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THE SPECIFIC BINDING OF IRON(III) AND COPPER(II) TO TRANSFERRIN AND CONALBUMIN

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SUMMARY

The binding of Fe^{3+} to transferrin has been studied by pH titrations and equilibrium dialysis. In the pH range studied (7.5–8.9), 3 H^+ are released for each Fe^{3+} bound. In the dialysis experiments, EDTA and citrate were used as competing chelating agents. It was found that the binding of Fe^{3+} to transferrin may be described as coordination to two equivalent and independent sites.

The nature of the binding sites in transferrin as well as conalbumin has been studied by measurements of ESR and visible spectra. The ESR spectra of the Fe complexes indicate that the metal is present as Fe^{3+}. Measurements of ESR and optical absorption as a function of the degree of binding in transferrin, coupled with the known binding constants, exclude strong interactions between the 2 Fe^{3+}, and indicate that the distance between them is greater than 9 Å. Attempts have been made to fit the experimental data to a spin-Hamiltonian and the results discussed in terms of the geometrical configuration of the complex. In the case of both Fe^{3+} and Cu^{2+}, the ESR spectra of transferrin and conalbumin are very similar. The Cu^{2+} spectra show hyperfine structure from 2–3 N nuclei.

Urea denaturation destroys the specific bonding of Fe^{3+} in transferrin, as shown by the parallel loss of color and ESR signal. The nature of the chelating sites in transferrin and conalbumin is discussed in relation to the present and earlier data. It is suggested that each chelating site involves 2 imidazole groups of the protein in addition to 3 tyrosyl residues.

INTRODUCTION

Transferrin, the iron-binding β_1-globulin from plasma, has been extensively studied by many investigators, and their findings have been comprehensively reviewed[1–3]. The protein has been crystallized and its molecular weight determined as 88000. Each molecule of the colorless apoprotein specifically binds 2 Fe^{3+} ions (*cf.* DISCUSSION) to yield an intensely colored complex with an absorption maximum at 470 mμ. However, Cu^{2+} and Zn^{2+} can also be bound to the protein in a ratio of 2 metal ions per molecule of transferrin, but the affinity is much less than that for Fe^{3+}. An

Abbreviation: ESR, electron-spin resonance.

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attempt to measure the dissociation constants for Fe^{3+}–transferrin has recently been reported[4]; the extremely strong binding indicates that highly specific polydentate chelate sites are involved.

Conalbumin is a protein from egg white, displaying a remarkable similarity to transferrin with respect to metal-binding activity[1]. Its specificity for metal ions as well as the visible spectra of its metal complexes are nearly identical with the corresponding properties of transferrin. Recent rotatory dispersion[5] and structural studies[6] give further indications of the close relation between the metal binding sites of the two proteins. WARNER AND WEBER[7], on the basis of pH titrations and spectral data, have proposed that Fe^{3+} in conalbumin is chelated to the phenolic groups of 3 tyrosine side chains of the protein as well as to 1 HCO_3^- ion from solution. It has generally been assumed that both metal-binding sites in conalbumin, as well as in transferrin, are identical, even if the data of WARNER AND WEBER[7] indicate a strong interaction between the sites.

In an attempt to get further information on the nature of the metal-binding sites in these two proteins, we have utilized the ESR technique as well as measurements of visible and ultraviolet spectra. Further measurements of the strength of binding by the equilibrium dialysis technique have also been performed in the case of transferrin, as its metal-binding properties have not been as extensively studied as those of conalbumin[7].

MATERIAL AND METHODS

Proteins

Transferrin samples prepared by two different methods were employed. Most experiments were carried out with protein supplied by AB Kabi, Stockholm (Sweden) through the generosity of Mr. H. BJÖRLING. It was prepared from Cohn Fraction IV by chromatography on DEAE-Sephadex, and was approx. 80 % pure, as determined from its ratio of absorbancy at 280 and 470 mμ. Some experiments were performed with transferrin kindly supplied by Drs. P. FLODIN and B. GELOTTE, AB Pharmacia, Uppsala (Sweden). It was prepared by direct gel filtration of serum, followed by chromatography on DEAE-Sephadex[8], and contained no contaminating protein, as judged from the absorbancy ratios.

Four times crystallized conalbumin, in the metal-free form, was obtained from Sigma Chemical Co.

