found above it is evident that the ionization of a proton from either ion is accompanied by about the same decrease in entropy. This finding is not necessarily in contradiction to the conclusion of Calvin and Bailes[24] regarding the increase in entropy attending the formation of chelates because the cases which they consider involve relatively little change in the structure of the chelate and most of the entropy change is due to the increase in the number of particles in the reactions which were considered.

Values have not been included in Table I for the stability constants of these chelates as usually defined because this calculation requires a knowledge of the dissociation constant of the hydroxyl group in citric acid. If this is estimated to be 10^{-16} the constant for the reaction $Ci^{4-} + Cu^{2+} = CiCu^{2-}$ will be about 10^{18} and that for the corresponding formation of $CiFe^{-}$ will be 10^{25}.

NEW YORK, N. Y.

(24) M. Calvin and R. H. Bailes, THIS JOURNAL, **68**, 949 (1946). See also reference 10, p. 149.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, NEW YORK UNIVERSITY COLLEGE OF MEDICINE]

The Metal Combining Properties of Conalbumin[1]

BY ROBERT C. WARNER AND IONE WEBER

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The association reactions of ferric, cupric and zinc ions with conalbumin from the chicken egg have been investigated. These ions combine with two specific sites in each molecule of protein and bind one bicarbonate ion per metal ion in forming the complex. Protons are displaced in this process. Evidence is presented to show that the phenolic groups of tyrosine are an essential part of the binding sites which coördinate the metal. Reversible dissociation curves for the ferric and cupric complexes as a function of *p*H have been obtained and equilibrium constants have been derived for a mechanism which accounts for these curves. A high degree of interaction between the two binding sites such that the second metal ion is associated more readily than the first has been demonstrated.

The egg white protein, conalbumin, has the property of combining stoichiometrically with ferric or cupric ions. Since the colored complexes contain two metal ions per mole of protein, there appear to be two specific sites on the protein, each of which will coördinate one metal ion.[2-5] This situation may be contrasted with the type of protein–metal association studied by Klotz and Curme,[6] in which the number of bound ions increased as the free metal ion concentration was increased without approaching any well defined maximum binding capacity. The latter type of binding may be termed non-specific in the sense that many or all of the carboxyl and amino groups may be involved.

Specific protein–metal interaction is of particular interest in the case of the binding of activating metals by enzymes.[7] However, few of these reactions have been investigated in detail, largely because of the unavailability of purified preparations in the quantity required for such a study. The striking similarity between the complexes formed by conalbumin and those formed by the metal binding globulin of serum[8] gives an added interest to this type of interaction. We have used our recently described preparation of crystalline conalbumin[5] to investigate the combination of this protein with iron and other metals.

Experimental

Crystalline iron conalbumin and metal-free conalbumin were prepared as previously described.[5] The crystalline zinc complex was prepared by adding a 30% excess of zinc sulfate to the metal-free, isoelectric protein and chilling the solution. The crystals which formed were recrystallized by dissolving them with dilute alkali and adjusting the solution to *p*H 6.4.

Combining Capacity.—The combining capacity of conalbumin for iron was previously reported to be equivalent to a minimum molecular weight of 38,300.[5] The copper capacity was determined by spectrophotometric titration similar to that employed for iron. The titration was conducted in a veronal buffer in the presence of 0.002 *M* sodium citrate and 0.1 *M* sodium bicarbonate. The color formed by adding increments of standard cupric sulfate to a series of flasks was measured at 440 mμ with a Beckman spectrophotometer. The titration must be carried out at a *p*H between 8 and 9. The lower limit is fixed by the shape of the dissociation curves described below. The upper limit is set by the fact that the absorption of the copper complex begins to decrease as the *p*H is raised above 9. The iron complex is not so sensitive to *p*H and the same results were obtained from *p*H 7.5 to 10. The curves obtained with both iron and copper are shown in Fig. 1. The combining capacity is located as the intersection of the linear rise in optical density and its subsequent maximal value. The capacity values calculated as the minimum molecular weight are given in Table I. The slope of the linear rise in optical density of the iron curve can be used to calculate a coefficient for the optical density of the complex per micromole of iron

TABLE I

MINIMUM MOLECULAR WEIGHT OF CONALBUMIN[a]

Iron titration	38,300[b]
Copper titration	38,100
Absorption of crystalline iron complex	38,500
Bound carbon dioxide	38,200
Zinc binding, equilibrium dialysis	36,000
Direct analysis of crystals Iron	35,100[b]
Direct analysis of crystals Zinc	34,800

[a] 38,300 was adopted as the minimum molecular weight and 76,600 as the molecular weight. These values were employed in all calculations in this paper. [b] Taken from Warner and Weber, reference 5.

(1) This work was carried out under contract with the Office of Naval Research. A preliminary report of this investigation was presented at the meeting of the American Chemical Society at Boston, April 4, 1951.

(2) G. Alderton, W. H. Ward and H. L. Fevold, *Arch. Biochem.*, 11, 9 (1946).

(3) A. L. Schade, R. W. Reinhart and H. Levy, *ibid.*, **20**, 170 (1949).

(4) H. Fraenkel-Conrat and R. E. Feeney, *ibid.*, **29**, 101 (1950).

(5) R. C. Warner and I. Weber, *J. Biol. Chem.*, **191**, 173 (1951).

(6) I. M. Klotz and H. G. Curme, THIS JOURNAL, **70**, 939 (1948).

(7) E. L. Smith, *Proc. Natl. Acad. Sci., U. S.*, **35**, 80 (1949).

(8) D. M. Surgenor, B. A. Koechlin and L. E. Strong, *J. Clin. Invest.*, **28**, 73 (1949); B. A. Koechlin, THIS JOURNAL, **74**, 2649 (1952).

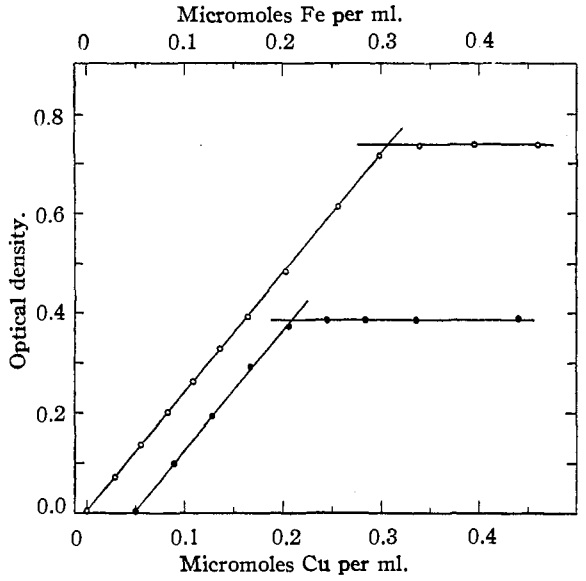


Fig. 1.—Spectrophotometric titration of conalbumin with iron and copper in the presence of citrate: O, 1.19% conalbumin titrated with iron at *p*H 9; ●, 0.634% conalbumin titrated with copper at *p*H 8.2.

