tions, buffer and carbon tetrachloride in the following manner: to $x$ ml. of the lead solution were added $15 - x$ ml. of buffer, $15 - x$ ml. of dithizone solution and $x$ ml. of carbon tetrachloride. The immiscible phases were shaken and the lead dithizonate extracted into the organic layer. Optical density measurements were made on the organic layer at 500, 510, 520, 530 and 540 m$\mu$.

The total concentration of lead plus dithizone in the organic phase would be $2 \times 10^{-5}$ $M$ if lead is completely extracted and any excess dithizone remains in this phase. In the solutions where lead is in excess and is not completely extracted, the contribution of lead in carbon tetrachloride to the optical density, were it possible to exist in this manner, is assumed to be zero.

### Results and Discussion

The optical densities measured, and those calculated from molar extinction coefficient data[8] on the basis of no interaction, are given in Table I.

Values of $Y$ obtained from observed and calculated data in Table I are plotted in Fig. 1 against mole fraction of dithizone.

It was found that above 0.667 mole fraction of dithizone, where the reagent is in excess, some of the dithizone was extracted into the aqueous phase. However, measurements of this partitioning showed the necessary mole fraction corrections to be very small, and the position of the minimum in Fig. 1 is not affected.

### Conclusions

From the formula $n = x/(1 - x)$, it can be seen that for the formula of the complex to be PbDz, the minimum, when $Y$ is plotted against $x$, must occur at $x = 0.5$, and for the formula to be $PbDz_2$, the minimum must occur at $x = 0.667$. Since the curves at all five wave lengths have minima at 0.667, the formula $PbDz_2$ is confirmed.

**Acknowledgment.**—The authors gratefully acknowledge the assistance of R. C. McIlhenny, of Carbide and Carbon Chemicals Co., in preparing and editing this manuscript, and for drawing the figure shown.

OAK RIDGE, TENN.

---

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

# The Cupric and Ferric Citrate Complexes[1]

BY ROBERT C. WARNER AND IONE WEBER

RECEIVED MARCH 12, 1953

The complexes formed between cupric or ferric ions and citric acid have been investigated as a function of *p*H by spectrophotometric and titrimetric methods. In each case a chelate having a ratio of citrate to metal ion of 1:1 is formed in which four protons have been displaced from the citric acid. This is achieved at a *p*H of about 3 in the case of the iron and at 6 to 7 in the case of copper. At *p*H values below these, other 1:1 complexes are also present in which fewer than four protons have been displaced by the metal ion. Values of the equilibrium constants for the formation of all species contributing significantly to the equilibria have been obtained.

In an investigation[2] of the formation of metal–protein complexes between ferric or cupric ions and conalbumin, citrate was used as a competitor for the metal in studying the dissociation of the complexes. A knowledge of the nature of the metal–citrate complexes and of their formation constants was required for the interpretation of the protein work. In spite of numerous investigations of these compounds, satisfactory information of this kind was not available, particularly over the *p*H range of interest to us. We have therefore reinvestigated these systems employing both spectrophotometric and titrimetric methods.

Bobtelsky and Jordan[3] have concluded that citrate and cupric ions form complexes in a molar ratio of 1:1 and that a proton is displaced from the hydroxyl group of citric acid in the formation of the complex at *p*H 7. These conclusions are in agreement with our results. However, their data do not define the nature of the complexes as a function of *p*H nor do they permit the derivation of formation constants. Other investigators have not correctly assessed the displacement of protons by the cupric ion[4a] or have limited their study to acid solutions.[4b]

The complexes formed between citrate and ferric ion have not been clearly defined in the neutral *p*H range. The proposals of Bobtelsky and his coworkers[5,6] for the existence of complexes with various ratios of citrate to iron have been criticized by Lanford and Quinan[7] and do not appear to be well founded. The latter workers demonstrated a 1:1 ratio in strongly acid solution and formulated their results in terms of a complex resulting from the displacement of two protons by a ferric ion. While our results confirm the 1:1 complex as the predominant species, three protons were found to be displaced by the iron in acid solution. At a higher *p*H this is followed by the displacement of a fourth proton as in the case of the cupric complex.

### Experimental

Reaction mixtures were prepared in a series of volumetric flasks from standard solutions of cupric nitrate or ferric nitrate and citric acid. Standard sodium hydroxide or nitric acid was added in varying quantity to cover the desired *p*H range. The ionic strength was calculated from a knowledge of the *p*H of each reaction mixture and the concentration of the various ionic species derived from preliminary experiments. It was then adjusted to 0.1 by the addition of sodium nitrate. This value was exceeded in some of the solutions in the iron series at the lowest and highest *p*H values. All experiments were run at a concentration of metal ion of 0.012 $M$ for the copper series and 0.008 $M$ for the iron series. These concentrations allowed

---

(1) This work was carried out under contract with the Office of Naval Research.

(2) R. C. Warner and I. Weber. THIS JOURNAL, **75**, 5094 (1953).

(3) M. Bobtelsky and J. Jordan, *ibid.*, **67**, 1824 (1945).

(4) (a) L. Meites, *ibid.*, **72**, 180 (1950); (b) R. W. Parry and F. W. DuBois, *ibid.*, **74**, 3749 (1952).

(5) M. Bobtelsky and A. E. Simchen, *Compt. rend.*, **208**, 1646 (1939).

(6) M. Bobtelsky and J. Jordan, THIS JOURNAL, **69**, 2286 (1947).

(7) O. E. Lanford and J. R. Quinan, *ibid.*, **70**, 2900 (1948).