Reagents

Deionized water was used in making up all solutions. Analytical-grade salts were used without purification as the source of metal ions, but buffer and base electrolyte solutions were purified by dithizone extraction. Reagent-grade urea was recrystallized from water before use.

Preparation of apotransferrin and method of reconstitution

Endogenous iron was removed by acidifying a solution of the protein (approx. 5 %) to pH 3.5 with dilute HCl in the presence of a 10-fold excess of citric acid. The Fe^{3+}–citrate chelate thus formed was removed by adding IRA-401 ion-exchange resin (approx. 25 g/100 ml). The apotransferrin solution was decanted and brought

to pH 7.5 with 1 N NaOH and dialyzed at 4° for 48 h against several changes of water or the buffer to be used in subsequent experiments. The protein contained less than 0.001% iron, as determined with the 1,10-phenanthroline method[9].

Fe^{3+} and Cu^{2+} were in general added to the apoproteins in the form of the citrate chelates; the low concentrations of free metal ions thus achieved eliminate non-specific binding. Standard 0.01 M $Fe(NO_3)_3$ and $Cu(NO_3)_2$ solutions were prepared in 0.1 N HNO_3 and mixed with equal volumes of 0.01 M citrate solution. The solutions were brought to pH 7.5 with 1 N NaOH and diluted to give a 0.004 M concentration of the chelates.

Liberation of H^+ on binding of Fe^{3+} to apotransferrin

About 4 ml of an unbuffered, approx. 3% solution of apotransferrin, was brought to various pH values between 7.5 and 9 by the addition of 0.1 N NaOH. The solution was placed at 25° in an autotitrator (bi-functional recording titrator, International Instrument Co., Canyon, Calif.), 250 μl of 1.00 mM $FeCl_3$ solution was added and the volume of 0.0100 N NaOH required to bring the pH back to its original value measured. A stream of N_2 was passed over the solution to minimize uptake of CO_2, but the HCO_3^- concentration in solution was sufficient to allow complex formation (*cf.* ref. 7), as judged from the color development. The excess of transferrin and CO_2 was sufficiently great so that the same consumption of NaOH and increase in color were recorded each time in two or three successive additions of Fe^{3+}.

Equilibrium dialysis technique

The binding of Fe^{3+} to transferrin was studied by the equilibrium dialysis method with radioactive iron (^{59}Fe, obtained as the citrate complex from Amersham Radiochemical Centre, Great Britain), utilizing the technique recently described in detail[10]. Most equilibrations involved the uptake of Fe^{3+} by metal-free transferrin but some experiments in which Fe^{3+} was dialyzed out from ^{59}Fe–transferrin were also performed. The transferrin concentration used was always about $2 \cdot 10^{-5}$ M and the total amount of Fe^{3+} was just sufficient to saturate the two specific metal-binding sites. To achieve partial dissociation, a competing chelating agent must be present, and citrate and EDTA were employed. To minimize non-specific binding, the concentration of free Fe^{3+} must be small. Thus, the total concentration of the chelating agent must be greater than that of Fe^{3+}; the lowest concentration used was 10^{-4} M. From preliminary measurements at different pH values and concentrations of chelating agent, approximate association constants were estimated. On the basis of these constants, it was calculated (see EQUILIBRIUM EQUATIONS) that, with concentrations of chelating agents in the range 10^{-4} M to 10^{-1} M, the pH should be between 6 and 7 in the case of citrate and between 8 and 9 in the case of EDTA to obtain binding values in the entire range between 0 and 2 metal ions per molecule of transferrin. No buffer was included but all solutions were adjusted to the desired pH with NaOH. At the lower pH, no HCO_3^- was added but the solutions were allowed to equilibrate with the atmospheric CO_2, and the equilibrium concentration of HCO_3^- was calculated (see EQUILIBRIUM EQUATIONS). At the higher pH, the equilibrium concentration of HCO_3^- is too high to allow equilibration in a reasonable time, so that the calculated concentration of HCO_3^- was added immediately after the adjustment of pH. In the case of citrate, 0.1 M $NaNO_3$ was included and the temperature was 25°, while with

EDTA the solutions contained 0.1 M KCl and the temperature was 20°, as the stability constants for the respective chelates have been determined under these conditions[11,12].