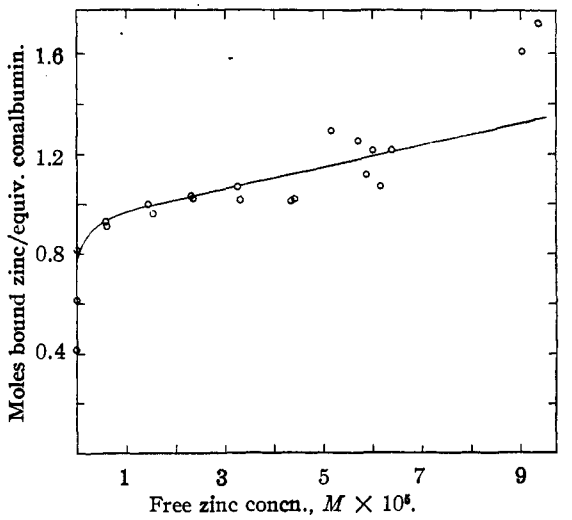


Fig. 2.—Binding of zinc by conalbumin determined by equilibrium dialysis.

per ml. Application of this coefficient to the absorption of several samples of the crystalline iron complex at 470 mμ gave the value shown in the third line of Table I.

The combining capacity for zinc was investigated by equilibrium dialysis because the complex with this metal is colorless. Zinc was determined essentially by the method of Vallee and Gibson.[9] This method was found to give an average deviation of 2.3% in the range of 2 to 30 μg. of zinc. In analyzing solutions containing conalbumin, the protein was removed by precipitation at *p*H 2.5 in 1.3 *M* sodium chloride. The experiments were performed at *p*H 9 in an ammonia buffer with an ionic strength of 0.1. The protein solution was placed in a bag of Visking casing which had been soaked in 0.1 *M* nitric acid for several days and then thoroughly rinsed with water to remove iron and other metals. The bags, containing 10 ml. of the solution inside, were equilibrated in glass stoppered tubes with an equal volume of outside solution by rocking in a water-bath at 30°. It was found that considerable zinc was adsorbed by the membrane so that it was necessary to analyze both the inside and outside solution for zinc. The results are plotted in Fig. 2 as the moles of zinc bound per equivalent of protein against the free zinc concentration. In the experiments in which 0.8 mole or less of zinc was bound, no free zinc could be detected in the outside solution. The curve rises to the capacity determined for iron and copper at a low concentration of free zinc. As the free zinc is increased additional zinc is bound, apparently by linkages other than those responsible for the specific binding. A value for the minimum molecular weight obtained by extrapolation of this secondary rise to zero free zinc concentration is included in Table I. The zinc content of the crystalline zinc complex was also determined. The result is included in Table I along with the previously reported analysis of iron conalbumin. The lower minimum molecular weight (higher zinc content) obtained by this method is probably a result of the additional non-specific binding shown in Fig. 2 since the crystals were originally separated from solutions containing excess zinc.

The Role of Carbon Dioxide.—The presence of carbon dioxide was reported by Schade, Reinhart and Levy[3] to be necessary for the formation of the metal complexes of conalbumin and the metal binding globulin of serum. Recently Fraenkel-Conrat[10] has concluded that carbon dioxide is unnecessary for the formation of the complexes although it will "favor color development." Because of this discrepancy we have reinvestigated the role of carbon dioxide in the process.

Iron conalbumin is sufficiently stable so that dissolved carbon dioxide can be removed from its solution without dissociating the complex provided no citrate, phosphate or other buffer anion capable of combining with iron is present. This fact permits the direct determination of bound carbon dioxide in the Van Slyke manometric apparatus. A 5% solution of the crystalline iron complex in water was adjusted to *p*H 6 and a sample was repeatedly evacuated in the Van Slyke apparatus. The color of the solution remained unchanged during this procedure. The bound carbon dioxide was then determined by acidification and extraction in the usual manner. The average of 6 determinations on 3 samples of iron conalbumin is shown in Table I.[11] The value is calculated as a minimum molecular weight and demonstrates that iron and carbon dioxide occur in a 1:1 ratio in the complex.

Experiments similar to those of Fraenkel-Conrat[10] were also performed. The reaction was carried out in the Van Slyke apparatus equipped with a small vessel containing concentrated sodium hydroxide and ammonia attached to the side arm. The air space in this vessel was flushed out with ammonia by evacuating the apparatus and was sealed off with mercury. A 5% solution of iron conalbumin containing 0.01 *M* citrate was adjusted to *p*H 5 and a sample was placed in the apparatus. It was repeatedly evacuated and the gas ejected. All of the red color of the iron conalbumin disappeared and was replaced by that of the yellow ferric citrate. Gaseous ammonia was then allowed to distil in through the side arm until the *p*H rose to 8 or 9 as checked after completion of the experiment. The side arm was sealed off and the formation of color was followed visually by comparison with a series of standards corresponding to 5, 10 and 20% formation of the iron complex. Color development did not exceed 10% over a period of 3 or 4 hours, but rose rapidly to the maximum on addition of bicarbonate. When similar experiments were performed in the cold room at 2 to 3°, the solution remained yellow for several hours and the reddish color developed only after overnight standing. Similar results were obtained with the copper complex except that even less color developed in the same time periods. We are inclined to attribute the small amount of color formed to leakage of carbon dioxide into the apparatus by diffusion since the color formed from the top downward. It is not possible to say whether the same was true of Fraenkel-Conrat's experiments. It may be noted however that

(9) B. L. Vallee and J. G. Gibson, 2nd., *J. Biol. Chem.*, **176**, 435 (1948); F. L. Hoch and B. L. Vallee, *ibid.*, **181**, 295 (1949). Contrary to the experience of these authors, the absorption of the reagent solution slowly changed during the exposure to light involved in the extraction procedure. Corrections for this fading were made by the use of suitable controls. The ratio of the optical density of the reagent at 625 mμ to that at 525 mμ averaged 5.5 or considerably higher than reported by the above authors. It was also found necessary to correct for the absorption of zinc dithizonate at 625 mμ.