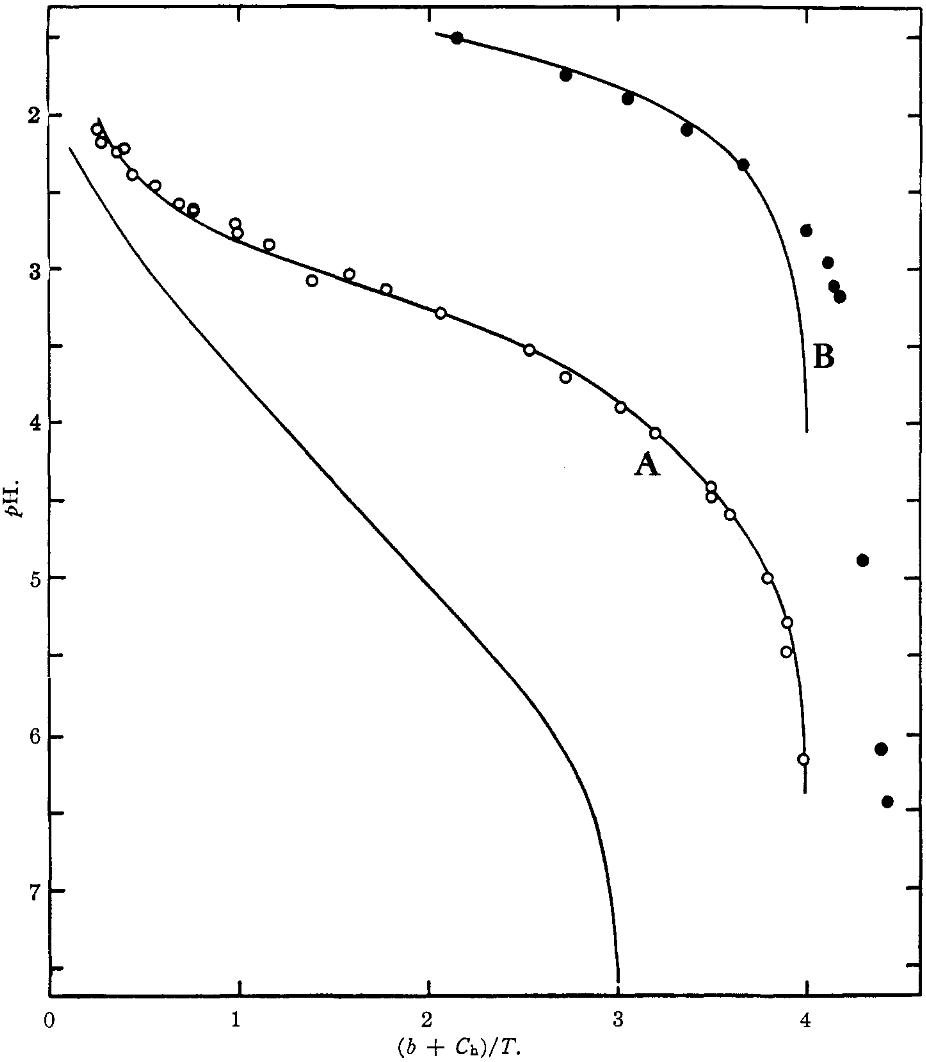

Fig. 1.—Titration of citric acid (lower curve); of 0.012 *M* citric acid plus 0.012 *M* cupric nitrate, A, ○; and of 0.008 *M* citric acid plus 0.008 *M* ferric nitrate, B, ●. In the latter two cases the solid lines are theoretical curves calculated from the constants given in Table I.

spectrophotometric measurements to be made on the same solutions employed for the titration curve.

The titration curves were calculated from the *p*H of each reaction mixture as measured with a Beckman Model G *p*H meter standardized with 0.05 *M* acid potassium phthalate (*p*H 4.00 at 25°). The curves are plotted in Fig. 1 in terms of $(b + C_h)/T$, the corrected equivalents of base added per mole of metal ion. This is equivalent to $b'$ as defined by Simms.[8] In making this calculation it was assumed that $-\log \gamma_{H^+} = 0.1$. Deviations from this assumption will not introduce appreciable errors into the copper series except at the beginning of the titration. Over the more acid *p*H range of the iron citrate titration the error may be greater, although in this range the assumption was in agreement with the direct determination of the *p*H of standard nitric acid in sodium nitrate at an ionic strength of 0.1.

The light absorption measurements were made on a Beckman Model DU spectrophotometer. They are reported in Figs. 2–4 and 6 in terms of the molar extinction coefficient at a given *p*H, $E_h$, based on the total metal concentration present.

Most of the determinations in the copper series were carried out at a temperature of 30°. A less extended series was run at 5° which included titration data and absorption measurements made with a photoelectric colorimeter operated in a cold-room using a 770 mμ filter. The determinations in the iron series were carried out at a room temperature of 24 ± 1°.

All solutions were prepared from reagent grade chemicals The ferric nitrate was standardized by the Zimmerman–Reinhardt method,[9] and the cupric nitrate by iodimetric titration.[9]

The solutions of the iron series were protected from light by preparing them in Pyrex low actinic glass volumetric flasks. When this precaution was not taken the optical density at first increased on exposure to light. This was followed by a slow decrease to an almost colorless solution with accompanying reduction to the ferrous state. The initial increase was negligible during exposure to the spectrophotometer beam provided the readings were taken rapidly.

At *p*H values greater than 2.4 in the iron series, a red color was present in some of the solutions due to the formation of ferric hydroxide. This disturbing factor was reduced to a minimum by arranging the order of addition of reagents so that the ferric nitrate was added last with vigorous shaking. This was effective up to a *p*H of 3.0 and above depending on the ratio of citrate to iron. In the more alkaline solutions in which the red color was initially present, it faded either completely or to a constant value in about 24 hours. The fading was always accompanied by a rise in *p*H. The presence of a permanent red color was noted for a ratio of citrate to iron of 1:1 at *p*H 5.2, for 2:1 at *p*H 6.2 and for 5:1 at *p*H 8.5. At *p*H values more acid than these,

---

[8] H. S. Simms, THIS JOURNAL, **48**, 1239 (1926).

[9] W. Rieman, J. D. Neuss and B. Naiman, "Quantitative Analysis," McGraw-Hill Book Co., Inc., New York, N. Y., 1942, pp. 182, 224.