The equilibrium dialysis experiments with citrate were carried out in a small, thermostated room which was shut off from the rest of the laboratory but whose air was rapidly exchanged with outside air through an efficient fan. The CO_2 content of the air in the room was determined by infrared absorption measurements in a URAS-1 gas analyzer (Hartmann and Braun, Frankfurt (Germany); we are indebted to Drs. P. HOLMGREN and O. BJÖRKMAN for carrying out this analysis).

Spectral measurements

Visible and ultraviolet spectra were measured at 22° in a Zeiss RPQ20A recording spectrophotometer with quartz cells; a 1-cm light path was generally employed.

The ESR spectra were recorded with a Varian V-4500 spectrometer using 100 kcycles/sec field modulation. Most spectra were obtained from frozen solutions; the spectrometer then operated at a microwave frequency close to 9170 Mcycles/sec.

Denaturation experiments

A series of urea solutions of different concentrations were made in 0.025 M phosphate buffer (pH 7.0) and 7.0 ml of approx. 5% Fe^{3+}–transferrin in the same buffer was added to 10 ml of each urea solution. After 2 and 4 h at 22°, the absorbancy of the solutions at 470 mμ was measured.

EQUILIBRIUM EQUATIONS

As 3 H^+ are released for each Fe^{3+} bound to metal-free transferrin (see RESULTS), it is convenient to designate the apoprotein as H_6Tr^n, so that the stepwise binding of Fe^{3+} can be described by the following equations*, in which the symbols given under the molecular species represent their molar concentrations:

$$\underset{p_0}{H_6Tr^n} + \underset{m}{Fe^{3+}} + \underset{b}{HCO_3^-} \rightleftharpoons \underset{p_1}{H_3TrFe\,(HCO_3)^{n-1}} + \underset{h}{3H^+} \qquad K_1 = \frac{p_1 \cdot h^3}{p_0 \cdot m \cdot b} \tag{1}$$

$$\underset{p_1}{H_3TrFe\,(HCO_3)^{n-1}} + \underset{m}{Fe^{3+}} + \underset{b}{HCO_3^-} \rightleftharpoons \underset{p_2}{TrFe_2\,(HCO_3)_2^{n-2}} + \underset{h}{3H^+} \qquad K_2 = \frac{p_2 \cdot h^3}{p_1 \cdot m \cdot b} \tag{2}$$

The experimentally determined quantity r, representing the average number of metal ions bound per molecule of protein, is described by the following relation (see ref. 1):

$$r = \frac{p_1 + 2p_2}{p_0 + p_1 + p_2} \tag{3}$$

At a given pH and concentration of HCO_3^-, apparent constants K_1' and K_2',

* In all calculations, the approximation of setting the activities equal to molar concentrations has been used. In view of the low concentrations, this is probably justified except in the case of the protein, since the charges around the binding site of a single protein molecule are not separated by dilution (see ref. 1; *cf.* DISCUSSION). In analogy with the results with conalbumin[7], 1 HCO_3^- has been assumed to partake in the binding of each Fe^{3+}.

not involving h and b, can be used, and these are related to r by the following equation, derived from Eqns. 1–3:

$$r = \frac{K_1'm + 2K_1'K_2'm^2}{1 + K_1'm + K_1'K_2'm^2} \tag{4}$$

The relation between K_1 and K_2 can be given as:

$$K_2 = \frac{1}{4} RK_1 \tag{5}$$

so that $R = 1$ if the two binding sites are identical and show no interaction (*cf.* ref. 1). K_1' is then given by the following equation, derived from Eqns. 4 and 5:

$$K_1' = \frac{2}{Rm(2-r)}\left[(r-1) \underset{(-)}{+} \sqrt{(r-1)^2 + r(2-r)R}\right] \tag{6}$$

To calculate K_1' from Eqn. 6, m must be known. It can be determined from the total Fe^{3+} concentration on the non-protein side of the dialysis cell with the aid of the known stability constants[11,12] of the chelates used. If EDTA is designated as H_4A, the pK values for the dissociation of the first two H^+ are so low[12] that these can be considered as fully dissociated in the pH range used (8–9). Thus, only the following two equilibria need to be considered in the binding of Fe^{3+} to EDTA:

$$\underset{a_1}{HA^{3-}} + \underset{m}{Fe^{3+}} \rightleftharpoons \underset{a_3}{FeA^-} + \underset{h}{H^+} \qquad K_3 = \frac{a_3 \cdot h}{a_1 \cdot m} \tag{7}$$

$$\underset{a_2}{A^{4-}} + \underset{m}{Fe^{3+}} \rightleftharpoons \underset{a_3}{FeA^-} \qquad K_4 = \frac{a_3}{a_2 \cdot m} \tag{8}$$