(10) H. Fraenkel-Conrat, *Arch. Biochem.*, **28**, 452 (1950).

(11) The authors are indebted to Seymour Ehrenpreis for carrying out these determinations.

a small leakage of carbon dioxide would cause a much greater percentage color development in the dilute conalbumin solutions used in his experiments. One other possibility may be mentioned. Oxalate can replace bicarbonate to some extent in the formation of a colored iron complex. We have not found any other anions capable of doing this, but some such effect might account for Fraenkel-Conrat's results.

The carbon dioxide-free solution at *p*H 8 obtained by the above method at 2° was used to demonstrate that the molecular species involved in the formation of the complex is HCO_3^- or CO_3^{2-} rather than unhydrated carbon dioxide. The addition of bicarbonate to such a solution resulted in one-half maximum color formation in 50 to 60 sec. However, if a solution of water saturated with carbon dioxide (same total carbon dioxide content as the bicarbonate solution) was used, the half time for color development was 22 min. When carbonic anhydrase[12] was added to each solution before the bicarbonate or carbon dioxide the half time was 50 to 60 sec. in each case. This result eliminates the possibility that CO_2 is directly involved in the formation of the complex. No decision can be made on this basis between HCO_3^- and CO_3^{2-} since equilibrium between these species is established essentially instantaneously in solution.

Displacement of Hydrogen Ions by the Metal.—Hydrogen ions are displaced from conalbumin when the metal complexes are formed. The number displaced per mole of metal was determined by direct titration. Metal-free conalbumin at *p*H 8.5 was mixed with freshly prepared ferric nitrate and the amount of standard base required to restore the *p*H to 8.5 was determined. The conalbumin was always present in excess over the added metal. Experiments were performed both in an atmosphere of nitrogen with only sufficient bicarbonate present to permit complex formation and in the presence of the concentration of bicarbonate calculated to be in equilibrium with atmospheric carbon dioxide at the *p*H of the end-point. Similar experiments were carried out with cupric sulfate. The number of equivalents of protons displaced was found to be 2.9 per mole of iron and 1.9 per mole of copper. The same value was found for iron in experiments in which the *p*H was 7.5 and 9.5. At *p*H 10.5 the value was lower, but the precision of the titration under these conditions was too poor to be conclusive. It is to be expected that slightly less than the number of equivalents of protons displaced would be titrated in these experiments because the complex bears a greater negative charge than the metal-free protein.[13] These results may be taken to indicate that three protons are displaced by iron and two by copper when the complex is formed at the expense of the bicarbonate ion. It is also clear that the groups on the protein involved in coördinating the metal must bear protons at least up to *p*H 9.5 and therefore must have *pK*'s greater than 10.

Dissociation Equilibria.—Solutions of the iron complex are stable between *p*H 6 and 11. They have a constant maximal light absorption for equivalent amounts of protein and iron in this range. Below *p*H 5.5, the color decreases, but does not disappear until the *p*H is below 4. In this *p*H range the color fades gradually with time and equilibrium is established only very slowly. This is probably because the hydrolysis equilibrium of the ferric ion is very slowly attained. In order to make measurements of the dissociation of the iron complex under conditions in which reversible equilibria could be established, it was necessary to add a competing, iron complexing ion. Citrate was the only competitor of a number tried that proved to be satisfactory. It was used in 4- to 100-fold excess over the metal in all experiments. Because of the participation of bicarbonate in the reaction, a constant partial pressure of carbon dioxide was maintained. Conalbumin, metal ion and citrate were present in known concentrations in 0.01 *M* cacodylate buffer and sodium hydroxide or nitric acid was added to adjust the *p*H. The ionic strength was made up to 0.1 by adding sodium nitrate. This ionic strength was exceeded in some of the experiments at high *p*H and high citrate concentration. The solutions were equilibrated in a water-bath at 30° in closed glass tubes fitted with a capillary inlet and outlet for gas. A mixture of nitrogen and carbon dioxide was bubbled through the solution at intervals and the tubes were rocked gently. Equilibrium was attained in a few hours in the experiments with copper, but 16 to 24 hours were required for the iron experiments. The gas inlet and outlet tubes were closed off and the light absorption was determined with the Beckman spectrophotometer at 470 mμ for iron and 440 mμ for copper. Tubes were included in each series at *p*H values above and below the range of the dissociation to determine, respectively, the absorption corresponding to completion of the reaction and to zero complex formation. The latter values were used as blanks. The fraction, α, of the maximum binding capacity of the conalbumin which was present as the metal complex was calculated. The *p*H of the sample was determined with a glass electrode assembly. In separate experiments the effect of varying the concentration of citrate, metal, carbon dioxide and conalbumin was explored. Experiments were also carried out at 5° in a similar manner except that the absorption measurements were made with a photoelectric colorimeter in a refrigerated room. Measurement of the *p*H was carried out at 5°. The curves obtained are shown in Figs. 3 and 4. The solid lines are theoretical curves derived as described below.

Effect of Other Metals.—Iron is so tightly bound by conalbumin that it cannot be displaced by any other metal. Copper is readily displaced by iron and can be partially displaced by zinc. In 0.01 *M* sodium citrate in the presence of 5% carbon dioxide in the gas phase, the bound copper was reduced 28% in the presence of an equimolar concentration of zinc ion at *p*H 7.1. Copper is thus more tightly bound than zinc. Other metals were tested for their ability to displace copper in a similar manner except that the competing ion was added in a 10-fold excess over copper. The results indicated a small combining ability for cobaltous and cadmium ions, but none for nickelous or other ions tested. These competition phenomona demonstrate that iron, copper and zinc are bound to the same sites on the conalbumin molecule.

Nature of the Binding Sites.—Fiala and Burk[14] have suggested that conalbumin contains an hydroxylamino group and binds iron in a complex similar to that formed by iron with hydroxamic acids. The experiments of Fraenkel-Conrat[10] on hydroxylamino derivatives of proteins indicate that conalbumin does not contain a stable group of this kind. In addition, the complex as formulated by Fiala and Burk involves the association of carbon dioxide as a carbamino compound. This possibility is eliminated by our experiments with carbonic anhydrase since carbamino compounds are formed by direct combination with unhydrated carbon dioxide.[15]

Since no unusual groups with metal coördinating properties have been found in conalbumin, we have limited our consideration to groups known to be present in the protein. It is probable that the specific properties of the binding sites are a result of the unique steric arrangement of the several groups comprising the site. Fraenkel-Conrat and Feeney[4] have explored the effect of chemical modification of such protein groups on the iron binding capacity of conalbumin. They found that this capacity was reduced by every type of modification employed. Since it appeared improbable that all of the groups investigated could be directly involved in coördinating the metal ion, these authors suggested that the introduction of the various substituents partially or wholly destroyed the essential steric arrangement of the binding site. Their results thus produced no evidence in favor of any particular group.