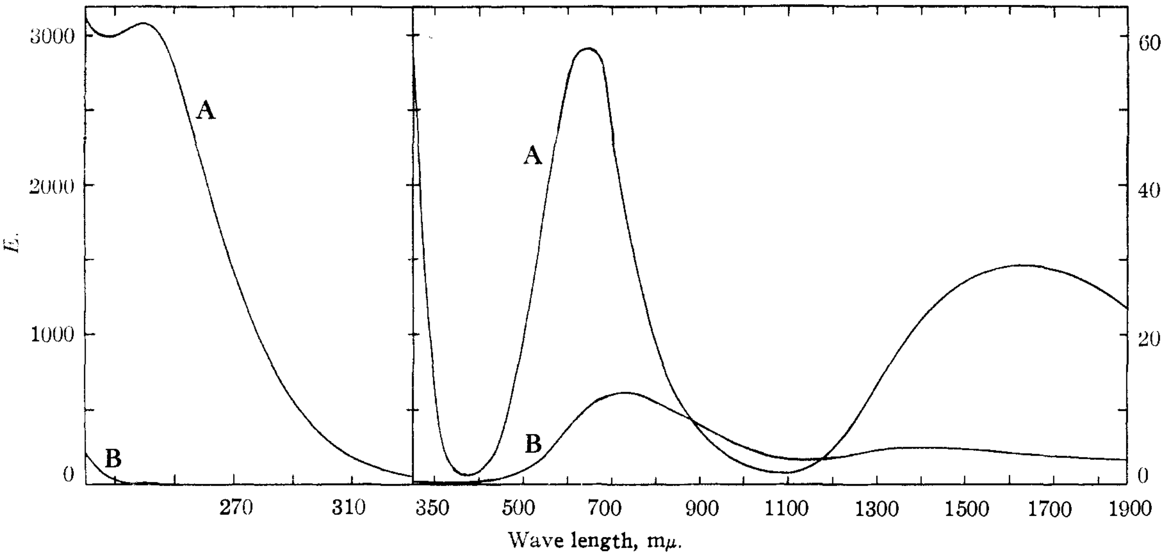

Fig. 2.—Absorption spectra: A, cupric citrate complex at *p*H 7; B, cupric nitrate. The molar extinction coefficients at the left refer to the spectra below 350 mμ and those at the right to the spectra above this wave length.

the optical densities were readily reproducible when the above precautions were taken.

In the copper series equilibrium was established immediately on mixing the solutions and no changes in optical density or *p*H occurred during four hours standing in the light or dark. Under different conditions Parry and DuBois[4] noted some time dependent changes in copper citrate solutions.

## Results

**Cupric Citrate.**—The titration curve of a solution containing equimolar quantities of citric acid and cupric nitrate (Fig. 1) shows that this mixture titrates as a tetrabasic acid. This is in agreement with the observation of Bobtelsky and Jordan[3] that the cupric ion displaces an equivalent of protons per mole from trisodium citrate. A solid cupric citrate, $Cu_2(C_6H_4O_7)$, corresponding to the copper salt of the complex indicated by these observations to be present at *p*H 6.5 and above has been isolated by Parry and DuBois.[4b] A high degree of overlapping of the successive equilibria which determine the curve is indicated by the small *p*H span over which complete neutralization takes place. This overlapping is so extensive that equilibrium constants could not be derived from the titration data above by the application of the usual methods.[10] The resolution of these constants was possible, however, when the spectrophotometric data were used in conjunction with the titration curve.

The absorption spectrum of cupric citrate at *p*H 6.5 is plotted in Fig. 2. Distinct maxima at 750 and 1700 mμ are evident. The peak at 750 mμ is characteristic of the complexes of copper with carboxyl groups.[11] The other two peaks are absent from the spectra of copper complexes of acids such as acetic and tricarballylic and must therefore be due to the properties of the chelate with citrate which evidently involves the hydroxyl group. The changes in extinction with *p*H at 350, 750 and 1700 mμ are recorded in Figs. 3 and 4.[12] These curves may be compared with the titration curve in Fig. 1. The increase in extinction at 750 mμ covers the entire *p*H range of the titration curve. Thus all intermediate ionic species corresponding to the displacement of less than the maxima of four protons must make some contribution to $E_h$. In contrast, the curves at 350 and 1700 mμ begin to rise at a slightly higher *p*H and have essentially reached their maximum values when three equivalents have been titrated. As a consequence these curves have a high slope with respect to the *p*H axis and their shape suggests that they are determined by the formation of a single complex species in which two protons are displaced or ionized in one step.

The following set of equilibria incorporates this assumption in reaction 2

$$H_4Ci + Cu^{2+} = H_3CiCu^+ + H^+ \qquad K_1 = \frac{(H_3CiCu^+)h}{(H_4Ci)(Cu^{2+})} \qquad (1)$$

$$H_3CiCu^+ = HCiCu^- + 2H^+ \qquad K_2 = \frac{(HCiCu^-)h^2}{(H_3CiCu^+)} \qquad (2)$$

$$HCiCu^- = CiCu^{2-} + H^+ \qquad K_3 = \frac{(CiCu^{2-})h}{(HCiCu^-)} \qquad (3)$$

Citric acid is represented as $H_4Ci$ because four protons must be accounted for over the entire span of the curve. The brackets refer to concentrations and *h* to the hydrogen ion activity as measured by the glass electrode. The contribution of the simple carboxylate complex formed in reaction 1 to the curve (Fig. 1) is shown by the displacement of the initial points from the citric acid curve and the concomitant rise in extinction at 750 mμ, but not at 350 mμ. The strength of this association is sufficient to cause a drop in *p*H when cupric nitrate is added to citric acid. The final ionization, reaction 3, results in an appreciable increase in extinction at 750 mμ, but not at 350 mμ.

---

(10) A. E. Martell and M. Calvin, "Chemistry of the Metal Chelate Compounds," Prentice-Hall, Inc., New York, N. Y., 1952, pp. 78–94.

(11) I. M. Klotz, I. L. Faller and J. M. Urquhart, *J. Phys. Colloid Chem.*, **54**, 18 (1950).

(12) The wave length 350 mμ was chosen rather than that of the peak at 260 mμ so that the measurements could be made on the same solutions used in obtaining the titration curves.