In Eqn. 7, $K_3 = K_4 \cdot K_{HA}$ where K_{HA} is the acid dissociation constant for HA^{3-}. However, at high pH the Fe^{3+} in the chelate undergoes hydrolysis, so that the following two reactions must also be included in a calculation of m:

$$\underset{a_3}{FeA^-} + \underset{o}{OH^-} \rightleftharpoons \underset{a_4}{FeA(OH)^{2-}} \qquad K_5 = \frac{a_4}{a_3 \cdot o} \tag{9}$$

$$\underset{a_4}{FeA(OH)^{2-}} + \underset{o}{OH^-} \rightleftharpoons \underset{a_5}{FeA(OH)_2^{3-}} \qquad K_6 = \frac{a_5}{a_4 \cdot o} \tag{10}$$

The total concentration of EDTA will be designated as a and that of Fe^{3+} in the non-protein cell as t:

$$a = a_1 + a_2 + a_3 + a_4 + a_5 \tag{11}$$

$$t = m + a_3 + a_4 + a_5 \tag{12}$$

In Eqn. 12, m is much smaller than the sum of the other terms under the conditions used and can be neglected. On the basis of this approximation, the following expression for m can be derived from Eqns. 7–12:

$$m = \frac{t(h + K_{HA})}{K_4K_{HA}(a-t)\{1 + K_{50}(1 + K_{60})\}} \tag{13}$$

In the calculations of m by Eqn. 13, the values of the constants determined by SCHWARZENBACH AND HELLER[12] have been used.

In the case of citrate, a somewhat different formulation must be used. If the acid is designated as $H_3Ci(OH)$, it is present as $HCi(OH)^{2-}$ and $Ci(OH)^{3-}$ in the pH range used (6–7). When Fe^{3+} is bound to $Ci(OH)^{3-}$, a proton is liberated[11,13]. WARNER AND WEBER[11] interpreted this as a displacement of the proton on the –OH of the acid, while HAMM *et al.*[13] attributed it to hydrolysis of Fe^{3+} in the chelate. While the latter alternative may seem more likely in view of the high pK value of the –OH group and of the hydrolysis constants for other Fe^{3+} chelates[14], it can be shown that both formulations lead to the same mathematical expression for m, the only difference being the physical meaning of the constants involved. As under the conditions used, the constants of WARNER AND WEBER[11] must be employed, their formulation will also be followed. The liberation of an additional proton is attributed to hydrolysis in both studies[11,13] but occurs only above pH 8 and can be neglected in the present experiments. Thus, the following reactions must be considered:

$$\underset{c_1}{HCi(OH)^{2-}} + \underset{m}{Fe^{3+}} \rightleftharpoons \underset{c_3}{FeCiO^-} + 2\,\underset{h}{H^+} \qquad K_7 = \frac{c_3 \cdot h^2}{c_1 \cdot m} \tag{14}$$

$$\underset{c_2}{Ci(OH)^{3-}} + \underset{m}{Fe^{3+}} \rightleftharpoons \underset{c_3}{FeCiO^-} + \underset{h}{H^+} \qquad K_8 = \frac{c_3 \cdot h}{c_2 \cdot m} \tag{15}$$

In Eqn. 14, $K_7 = K_8 \cdot K_{HCi(OH)}$, where $K_{HCi(OH)}$ is the acid dissociation constant of the third carboxyl group in citric acid. If the total concentration of Fe^{3+} is again designated as t and that of citrate as c (*cf.* Eqns. 11, 12), the following expression for m can be derived from Eqns. 14, 15:

$$m = \frac{th\,(h + K_{HCi(OH)})}{K_8 K_{HCi(OH)} \cdot (c - t)} \tag{16}$$

In the calculations of m by Eqn. 16, the values of the constants determined by WARNER AND WEBER[11] have been used.

The concentration of HCO_3^- (b) at a given pH can be calculated by the following relation[15], if the partial pressure of CO_2 in the air (p_{CO_2}) is known:

$$\frac{h \cdot b}{Q p_{CO_2}} = K \tag{17}$$

where Q is the coefficient of Henry's law and K a constant (see ref. 15).