The equations derived below which describe the experimental dissociation curves do not place any requirements on the nature of the groups except that protons must be dis-

(12) F. J. W. Roughton and V. H. Booth, *Biochem. J.*, **40**, 309 (1946).

(13) The magnitude of this effect was estimated by calculating the change in the apparent ionization constants of conalbumin caused by this difference in net charge. For this purpose the equation used by I. M. Klotz and H. A. Fiess, *J. Phys. Colloid Chem.*, **55**, 101 (1951), for calculating the electrical contribution to the partial molal free energy of an ionic species was employed. Using 30 Å. for the radius of the conalbumin molecule and reasonable values for the number and *p*K of the groups dissociating in this *p*H region, it was estimated that the maximum reduction in titer from the theoretical value would be 0.2 equiv. per metal ion and probably less. The same equation was used for the calculation of other electrostatic effects elsewhere in this paper. A similar effect operates to decrease the net charge difference between conalbumin and iron conalbumin at *p*H 8.6 below that predicted by equation 1. In the isoelectric region this difference should disappear.

(14) S. Fiala and D. Burk, *Arch. Biochem.*, **20**, 172 (1949).

(15) C. Fuerholz, *J. chim. phys.*, **21**, 400 (1924).

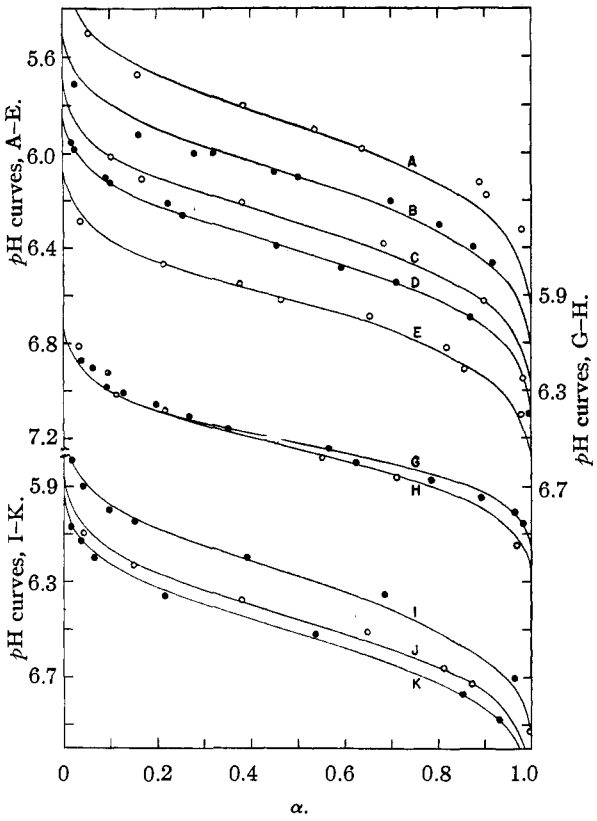


Fig. 3.—Dissociation curves for iron conalbumin. The experimental conditions for each curve are given in Table II. The solid lines are theoretical curves derived as explained in the text.

placed from them over the *p*H range in which dissociation was observed. The data on the displacement of protons by the metal as a function of *p*H place the *pK* of the groups concerned at a minimum of 10 and probably higher. We have confirmed the reported absence of sulfhydryl groups in conalbumin. Therefore amino, phenolic, guanidyl and possibly primary hydroxyl groups remain to be considered.

In attempting to gain a better estimate of the *pK* of the pertinent groups, we have examined conalbumin electrophoretically in the very alkaline *p*H range. At a *p*H sufficiently greater than the *pK* of the combining groups, these will have lost their protons by ionization. Conalbumin should then have a higher net negative charge than the iron complex and hence a greater mobility. In a glycine buffer having a *p*H of 11.2 at 0° the mobility of conalbumin was 0.02×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} less than that of the iron complex whereas at *p*H 8.6 the difference was 0.23×10^{-5}. The net charge on the two species has thus become essentially equal at *p*H 11.2. This result indicates that the combining groups are partially ionized at *p*H 11.2 and must have *pK*'s in this range. This experiment cannot be performed at a higher *p*H because irreversible changes take place as *p*H 12 is approached as shown by changes in the absorption at 470 mμ and in the ultraviolet.

This approximate *pK* is consistent with that of the protein phenolic groups. Guanidyl and primary hydroxyl groups can be eliminated because their *pK*'s are too high to permit significant ionization at *p*H 11.2. Lysine amino groups remain a possibility although they would be expected to be largely in the basic form at this *p*H. The ionization of tyrosine phenolic groups in proteins has been investigated by several authors.[16-18] Their results indicate that the phenolic ionizations in proteins take place at a much higher *p*H than in free tyrosine. The apparent *pK* may be

(16) J. L. Crammer and A. Neuberger, *Biochem. J.*, **37**, 302 (1943).
(17) I. W. Sizer and A. C. Peacock, *J. Biol. Chem.*, **171**, 767 (1947).
(18) C. Tanford and G. L. Roberts, Jr., THIS JOURNAL, **74**, 2509 (1952).

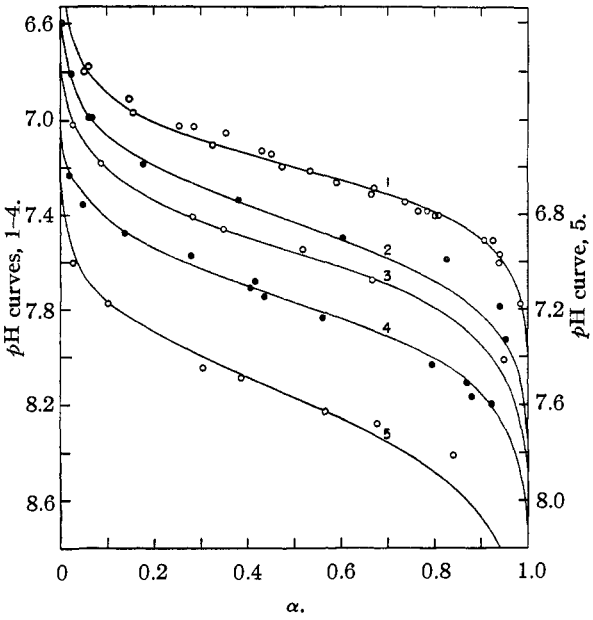


Fig. 4.—Dissociation curves for copper conalbumin. The experimental conditions for each curve are given in Table III. The solid lines are theoretical curves derived as explained in the text.