Before arriving at this scheme all other possible ways of assigning the titratable protons either singly or in pairs among reactions similar to (1), (2) and (3) were tried. Calculations of the type indicated below, based on these alternative equilibria were made. Without considering these in detail, it can be stated that some of the other schemes could account reasonably well for the titration curve. However, the spectrophotometric curve at 350 mμ could not be accounted for on the basis of the concentration of any single species or combination of species predicted by formulations other than reactions 1 to 3. In assembling these alternative schemes no consideration was given to complexes in which the ratio of citrate to cupric ion was other than 1:1. Evidence for this ratio has been presented by Bobtelsky and Jordan.[3] We have investigated the combining proportions by the method of continuous variations.[13] Curves were obtained at 750 mμ at a total concentration of citrate plus copper of 0.024 $M$ and at constant $p$H values of 3.1 and 6.1. Although the precipitation of cupric hydroxide precluded the use of those solutions containing a large excess of copper at $p$H 6.1, each curve had a maximum extinction at a citrate to copper ratio of 1:1 thus supporting this combining ratio for the complex.[14]

In addition to the mass law equations, the following relations will hold

$$T_{\mathrm{Ci}} = (\mathrm{H_4Ci})/p + (\mathrm{H_3CiCu^+})/r \qquad (4)$$

$$T_{\mathrm{Cu}} = (\mathrm{Cu^{2+}}) + (\mathrm{H_3CiCu^+})/r \qquad (5)$$

where

$T_{\mathrm{Ci}}$ = total concn. of citrate

$T_{\mathrm{Cu}}$ = total concn. of copper

$$r = \frac{h^3}{h^3 + K_2h + K_2K_3} \text{ and}$$

$$p = \frac{h^3}{h^3 + K_{a1}h^2 + K_{a1}K_{a2}h + K_{a1}K_{a2}K_{a3}}$$

$K_{a1}$, $K_{a2}$ and $K_{a3}$ are the dissociation constants of citric acid.[15] The acid ionization of the hydrated cupric ion has been neglected.[16] The first term on the right in equation 4 is thus the sum of the concentrations of the several citrate ions while the second term is the sum of the concentrations of the complex species. The relative concentrations of the various species may be defined by

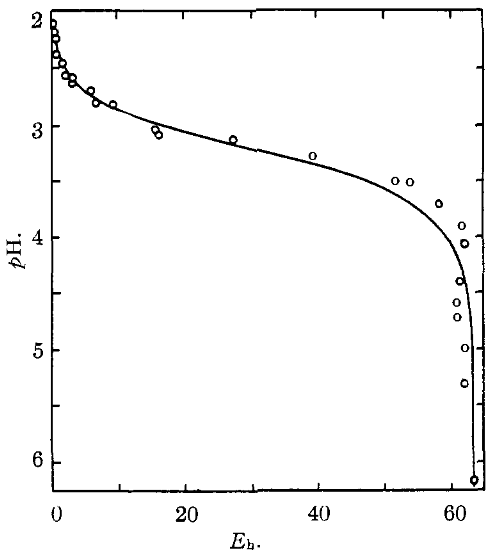

Fig. 3.—Molar extinction coefficient at 350 mμ of 0.012 $M$ citric acid plus 0.012 $M$ cupric nitrate as a function of $p$H. The solid line is a theoretical curve calculated from the constants given in Table I.

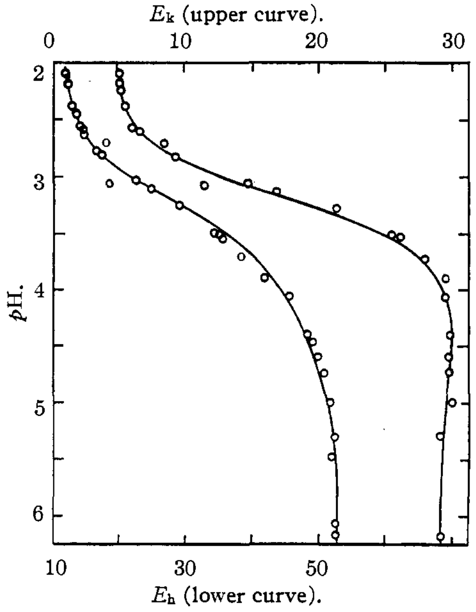

Fig. 4.—Molar extinction coefficients at 1700 mμ (upper curve) and at 750 mμ (lower curve) of 0.012 $M$ citric acid plus 0.012 $M$ cupric nitrate as a function of $p$H. The solid lines are theoretical curves calculated from the constants given in Table I.

$$\alpha = \frac{(\mathrm{H_3CiCu^+})}{T_{\mathrm{Cu}}},\quad \beta = \frac{(\mathrm{HCiCu^-})}{T_{\mathrm{Cu}}}$$

$$\gamma = \frac{(\mathrm{CiCu^{2-}})}{T_{\mathrm{Cu}}} \text{ and } \delta = \frac{(\mathrm{Cu^{2+}})}{T_{\mathrm{Cu}}}$$

Equations 1 to 5 may be combined and solved for any of these relative concentrations. Solving for $\delta$ for the case in which $T_{\mathrm{Ci}} = T_{\mathrm{Cu}}$

$$\delta^2 + \delta\frac{hr}{K_1pT_{\mathrm{Cu}}} - \frac{hr}{K_1pT_{\mathrm{Cu}}} = 0 \qquad (6)$$

---

(13) W. C. Vosburgh and G. R. Cooper, THIS JOURNAL, **63**, 437 (1941); P. Job, *Ann. chim.* (*Paris*), **9**, 113 (1928).

(14) Measurements were also made at 350 mμ on the same solutions. Greater extinctions were found in the presence of excess copper than at a ratio of 1:1. This may indicate the formation of a species containing more copper than citrate. However, in view of the quantitative displacement of four protons when the ratio is 1:1, it is improbable that such a species is significant under the conditions of our experiments. There was no indication in any of the curves for a complex containing more citrate than copper and these also have been neglected. The evidence of Parry and DuBois[4b] for a 2:1 complex was derived from solutions containing a large excess of citrate. In the presence of less citrate, the predominance of a 1:1 complex is indicated by their data. A quantitative comparison of our results with those of Parry and DuBois cannot be made because of the large difference in ionic strength between the two sets of measurements.

(15) These constants are defined in the same way as $K_1$, $K_2$ and $K_3$ in terms of $h$ and the concentration of the appropriate ions. Values at 30° of $pK_{a1} = 2.94$, $pK_{a2} = 4.44$ and $pK_{a3} = 5.82$ were derived from the citric acid titration curve shown in Fig. 1 and were used in the calculations. They are in agreement with similarly defined constants interpolated from the data of R. G. Bates and G. D. Pinching, THIS JOURNAL, **71**, 1274 (1949), for an ionic strength of 0.1. At 5°, $pK_{a1} = 3.02$, $pK_{a2} = 4.50$ and $pK_{a3} = 5.80$ were used.

(16) K. J. Pedersen, *Kgl. Danske Videnskab. Selskab., Mat. fys. Medd.*, **20**, No. 7 (1943).