RESULTS

Number of H^+ released on binding of Fe^{3+} to apotransferrin

The experiments were carried out as described under METHODS, where details of volumes and concentrations are given. At pH 7.5, two successive additions of Fe^{3+} lead to the consumption of 70.5 and 71.1 µl of NaOH, respectively, corresponding to 2.82 and 2.87 H^+ liberated per Fe^{3+} bound. At pH 7.7, the corresponding values were 71.2 and 71.6 µl or 2.85 and 2.86 H^+, and at pH 8.9, 73.1 µl or 2.92 H^+. The

slight increase at higher pH is probably related to a small uptake of CO_2. The measured release of H^+ required 3–5 min in all cases. It should be noted that the H^+ released cannot be attributed to hydrolysis of the Fe^{3+} added, since any H^+ thus derived from H_2O are again neutralized by OH^- liberated from the $Fe(OH)_3$ on binding to the protein.

The strength of binding of Fe^{3+} to transferrin

EDTA was the first competing chelating agent tried, since it had been used in an earlier study of Fe^{3+} binding to transferrin[4]. However, when the equilibrium was approached in different directions, the same equilibrium constants were not obtained, indicating that true equilibria had not been attained. When the dissociation of Fe^{3+} from the metal-containing protein was measured at pH about 9, a measurable dissociation after 1 week was only obtained with 0.1 M EDTA. The in-velocities were found to be greater, but even in this direction several weeks were required for equilibration, as shown by data in Table I. Apparent binding constants at different times, calculated from Eqns. 1, 6, 13 and 17, are also included. The CO_2 content of the air was assumed to be 0.04 vol. % in the calculations (*cf.* below). The following values of the constants were used: $pK_{HA} = 10.26$, $\log K_4 = 25.1$, $\log K_5 = 6.45$, $\log K_6 = 4.53$, $pK_w = 14.17$, $Q = 0.0383$ and $pK = 6.148$ (refs. 12 and 15). The protein was not denatured during the experiment despite the long time and high pH, as the color corresponding to the binding of 2 Fe^{3+} always developed when excess Fe^{3+} was added to protein solution from the dialysis cells.

With citrate, equilibrium was reached more rapidly (*cf.* ref. 7), and a 5-day

TABLE I

THE BINDING OF Fe^{3+} TO TRANSFERRIN WITH EDTA AS THE COMPETING CHELATING AGENT

Various concentrations of EDTA, Fe^{3+} and 0.1 M KCl were present in both cell compartments (volume, 2 ml each). The transferrin concentration was $2.0 \cdot 10^{-5}$ M. The cell contents were analyzed after 1, 2 and 3 weeks of dialysis time at 20° (in the calculations, all concentrations have been corrected for small volume changes at the long times).

Dialysis time (weeks)	*Concn. of EDTA (M)*	*Concn. of Fe^{3+} ($M \times 10^6$)*		*pH*	*r*	*$-\log m$*	*Apparent*	
		Protein comp.	*Non-protein comp.*				*$\log K_1$*	*R*
1	10^{-4}	2.93	0.76	9.08	1.09	26.5	3.2	
1	10^{-3}	2.74	1.03	9.02	0.86	27.2	2.9	
1	10^{-2}	2.43	1.28	9.05	0.58	28.2	3.1	0.003
1	$3 \cdot 10^{-2}$	2.30	1.42	9.05	0.44	28.6	3.3	
1	10^{-1}	2.19	1.61	9.08	0.29	29.2	3.4	
2	10^{-4}	3.31	0.49	9.14	1.41	26.7	3.5	
2	10^{-3}	3.04	0.70	9.14	1.17	27.6	3.8	
2	10^{-2}	2.69	1.03	9.15	0.83	28.5	3.5	0.015
2	$3 \cdot 10^{-2}$	2.54	1.26	9.13	0.64	28.9	3.6	
2	10^{-1}	2.32	1.46	9.08	0.43	29.2	3.7	
3	10^{-4}	3.32	0.38	9.10	1.47	26.8	3.5	
3	10^{-3}	3.04	0.63	9.13	1.21	27.7	3.8	
3	10^{-2}	2.80	0.95	9.12	0.93	28.5	3.8	0.03
3	$3 \cdot 10^{-2}$	2.56	1.17	9.10	0.70	28.8	3.7	
3	10^{-1}	2.32	1.47	9.05	0.43	29.1	3.8	

TABLE II

THE BINDING OF Fe^{3+} TO TRANSFERRIN WITH CITRATE AS THE COMPETING CHELATING AGENT

Various concentrations of citrate, Fe^{3+} and 0.1 M $NaNO_3$ were present in both cell compartments (volume, 2 ml each). The transferrin concentration was $2.0 \cdot 10^{-5}$ M. The cell contents were analyzed after 5 days of dialysis at 25°.