placed above 11 from the results of these investigations.[19] The change in the ultraviolet absorption of conalbumin with *p*H is similar to that of bovine serum albumin. The tyrosine content as calculated from the extinction coefficients given by Sizer and Peacock[17] at 240 mμ in acid solution and at *p*H 12 was 20 groups per mole as compared with the analytical value of 19.[20] Calculation from the shift in the curve with *p*H indicates that less than one-third of these are ionized at *p*H 11. These considerations favor the assumption that the combining groups are phenolic. However, they do not decisively eliminate amino groups as a possibility. Additional evidence on this point is obtained from comparison of the absorption spectra of the conalbumin complexes and some simpler complexes of the same metals. Most copper complexes involving amino or carboxyl groups are blue. The yellow copper complexes are those involving a phenolic hydroxyl group such as *o*-dihydroxybenzene, *o*-aminophenol and 8-hydroxyquinoline. The first of these forms a copper complex which has a broad absorption maximum at 400 mμ as compared with that of the copper conalbumin at 440 mμ. It also forms a red iron complex when three moles of the phenol are present per mole of iron at *p*H 7 or higher.[21,22] Protons are displaced from the phenolic groups when the complex is formed.

The ultraviolet absorption spectra of conalbumin and the iron complex at *p*H 6 are shown in Fig. 5. The absorption curve of the iron complex parallels that of conalbumin at a higher optical density particularly in the region of the maximum absorption of tyrosine. The same qualitative relation obtains between the absorption of *o*-dihydroxybenzene and its iron and copper complexes. The increase in extinction coefficient per metal ion is somewhat higher, but of the same order of magnitude as for conalbumin.

Dissociation Curves

Mechanism of the Reaction.—The over-all reaction of conalbumin with iron can best be described by the equation

(19) Tanford and Roberts have described the dissociation in terms of an intrinsic constant $pK_0 = 10$. The higher *p*H range of ionization of the phenolic groups (50% at *p*H 11.5) was accounted for in terms of the electrostatic effect of the high net negative charge on the protein.
(20) J. C. Lewis, N. S. Snell, D. J. Hirschmann and H. Fraenkel-Conrat, *J. Biol. Chem.*, **186**, 23 (1950).
(21) R. F. Weinland and K. Bindu, *Ber.*, **45**, 148, 1113 (1912).
(22) G. Schwarzenbach and A. Willi, *Helv. Chim. Acta*, **34**, 528 (1951).

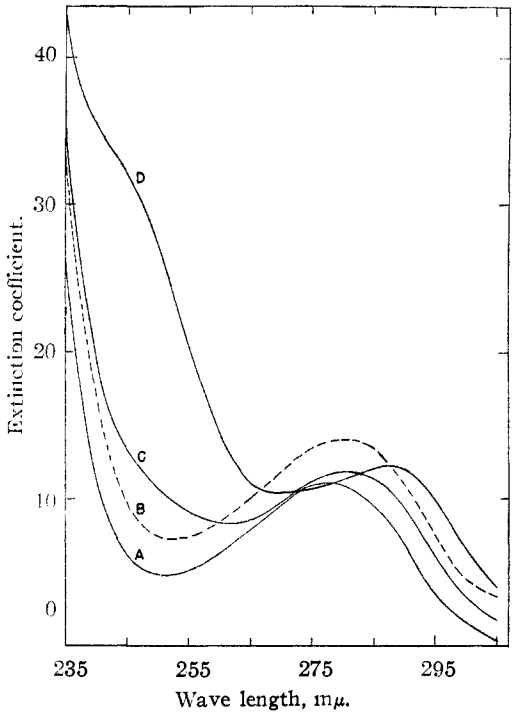
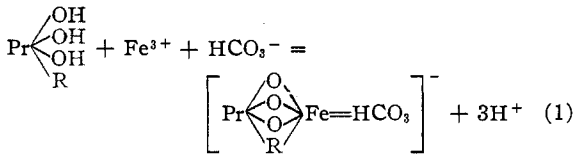


Fig. 5.—Absorption spectra of conalbumin and iron conalbumin: A, conalbumin at *p*H 6; B, iron conalbumin at *p*H 6; C, conalbumin at *p*H 11; D, conalbumin at *p*H 12.



where Pr represents the protein. Only the groups combining with one ferric ion are shown. This formulation is based on the following considerations.

(1) The groups combining with the iron are represented as being tyrosine phenolic groups. The evidence for this has been discussed.

(2) The participation of three such groups is required by the direct determination of the three protons displaced in the reaction.

(3) The carbon dioxide was shown to combine as HCO_3^- or CO_3^{--}. A decision between these possibilities was made on the basis of the electrophoretic mobilities previously reported.[5] The iron complex was found to be more negative than the metal-free protein between *p*H 5 and 8.6. When the magnitude of the difference between the two curves was compared with the hydrogen ion titration curve of either preparation it was evident that this difference corresponds to about one charge per iron atom in the region of the isoelectric point. Although the difference decreases somewhat at *p*H 8.6 we concluded that the net charge difference between conalbumin and iron conalbumin is equal to −1 per metal combining equivalent.[13] In order to achieve this difference the complex must contain HCO_3^- rather than CO_3^{--}. The additional proton may ionize from the complex at *p*H's higher than those involved in the present equilibrium studies.

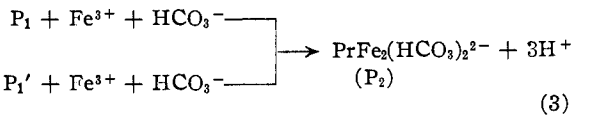
(4) The iron is assumed to coördinate with an additional group in the protein, R in equation 1, such as a carboxyl anion. No proton is displaced from this group between *p*H 6 and 9.5. This assumption is made because iron normally coördinates six groups. The sixth might be a water molecule. However no change in the absorption spectrum of iron conalbumin could be detected on the addition of cyanide which would be expected to replace water under such circumstances.[23] If the complex were formulated similarly to the cobaltipentammine bicarbonato[24,25] complex, two such R groups would have to be assumed for iron and one for copper conalbumin.