The other relative concentrations can be obtained from $\delta$ and equations 1 to 5.

The quantity $(b + C_h)$ determined experimentally can be defined in terms of the species in equations 1 to 3 by

$$b + C_h = (H_4Ci)/p' + (H_3CiCu^+)/r' \qquad (7)$$

where

$$r' = \frac{h^3}{h^3 + 3K_2h + 4K_2K_3} \text{ and } p' = \frac{h^3}{K_{a1}h^2 + 2K_{a1}K_{a2}h + 3K_{a1}K_{a2}K_{a3}}$$

This can be simplified using equations 1 to 6 to give

$$\frac{b + C_h}{T_{Cu}} = p/p'\delta + r/r'(1 - \delta) \qquad (8)$$

Similarly the observed extinction coefficient, $E_h$, can be described by

$$E_h = \alpha E_{H_3CiCu} + \beta E_{HCiCu} + \gamma E_{CiCu} + \delta E_{Cu} \qquad (9)$$

Equations 8 and 9 define the experimental data in terms of the constants $K_1$, $K_2$ and $K_3$ and the extinction coefficients. The equations are not suitable for the direct estimation of these constants and cannot be modified to obtain them one at a time in restricted regions of $p$H because of the high degree of overlapping, particularly between $K_1$ and $K_2$. Preliminary values were therefore substituted into equations 9 and 10 and $(b + C_h)/T_{Cu}$ and $E_h$ at 350 mμ were obtained for comparison with the experimental data. In these calculations it was assumed that at 350 mμ, $E_{Cu} = E_{H_3CiCu} = 0$ and that $E_{HCiCu} = E_{CiCu}$ = maximum extinction observed at $p$H 6 to 7. The preliminary values for the constants were obtained as follows. $K_3$ was estimated by the method of Simms[8] since this ionization is reasonably well isolated from that of equation 2 and $\delta$ is very small in this $p$H region. $K_1$ was approximated from the data at the low $p$H end of the titration curve by assuming $r = r' = 1$ in equations 6 and 8. $K_2$ was obtained from Fig. 3 by means of the approximate relation, $pK_2 = 2p\text{H}_{0.5}$, where $p\text{H}_{0.5}$ is the $p$H at which $E_h$ has attained half of its maximum value. $K_1$ and $K_2$ were then varied systematically to achieve the best fit with the experimental data, most weight being given to the titration curve.

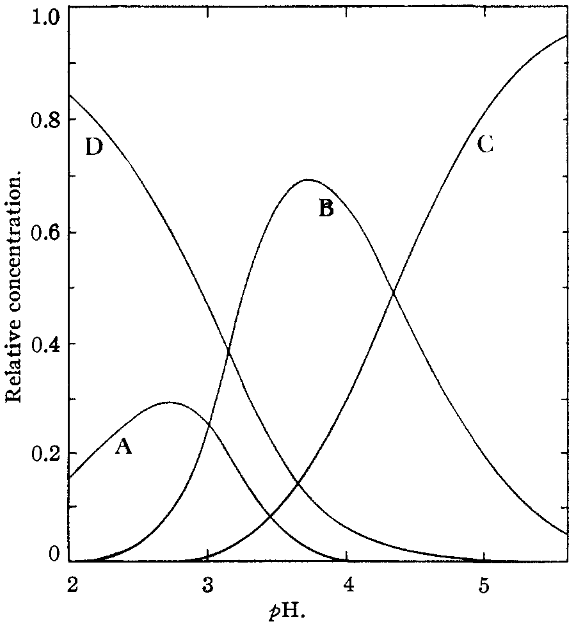

Fig. 5.—Relative concentrations of the various species present in a solution of 0.012 *M* citric acid plus 0.012 *M* cupric nitrate as a function of $p$H. The calculated curves represent the relative concentrations as follows: A, $\alpha = H_3CiCu^+/T_{Cu}$; B, $\beta = HCiCu^-/T_{Cu}$; C, $\gamma = CiCu^{2-}/T_{Cu}$; D, $\delta = Cu^{2+}/T_{Cu}$.

The constants obtained as a result of these calculations were used to determine the extinction coefficients which would account for the spectrophotometric curves at 750 and 1700 mμ. This involved estimating $E_{H_3CiCu}$ and $E_{HCiCu}$ since $E_{Cu}$ and $E_{CiCu}$ were known in both cases. The fit obtained at 1700 mμ is a confirmation of the value derived for $K_2$ since the contribution of $E_{H_3CiCu}$ is small and $E_{HCiCu}$ is close to $E_{CiCu}$. The fit at 750 mμ is arbitrary because all species make significant contributions and no independent estimates can be made of the intermediate extinction coefficients. The final values for the equilibrium constants and extinction coefficients are given in Table I. The solid lines in Figs. 1, 3 and 4 have been calculated from these values. High precision cannot be claimed for the constants derived by this method. We feel, however, that the results adequately account for the data and clearly establish the correctness of the equilibria assumed in reactions 1 to 3. The calculated relative concentrations of the various ionic species as a function of $p$H are plotted in Fig. 5.

TABLE I

EQUILIBRIUM CONSTANTS AND EXTINCTION COEFFICIENTS

| | 30° | | 5° |
|---|---|---|---|
| | Cupric citrate | | |
| $pK_1$[a] | 0.70 | | 0.70 |
| $pK_2$ | 6.00 | | 6.12 |
| $pK_3$ | 4.35 | | 4.46 |
| $pK_4$ | −2.15 | | −2.04 |
| | 350 mμ | 750 mμ | 1700 mμ |
| $E_{Cu}$[b] | 0.3 | 10.2 | 4.2 |
| $E_{H_3CiCu}$ | 0.0 | 19.4 | 7.6 |
| $E_{HCiCu}$ | 63.3 | 43.3 | 32.0 |
| $E_{CiCu}$ | 63.3 | 53.2 | 29.1 |
| | Ferric citrate | | |
| | | | 470 mμ |
| $pK_{1,2}$ | 1.82 | $E_{HCiFe}$ | 14 |
| $pK_3$ | 1.92 | $E_{CiFe}$ | 95 |
| $pK_4$ | −9.46 | | |

[a] $pK = -\log K$. [b] Molar extinction coefficient.

