with the colored copper-protein complexes (absorption bands near 440 mμ). This significant difference between the iron and copper complexes suggests that the optical asymmetry which generates the Cotton effect is controlled by limited configuration of the metal-protein ligand binding site and that the difference between the rotatory dispersion for the copper and iron proteins may be related to the coordination properties of these metals

[6] Other symmetries will give $S_x^2 - S_y^2$ coupling. For example, in glass equal charges on two corners of a tetrahedron with Fe^{+3} at the center (or any charge distribution obtained by superimposing four charges equal to each other but not necessarily equal to the first two) will give the $g = 4.27$ case (Castner *et al.*, 1960). This arrangement seems to us to be less likely in the case of proteins than the one discussed in the text.

(Vallee and Ulmer, 1962). The schematic diagrams proposed in the present work (Fig. 6) are in accord with this interpretation of the optical results; the iron forms an asymmetric center which is optically active but the copper-protein ligand site possesses a plane of symmetry and is therefore optically inactive. The formulation for copper will require alteration if bicarbonate is involved in the copper-protein complexes. Although bicarbonate has been shown to be necessary for the formation of the colored iron complex (Warner and Weber, 1953), it has never been shown to be necessary for copper binding. The binding of HCO_3^- to copper in the 5th or 6th coordination position can be ruled out on the basis of optical rotatory dispersion results. Therefore, if HCO_3^- is bound it must replace one of the tyrosyl groups.

Despite the proposed models, little is known about the protein nature of the binding sites. The most probable protein side chains forming the metal protein complex appear to be tyrosyl groups for the oxygen ligands and imidazole groups of histidine or guanidyl groups of arginine for the nitrogen ligands. The possibility that ϵ-amino groups of lysine are involved in the nitrogen binding appears to be less likely in view of preliminary results of chemical modification which indicate that such amino groups can be modified without loss of the iron-binding properties.[1]

Although the EPR results for the metal complexes of these proteins indicate similar binding sites, the relatively low pK values of dissociation of the metal complexes of human lactotransferrin and bovine "red protein" (Montreuil *et al.*, 1960; Groves, 1960) shows that the metal-binding properties of these proteins are not identical.

Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

Acknowledgement

The authors express their appreciation to Drs. J. Corse, L. L. Ingraham, R. E. Lundin, and K. J. Palmer for suggestions regarding the possible structures for the iron and copper complexes of conalbumin and transferrin.

Added in Proof

Dr. Bo G. Malmström now thinks that the nitrogen hf pattern on the copper comes from imidazole and not guanidyl groups (private communication from Dr. Malmström).

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