(5) The copper complex was found to have the same mobility as the iron complex at *p*H 8.6 and hence is also more negative than conalbumin. It is not sufficiently stable to be examined electrophoretically at *p*H 6. The formation of this complex may be written as in equation 1 except that the participation of the R group is unnecessary and the copper must combine with only two of the phenolic groups. This is required by the measured displacement of two protons by copper and is consistent with the normal coördinating capacity of four for the cupric ion.

Equation 1 describes the equilibrium for a single iron binding site. However, there are two sites per molecule and the evidence presented below indicates that there is interaction between them. The two sites must therefore be distinguished in formulating the equilibrium. The reaction for the association of the first ferric ion becomes

(2)

and for the second ferric ion



(3)

The letters in parentheses will serve as abbreviations for the species indicated. The mass law equations formulated in a manner similar to those for dibasic acids[26] are

$$K_1 = \frac{[(P_1) + (P_1')]h^3}{(P)(Fe^{3+})(HCO_3^-)} \quad (4)$$

$$K_2 = \frac{(P_2)h^3}{[(P_1) + (P_1')](Fe^{3+})(HCO_3^-)} \quad (5)$$

where h is the hydrogen ion activity as determined by the glass electrode. The activities of the other species have been assumed to be equal to their concentrations. In this formulation the concentration of intermediate ionized species corresponding to the loss of protons from the phenolic groups without association with iron is neglected in comparison with the concentration of the complex and of ferric citrate. This is reasonable in view of the extremely unfavorable *p*H (5.5 to 7.0) for the existence of such species and is required by the shape of the experi-

(23) Copper conalbumin is completely dissociated by cyanide.

(24) A. B. Lamb and K. J. Mysels, THIS JOURNAL, **67**, 468 (1945).

(25) A. Werner, *Ber.*, **40**, 4108 (1907).

(26) D. A. MacInnes, "The Principles of Electrochemistry," Reinhold Publ. Corp., New York, N. Y., 1939, p. 396.

mental dissociation curves. The three protons are thus lost in one step in which they are displaced by the iron. In the resulting complex the anionic phenolic groups are stabilized below their usual pH range by the energy of binding of the iron.

Let a = the molar concentration of combining sites. Then

$$a/2 = (P) + (P_1) + (P_1') + (P_2) \quad (6)$$

In these terms α, as previously defined, becomes

$$\alpha = \frac{(P_1) + (P_1') + 2(P_2)}{a} \quad (7)$$

From the equilibrium for the first dissociation of carbonic acid

$$(HCO_3^-) = \frac{K_3 k p_{CO_2}}{h} = \frac{p}{h} \quad (8)$$

where K_3 is the equilibrium constant and k is the molar solubility coefficient of carbon dioxide per mm. of partial pressure (p_{CO_2}). (Fe^{3+}) is obtained from the ferric citrate equilibrium[27]

$$HCi^{3-} + Fe^{3+} = CiFe^- + H^+, K_4 = \frac{(CiFe^-)h}{(HCi^{3-})(Fe^{3+})} \quad (9)$$

Since the dissociation of the complex is measured in the pH range of the third ionization of citric acid, this equilibrium must also be included.

$$K_{a3} = \frac{(HCi^{3-})h}{(H_2Ci^{2-})} \quad (10)$$

Let the total citrate concentration equal

$$t = (HCi^{3-}) + (H_2Ci^{2-}) + (CiFe^-) \quad (11)$$

and the total iron concentration equal

$$r = (CiFe^-) + (P_1) + (P_1') + 2(P_2) \quad (12)$$

Equations 9 to 12 can be rearranged to give

$$(Fe^{3+}) = \frac{(K_{a3} + h)h}{K_{a3}K_4}\phi(\alpha) \quad (13)$$

where

$$\phi(\alpha) = \frac{r - a\alpha}{t - (r - a\alpha)}$$

Combining equations 4–8 and 13 and rearranging a relation between h and α is obtained

$$2\frac{\alpha h^6}{1-\alpha} + K_1'\frac{(K_{a3} + h)}{K_{a3}}\left[\frac{\alpha}{1-\alpha} - 1\right]h^3 - 2K_1'K_2'\left[\frac{K_{a3} + h}{K_{a3}}\right]^2 p^2 [\phi(\alpha)]^2 = 0 \quad (14)$$

where

$$K_1' = \frac{K_1}{K_4} \text{ and } K_2' = \frac{K_2}{K_4} \quad (15)$$

An exactly similar equation is obtained for the copper conalbumin equilibrium except that the exponents of h in the first and second terms become 4 and 2, respectively.

This equation should describe both the shape of an experimental curve and its position on the pH axis when a, t, r and p are varied. K_3 and K_{a3} as defined above at an ionic strength of 0.1 at 30 and 5° were estimated from results of Harned and Bonner[28] and Bates and Pinching,[29] respectively. All other parameters are known except K_1' and K_2'. For any curve the pH at which $\alpha = 0.5$ (middle term vanishes) was used to calculate a preliminary value for $K_1'K_2'$. Using this value, various ratios of K_1'/K_2' were assumed and the pH was calculated for a series of values of α and compared with the shape of the experimental curves. This must be done by successive approximation because the equation is not explicit in h. It was found that for the iron equilibria the experimental curve could be accounted for only by assuming $K_2' >> K_1'$. This assumption simplifies equation 14 in that the middle term will now become negligible for all values of α. In the case of the copper equilibria, the condition necessary to account for the shape of the experimental curve was $K_2' = 20K_1'$. Using these relations, the preliminary values of $K_1'K_2'$ were adjusted if necessary to give the best fit with the experimental points at all values of α. The final values are shown as $pK_1'K_2'$ in Tables II and III and were used to calculate the solid curves in Figs. 3 and 4 from equation 14. The data in these figures demonstrate the adequacy of equation 14 as a description of the dissociation. It should be noted that

TABLE II

EQUILIBRIUM CONSTANTS FOR IRON CONALBUMIN

Curve	t, citrate concn., M	a, conalbumin concn., equiv. $\times 10^4$	r/a	pH at $\alpha = 0.5$ Exptl.	Calcd.	$1/6\ pK_1'K_2'$[c]
A	0.002	2.46	1.20	5.88	5.90	2.78
B	.005	2.46	1.20	6.10	6.08	2.80
C	.01	1.94	1.20	6.29	6.26	2.82
D	.02	2.46	1.20	6.41	6.36	2.84
J	.02	1.23	1.20	6.46	6.46	2.79
I[a]	.005	2.46	1.20	6.28	6.35	2.74
K[a]	.02	2.46	1.20	6.52	6.59	2.73
H	.02	2.46	2.18	6.35	6.18	2.92
G	.02	2.46	11.8	6.31	5.80	3.18
E[b]	.02	2.34	1.20	6.63	(6.63)	3.09
				Av. of A, B, C, D, J, I and K		2.79

[a] The N_2–CO_2 gas mixture used in these experiments contained 1.20 vol. % CO_2. In all other experiments, 5.01 vol. % CO_2 was used. [b] Curve E was obtained at a temperature of 5°. All others were obtained at 30°. [c] $K_2' >> K_1'$ was assumed in all cases.