In order to utilize these data in our study of the copper binding of conalbumin[2] it was necessary to have the equilibrium constant for the reaction

$$HCi^{3-} + Cu^{2+} = CiCu^{2-} + H^+, K_4 = \frac{(CiCu^{2-})h}{(HCi^{3-})(Cu^{2+})} \qquad (10)$$

$K_4$ is readily obtained from the relation $K_4 = K_1K_2K_3/K_{a1}K_{a2}K_{a3}$ and is included in Table I.

The constants derived in a similar manner from the titration curve at 5° are also recorded in Table I.

**Ferric Citrate.**—The titration curve of an equimolar mixture of citric acid and ferric nitrate is

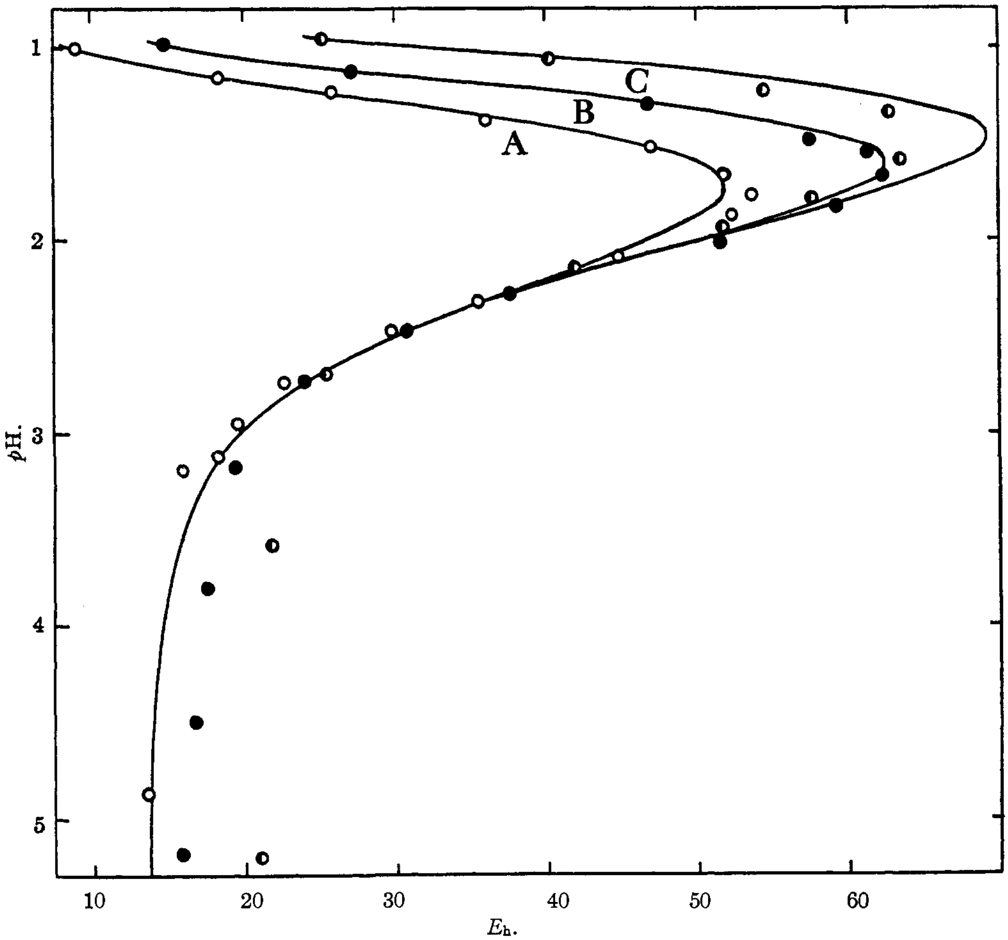

Fig. 6.—Molar extinction coefficients at 470 mμ of 0.008 *M* ferric nitrate plus 0.008 *M* citric acid (curve A), plus 0.016 *M* citric acid (curve B) and plus 0.040 *M* citric acid (curve C) as a function of *p*H. The solid lines are theoretical curves calculated from the constants given in Table I.

shown in Fig. 1. Somewhat more than two equivalents of protons have been ionized before the addition of any base. The first complex is thus so strong an acid that the determination of its formation constant cannot be made by titration. Because of this limited usefulness of the titration data and the experimental difficulties previously mentioned, it has not been possible to define this system as precisely as the cupric citrate equilibria. The curve (Fig. 1) indicates the neutralization of a little more than 4 equivalents per mole of metal ion at about *p*H 3. The end-point is not so well defined as for the copper, but the titer does not rise above 4.2 equivalents except at high *p*H values where $Fe(OH)_3$ is present. The formation of a complex, $CiFe^-$, similar to that of the cupric ion is evidently completed at a *p*H of about 3. The origin of the excess 0.2 equivalent is uncertain, but may be due to some contribution from a ferric citrate hydroxyl complex. The spectrophotometric curves in Fig. 6 were obtained on the same solutions employed in the titration as well as on others with added nitric acid or excess citric acid. In the absence of absorption maxima, the measurements were made at 470 mμ. Although there are also increases in extinction in the ultraviolet corresponding to the formation of the complexes, no detailed studies were made at these wave lengths because of the large interfering absorptions of nitric acid, $Fe^{3+}$ and $FeOH^{2+}$.[17]

These data can be accounted for by assuming that 3 protons are displaced by $Fe^{3+}$ in the formation of the first complex, HCiFe, with a corresponding increase in extinction. The ionization of the fourth proton would then yield $CiFe^-$ and would correspond to the decrease in extinction (Fig. 6). The ratio of citrate to iron of 1:1 established by Lanford and Quinan[7] was confirmed in a separate experiment by the method of continuous variation[14] carried out at a constant *p*H of 2. The possible formation of some 2:1 complex is discussed later. These processes can be described by equations analogous to those for the copper system except that the reaction corresponding to equation 1 is negligible.

$$H_4Ci + Fe^{3+} = HCiFe + 3H^+, \quad K_{1,2} = \frac{(HCiFe)h^3}{(H_4Ci)(Fe^{3+})} \tag{11}$$

$$HCiFe = CiFe^- + H^+ \qquad K_3 = \frac{(CiFe^-)h}{(HCiFe)} \tag{12}$$

$$T_{Ci} = (H_4Ci)/p + (HCiFe)/r \tag{13}$$

$$T_{Fe} = (Fe^{3+})/s + (HCiFe)/r \tag{14}$$

$$(b + C_h) = (H_4Ci)/p' + (Fe^{3+})/s' + (HCiFe)/r' \tag{15}$$

---

[17] E. Rabinowitch and W. H. Stockmayer, THIS JOURNAL, **64**, 335 (1942).