TABLE III

EQUILIBRIUM CONSTANTS FOR COPPER CONALBUMIN

Curve[a]	t, citrate concn., M	r/a	pH at $\alpha = 0.5$ Exptl.	Calcd.	$1/4\ pK_1'K_2'$[d]
2	0.01	1.55	7.43	7.41	2.30
3	.02	1.55	7.56	7.56	2.28
5	.01	0.776	7.70[c]	7.71[c]	2.27
1	.01	15.5	7.20	6.71	2.76
4[b]	.02	1.55	7.78	(7.78)	2.39
			Average of 2, 3 and 5		2.28

[a] The N_2–CO_2 gas mixture used in all experiments contained 5.01 vol. % CO_2. The conalbumin concentration, a, was 2.55×10^{-4} metal combining equivalents per liter in all experiments. [b] Curve 4 was obtained at a temperature of 5°. All others were obtained at 30°. [c] When $r/a < 1$, equation 14 must be altered by substituting $r\alpha/a$ for α. When $r/a = 0.776$ the middle term of this equation disappears at $\alpha = 0.645$. This point was used in the calculations for curve 5 in place of $\alpha = 0.5$. [d] $K_2' = 20K_1'$ was assumed in all cases.

(27) R. C. Warner and I. Weber, THIS JOURNAL, 75, 5086 (1953). Citric acid is represented as H_4Ci since four protons are displaced from it in the formation of the metal citrate complexes, $CiFe^-$ and $CiCu^{2-}$.

(28) H. S. Harned and F. T. Bonner, *ibid.*, 67, 1026 (1945).

(29) R. G. Bates and G. D. Pinching, *ibid.*, 71, 1274 (1949). See also reference 27, footnote 15.

the primary factor determining the shape of the curves is the net number of protons displaced in the exchange reaction between conalbumin and metal citrate. The shape also varies with t/a, r/a and K_2'/K_1'. Of these factors, only the last has been treated as a parameter and arbitrarily adjusted to fit the experimental data.

In addition to determining the shape of a curve, equation 14 also predicts its position on the pH axis. This position will depend on t, r, a and p. These quantities were varied as shown in Tables II and III which also give the experimental mid-point and $pK_1'K_2'$ for each curve. The calculated mid-point was obtained using the average $pK_1'K_2'$ indicated in the tables. This was included in order to show more clearly the comparison of the observed and computed shifts in the curves on the pH axis.[30] Table II shows that good agreement was obtained for variation in the citrate concentration over a wide range (curves A–D). The shift on reducing p (curves I and K) was in the expected direction, but was not so large as predicted. In obtaining curves G and H, r/a was increased in an attempt to buffer the iron concentration. This change had the expected effect on the shapes of the curves in that they were flatter than any of the others. However, they were not displaced up the pH axis the required amount. This is probably due to participation of hydrolysis products of the ferric ion in the equilibria as the total iron concentration is increased. Thus free (Fe^{3+}) would not rise as predicted, but a buffering of its concentration would still be produced. In a series of blanks prepared for curve G without conalbumin, a light brownish color was present in increasing intensity as the pH was increased. This color is characteristic of the hydrolysis products of the ferric ion and was not present in the blanks at lower iron concentrations. The behavior of the equilibria with change in citrate concentration demonstrates that these hydrolysis effects are not significant in the experiments at $r/a = 1.2$.

In the case of the copper dissociation (Table III), curve 1 with a 10-fold increase in r/a showed a large displacement up the pH axis, as contrasted with the iron curves. However, this shift was still only about one-third of that required by equation 14. The explanation offered for the iron curves is not valid here since the copper concentration can be increased well above this level without any evidence of competition by hydroxyl ion for free Cu^{2+} in the presence of citrate. It has been shown by Klotz and Fiess[13] that the carboxyl and amino groups of serum albumin can combine with copper in competition with citrate. A similar binding of zinc beyond that required to saturate the specific linkages is indicated in Fig. 1. Non-specific binding of this kind will prevent the (Cu^{2+}) from rising in proportion to the total copper concentration and will result in a smaller displacement of the dissociation curve than is predicted by equation 14. It is possible that non-specific binding of iron also contributes to the similar situation in curves G and H, but we have no evidence for this. In view of this difficulty only the values of $pK_1'K_2'$ obtained at low metal concentrations have been included in the averages shown in Table II and III.

Interaction between the Sites.—From the result that $K_2' > K_1'$ it must be concluded that the second metal ion associates more readily than the first. The free energy of interaction may be calculated by considering the equilibrium

$$P^z + P_2^{z-2} = 2P_1^{z-1}$$

where z is the charge on the metal-free protein ion and the subscripts refer to the species identified by equations 2 and 3. The equilibrium constant for this reaction equals $K_1/K_2 = K_1'/K_2'$ and we may write

$$\Delta F^\circ = -RT \ln K_1'/K_2' = -RT \ln 4 + \Delta F^\circ_{int} \quad (16)$$

where 4 is the statistical factor for a divalent dissociation. Using equation 16, ΔF°_{int} for the copper equilibrium is found to be 2600 cal. This value includes a ΔF° of about -85 cal. for electrostatic interaction due to the increase in negative charge as metal ions are bound.[13] In the case of the iron dissociation, K_2' is so much greater than K_1' that only the product of these constants could be derived. However, for this situation to obtain, K_2' must be greater than K_1' by a factor of at least 100. If this minimum ratio is used, equation 16 yields $\Delta F^\circ_{int} = 3600$ cal. No change in K_1'/K_2' could be detected for either metal when the temperature was decreased to 5°. The shape of the curves, however, is not very sensitive to small changes in this ratio.