where

$$r = \frac{h}{h + K_3} \text{ and } r' = \frac{h}{3h + 4K_3}$$

$s$ and $s'$ are exactly analogous functions to $p$ and $p'$ except that the constants appearing in them are those for the three steps in the acid ionization of the hydrated ferric ion as defined by Lamb and Jacques.[18] They are included because the hydrolysis products, particularly $Fe(OH)^{2+}$, may be present at appreciable concentrations in some $p$H regions. The relative concentrations of the various species are defined as for the copper system except that there is no species corresponding to $H_3CiCu^+$ and therefore $\alpha = 0$. Rearrangement of equations 11 to 15 yields

$$(\beta/r)^2 - (\beta/r)\left[\frac{T_{Ci}}{T_{Fe}} + 1 + \frac{h^3 r}{K_{1,2}T_{Fe}ps}\right] + \frac{T_{Ci}}{T_{Fe}} = 0 \tag{16}$$

and

$$\frac{b + C_h}{T_{Fe}} = \left(\frac{T_{Ci}}{T_{Fe}} - \frac{\beta}{r}\right)(p/p') + \left(1 - \frac{\beta}{r}\right)(s/s') + \frac{\beta}{r'} \tag{17}$$

At 470 m$\mu$ the absorption of the free ferric[17] ion can be neglected and the spectrophotometric equation can be written

$$E_h = \beta E_{HCiFe} + \gamma E_{CiFe} \tag{18}$$

The constants were derived from these equations as described above except that most weight was necessarily given to the spectrophotometric data. $E_{CiFe}$ was calculated from the extinction approached above $p$H 3 where $\gamma = 1$. $E_{HCiFe}$ could not be obtained independently and was treated as a parameter except that a preliminary value was estimated from the maximum extinction observed in separate experiments at low ionic strength in excess citric acid. Preliminary values for $K_{1,2}$ and $K_3$ were estimated, respectively, from the extreme acid end of the curves assuming $r = 1$ and $\gamma = 0$ and from the $p$H region from 2.2 to 3 assuming $\beta = r$. These estimates were adjusted to obtain $(b + C_h)/T_{Fe}$ and $E_h$ in best agreement with the experimental data. The final values are entered in Table I and were used to calculate the solid lines in Figs. 1 and 6. These theoretical curves account for the extinction measurements except for the deviations in excess citrate when the $p$H rises above 3. This increased extinction may be a result of the formation of some dicitrate complex when appreciable concentrations of the mono- and di-anions of citric acid accumulate. When the experiments for ratios of 2:1 and 5:1 were extended to a $p$H of 6 to 8, $E_h$ dropped to the value of 14 found for the 1:1 ratio at $p$H 3 to 4. The drop was succeeded at higher $p$H's by the rise in extinction described above which was associated with the appearance of a permanent red color. The drop may coincide with a repression of the dicitrate complex due to the increasingly large negative charge which such a species would bear as the $p$H increased. While these observations do not define the contribution of a dicitrate complex, it is concluded that the 1:1 complexes are the predominant species not only below $p$H 3, but also near neutrality as long as sufficient citrate is present to prevent the formation of ferric hydroxide (about 5-fold excess over iron).

The possibility that more than two complex species are formed has been neglected. The curves could undoubtedly be fitted on the basis of such assumptions since two additional parameters would be introduced for each species assumed. The values assigned to these parameters would necessarily be somewhat arbitrary and it is unlikely that such a possibility could be distinguished from the simpler scheme of reactions 11 and 12 by means of the experimental data at hand.

Calculations were made based on other ways of assigning protons between the two complex species. None of these was satisfactory. When only two protons were assumed to be displaced in the first step and the parameters were adjusted to the best fit at one ratio of citrate to iron, the calculated extinctions at the other ratios differed from the experimental by as much as 100%. The calculated titration curve also fell on the alkaline side of the experimental curve. Our data are thus not in agreement with the conclusion of Lanford and Quinan[7] that the formation of the first complex involves the displacement of two protons. They drew this conclusion from the dissociation of the complex as a function of $p$H, as determined by indirect spectrophotometric measurement of the free ferric ion concentration. Since the $p$H range in their experiment was only 0.3 of a unit and the calculation depends so critically on the difference between two large numbers, not much weight can be given to this determination.[19] Our data do not permit a direct determination of the number of displaced protons by means of a linear plot such as that used by Lanford and Quinan without prior assignment of values for $K_3$ and $E_{HCiFe}$. Our conclusion is thus based on the fact that the results at all ratios of citrate to iron can be accounted for only when reactions 11 and 12 are assumed. Additional experiments at an ionic strength of 0.5 were performed to examine the acid end of the curves over the wider $p$H range permitted by this change in conditions. Ratios of citrate to iron both above and below 1:1 were used. These data are not presented in detail, but are consistent with the above experiments in the choice of the number of protons displaced and the value derived for $E_{HCiFe}$. Some allowance was made for changes in the equilibrium constants due to the increase in ionic strength.

The equilibrium constant for the reaction

$$HCi^{3-} + Fe^{3+} = CiFe^- + H^+,\ K_4 = \frac{(CiFe^-)h}{(HCi^{3-})(Fe^{3+})} \tag{19}$$

was derived for use elsewhere.[2] $K_4$ was obtained as in the case of the copper series from $K_4 = K_{1,2} \cdot K_3/K_{a1}K_{a2}K_{a3}$ and is included in Table I.

## Discussion

The interpretation presented here of the reaction of ferric and cupric ions with citrate indicates a

---

[18] A. B. Lamb and A. G. Jacques, THIS JOURNAL, **60**, 1215 (1938). Values of $k_1 = 2.5 \times 10^{-3}$, $k_2 = 2 \times 10^{-5}$ and $k_3 = 4 \times 10^{-7}$ were used in the calculations. The last two may be neglected over most of the $p$H range of the experiments.