The interaction energy between the two binding sites is of the same magnitude as the 3500 cal. calculated by Wyman[31] to obtain between the two heme–oxygen associations in a half hemoglobin molecule. Two possibilities in such a situation were discussed by Wyman. In terms of the conalbumin equilibria, these are: (1) The interaction may be between the two sites with the metal bound to them. The interaction energy would then reflect a stabilization of the molecule with two bound ions and no interaction energy would be involved in the association of the first metal ion. (2) The interaction may be between the two sites without metal bound to them. In this case the interaction energy would represent a barrier that would be overcome by the first metal ion bound. There would then be no interaction energy involved in the dissociation of the first metal ion. No choice can be made between these alternatives on the basis of equilibrium data. Wyman has adduced other evidence in favor of the second possibility in the oxygenation of hemoglobin. Additional evidence supporting this choice has been given by St. George and Pauling.[32] They have interpreted this interaction in terms of a steric hindrance to the combination of the first oxygen which is decreased as successive molecules are bound. This steric hindrance was suggested to be a result of the heme groups

(30) The electrostatic effect on $pK_1'K_2'$ due to the change in the net charge on the protein over the pH span of a single curve is small and has been neglected. The effect on $pK_1'K_2'$ derived from curves at different points on the pH scale may be appreciable, but because of the uncertainty in such calculations no corrections have been applied for this effect.

(31) J. Wyman, Jr., in "Hemoglobin," ed. by F. J. W. Roughton and J. C. Kendrew, Interscience Publishers, Inc., New York, N. Y., 1949, p. 295.

(32) R. C. C. St. George and L. Pauling, *Science*, **114**, 629 (1951).

being "buried" in the protein in such a way that the initial oxygenation step makes them more readily available for successive steps. The interaction energy for the conalbumin complexes is of a similar order of magnitude and the same type of explanation may apply. An alternative corresponding to the first possibility can also be suggested. The relative position of the phenolic groups must satisfy certain steric requirements in order that they may coördinate a single metal ion. There may, however, be some "looseness" in this arrangement which disappears when the first metal ion is bound. This would fix the protein structure in the neighborhood of the site more rigidly. Because of this, the steric arrangement of the groups in the second site might also become fixed in such a way as to permit easier association of the second metal ion. While this suggestion is purely speculative, the greater stability of the protein structure in iron conalbumin as compared with the metal-free protein is indicated by its much lower rate of denaturation at 25° and *p*H 12. An analogy to the steric relationship of the phenolic groups comprising the site may be seen in the stability of the metal complexes of *o*-dihydroxybenzene as compared with those of the para and meta isomers.

Intrinsic Constants.—It is convenient in making further calculations to employ an intrinsic constant for the association of a single metal ion. This constant, K_0', may be defined to be equal to $^1/_2K_1'$ or to $2K_2'$ corresponding, respectively, to the two alternatives mentioned above. This choice has little effect on the results and $K_0' = {}^1/_2K_1'$ has been assumed. When this relation is combined with equation 16, it is found that for the copper equilibrium, $pK_0' = {}^1/_2pK_1'K_2' + 0.85$. For the iron equilibrium, assuming $K_2'/K_1' = 100$, $pK_0' = {}^1/_2pK_1'K_2' + 1.3$. Values for pK_0' obtained from these equations for both complexes at 30 and 5° are given in Table IV. This constant (pK_0') may be termed an intrinsic exchange constant for the metal between conalbumin and citrate. In order to obtain an association constant (pK_0) referred to the free metal ion concentration, the previously measured association constants[27] for the formation of the metal citrate complexes (pK_4) must be employed. Utilizing the values for pK_4[33] indicated in Table IV, pK_0 was calculated from the relation $K_0' = K_0/K_4$, analogous to equation 15.

TABLE IV

INTRINSIC EQUILIBRIUM CONSTANTS

	Copper		Iron	
	30°	5°	30°	5°
pK_0'	5.41	5.63	9.67	10.57
pK_4[a]	−2.15	−2.05	−9.46	(−9.46)
pK_0	3.26	3.58	0.21	(1.11)
pK_a	19.8	21.2	29.7	31.8
pK_b	−16.5	−17.6	−29.5	(−30.7)

[a] See footnote 33.

K_0 can be divided into two factors corresponding to considering separately, in equation 1, the ionization of the protons and the association of the metal ion. K_a may be defined as the constant for the dissociation from a single site of the displaceable protons and K_b as the association constant for the protein ion thus formed with the metal so that $K_0 = K_aK_b$. For the iron complex, K_a will equal the cube of the dissociation constant of a single phenolic group. For the copper complex it will equal the square of this value. If the intrinsic constant of the phenolic group ($pK = 10$) found by Tanford and Roberts[18] for bovine serum albumin is used together with their estimate of $\Delta H° = 11{,}500$ cal. for this ionization, the values of pK_b given in Table IV are obtained. These constants correspond to the stability constants for metal complexes as usually defined.

Alternative Formulations.—Some evidence has been presented to show that the proton binding groups are phenolic and they have been formally described as such in the equations and in Table IV. The analysis of the curves in terms of equation 14 does not depend on this assumption and would not be changed if groups of a different chemical nature participated. Only the assumed properties of pK_b would change with the nature of the groups. The distinction made in equation 1 between a bicarbonato and a carbonato complex depends on the assignment of all of the displaceable protons to groups attached to the protein. If a carbonato complex were formed, one of the protons would necessarily come from the HCO_3^- of the solution and only one phenolic group would be assumed to enter into the copper complex and two into the iron complex. An alternative possibility is that two phenolic groups are bound to the metal in each complex, but that iron forms a carbonato complex while copper forms a bicarbonato complex. While the formation of a carbonato complex would be consistent with the determination of the displaceable protons and the shape of the dissociation curve, it would place a different interpretation on the overall exchange constant ($K_1'K_2'$) in that this would contain the second dissociation constant of carbonic acid as a factor.

It has been implicitly assumed that the two binding sites are equivalent and that the extinction coefficient for the complex with two metal ions is twice that for the complex with a single metal ion. If the two sites are not equivalent, the first metal ion bound would always be associated with one particular site. Equation 14 would be unchanged for this case. An interaction between the two sites might still prevail, but the use of the statistical factor in the relation between the two constants would have to be omitted. A difference in the extinction coefficients for the first and second metal ions bound could not be detected in the iron dissociation curves since the complex with a single bound ion makes a negligible contribution to the absorption. Even in the case of the copper dissociation where K_1' and K_2' are not so widely separated, the difference in extinction coefficients would have to be large to have an appreciable effect on the curve.

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(33) See ref. 27. No measurements of the temperature coefficient of pK_4 for iron citrate were made. The value obtained at room temperature has been used in Table IV for both 30 and 5° on the assumption that the temperature coefficient is small.