[19] It can be calculated readily that an error in the optical densities reported by Lanford and Quinan of ±0.002 or a little over ±1% will introduce an uncertainty of ±18% into the slope of the linear plot from which the number of displaced protons was deduced.

similarity in the behavior of these two metal ions. In both cases the first stable chelate formed as the *p*H is increased is a species, HCiFe or $HCiCu^-$, in which three protons have been displaced by the metal. This species is formed in the case of copper only after a *p*H is reached at which citric acid is appreciably ionized. Under these circumstances the simple copper carboxylate complex, $H_3CiCu^+$, is formed in significant concentration.[20] The corresponding iron complex is negligible because the equilibria involving HCiFe are established at a much lower *p*H. A fourth proton is ionized in both cases leading to $CiFe^-$ or $CiCu^{2-}$. The existence of ions of this type has previously been suggested in the case of copper[2,4] but not of iron citrate. In addition to the analysis above, evidence for such ions is provided by the fact that ferric citrate can be removed from solution by ion exchange with the chloride form of an anion-exchange resin even at a *p*H as low as 2. No anionic complexes can be formulated at any ratio of citrate to iron at a *p*H below the region of ionization of citric acid without displacing more than three protons per ferric ion. Similarly, ferric citrate migrates electrophoretically as an anion over a wide *p*H range.

One of the four protons presumably is displaced from the hydroxyl group of citric acid. This is not necessarily the last proton displaced as suggested by Bobtelsky and Jordan.[3] No decision of this nature can be made from titration data. However, it seems reasonable to associate the formation of the ultraviolet absorbing chelate with this proton displacement since the copper carboxyl complexes do not have this property. The chelate, HCiFe or $HCiCu^-$, would thus involve coördination of two carboxylate groups and one hydroxylate group with the metal. Exploration of the various possibilities with the Fisher–Hirshfelder molecular models also suggests that this is the most reasonable formulation. That the final ionization leading to $CiFe^-$ or $CiCu^{2-}$ is accompanied by coördination of the third carboxylate ion with the metal is suggested by the concomitant change in light absorption and the fact that the ionization constant for this reaction is greater than $K_{a_3}$ for citric acid. Because of the small size of the citrate anion as a quadridentate chelating agent, the species $CiCu^{2-}$ probably cannot be formulated as a square, coplanar complex which is characteristic of most cupric complexes. It might, however, have a pyrimidal structure such as that suggested for some divalent platinum complexes.[21] Possible structures for the iron chelate are not clear. The fact that the coördination number of the ferric ion is six, may be responsible for the tendency noted above toward formation of dicitrate complexes.

While complexes with a citrate to metal ratio of 1:1 appear to be the only ones significant under the conditions of our experiments, it is possible that in solutions with a large excess of either citrate or metal, other complexes may be of importance. This is particularly true of the copper system in the *p*H range where $H_3CiCu^+$ is the predominant species and evidence for dicitrate complex has been found by Parry and DuBois.[4b] Evidence has been presented for other ratios in the case of iron by Bobtelsky and Jordan.[6] However, the latter authors have failed to appreciate the effect of the displacement of protons and the accompanying changes in *p*H in their various titrations. All of their conductance and extinction titrations can be qualitatively understood in terms of reactions 11 and 12 and the fact that the extinction coefficient for $CiFe^-$ is much lower than for HCiFe. The breaks in their curves therefore do not correspond to ratios of citrate to iron in the complexes, but rather to arbitrary points at which the *p*H goes through a minimum.

The determination of the equilibrium constant for the copper series at two temperatures permits the calculation of approximate values of the thermodynamic functions for the reactions involved. These have been assembled in Table II, based on calculations from the constants given in Table I and the dissociation constants of citric acid used previously. Reactions 1, 2 and 3 are seen to be similar to most ionization reactions of carboxyl groups in that $\Delta H^0$ is small and the magnitude and sign of $\Delta F^0$ is primarily determined by the entropy change. The comparison of the effect of dissociating $Cu^{2+}$ from the chelate and $H^+$ from citric acid is most easily made by considering the reaction

$$H_2Ci^{2-} + Cu^{2+} = CiCu^{2-} + 2H^+ \qquad (20)$$

for which calculated values have been included in Table II. The reaction is formulated in this way to make it as nearly "isoelectric" as possible thus reducing electrostatic contributions to the thermodynamic functions and minimizing the effect of employing equilibrium constants based on the activity of $H^+$ and on the concentration of other species.[22] Employing the relative molal entropies of $Cu^{2+}$ and $H^+$ in solution −26.5 and 0.0 e.u., respectively,[23] the relative entropy of the ion $CiCu^{2-}$ can be estimated to be approximately 38 e.u. lower than that of $H_2Ci^{2-}$. The small effect of size of the ion should be in the opposite direction. This decrease is probably to be correlated with the structural restrictions imposed on the ion by the formation of the chelate. Since the calculated difference between the entropy of $HCiCu^-$ and $H_3Ci^-$ is or almost is the same as

TABLE II

THERMODYNAMIC FUNCTIONS—CUPRIC CITRATE

| Reaction | Equilib. constant | $\Delta F^0$, cal. mole$^{-1}$ at 30° | $\Delta S^0$, e.u. | $\Delta H^0$, cal. mole$^{-1}$ |
|---|---|---|---|---|
| 1 | $pK_1$ | 970 | − 3 | 0 |
| 2 | $pK_2$ | 8320 | −21 | 1850 |
| 3 | $pK_3$ | 6030 | −14 | 1700 |
| 10 | $pK_4$ | −2980 | 15 | 1700 |
| 20 | | 5090 | −12 | 1390 |

---

(20) The constant for the reaction, $H_3Ci^- + Cu^{2+} = H_3CiCu^+$, leading to the formation of this species is equal to $pK_1 - pK_{a_1} = -2.24$. This may be compared with the $pK$ of −2.164 obtained by K. J. Pedersen, *Kgl. Danske Videnskab. Selskab., Mat. fys. Medd.*, **22**, No. 12 (1945), for the corresponding acetate complex at zero ionic strength.

(21) Reference 10, p. 261.

(22) The error in $\Delta S^0$ due to the use of concentrations may be estimated in equation 20 by the use of the Debye–Hückel extrapolation to be not over 2 e.u.

(23) W. M. Latimer, K. S. Pitzer and W. V. Smith, THIS JOURNAL, **60**, 1829 (1938).

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

RECEIVED MARCH 12, 1953

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 m$\mu$ 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] |
| | 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).