Concn. of citrate (M)	Concn. of Fe^{3+} ($M \times 10^3$)		pH	r	$-\log m$	$\log K_1$ ($R = 1$)
	Protein comp.	Non-protein comp.				
$1.05 \cdot 10^{-4}$	3.40	0.22	6.62	1.51	17.72	3.06
10^{-4}	2.78	0.17	6.59	1.30	17.77	3.02
10^{-3}	2.88	0.72	6.70	1.08	18.28	2.89
$3 \cdot 10^{-3}$	2.52	1.24	6.65	0.64	18.46	2.87
$6 \cdot 10^{-3}$	2.28	1.51	6.56	0.39	18.58	3.05
10^{-2}	1.88	1.71	6.42	0.08	18.58	2.86
$3 \cdot 10^{-2}$	1.83	1.74	6.35	0.045	18.96	3.27

equilibration period was used. The primary data from a set of dialysis cells are given in Table II, which also includes binding constants calculated on the assumption that $P = 1$ by use of Eqns. 1, 6, 16 and 17. The following values of the constants were used: $pK_{HCl(OH)} = 5.82$, $\log K_8 = 9.49$, $Q = 0.0337$ and $pK = 6.116$ (refs. 11 and 15). The effect of different R values on the calculated K_1 values (Eqn. 6) is shown in Fig. 1.

The CO_2 content of the air of the room used for citrate experiments was found to be 0.0360 vol. % on the final day of the equilibration period. This corresponds

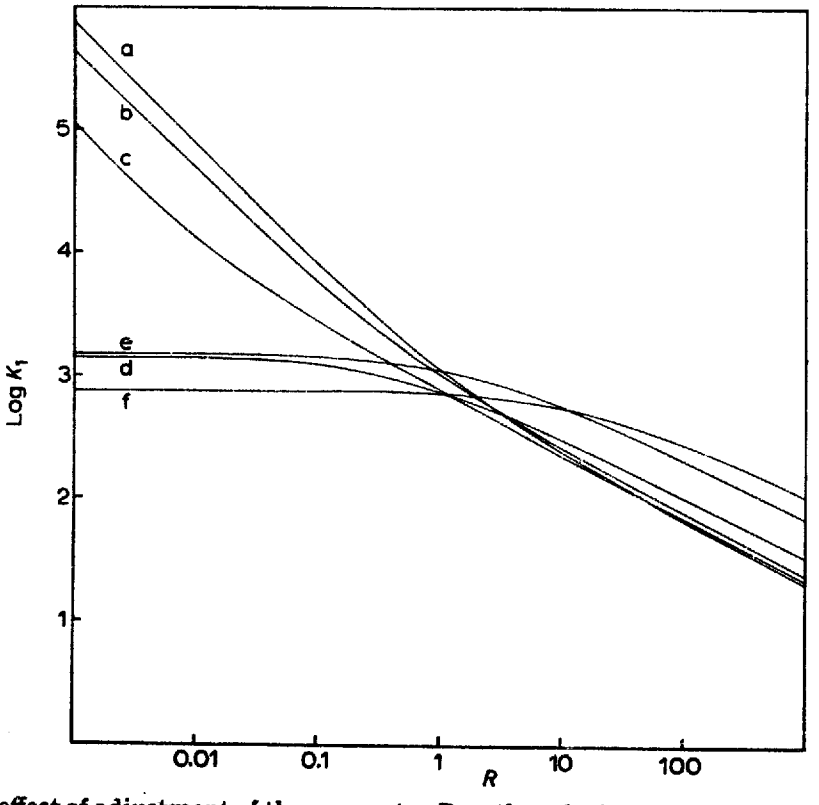

Fig. 1. The effect of adjustment of the parameter R on the calculated value of $\log K_1$ for the binding of Fe^{3+} to transferrin. The binding data of Table II have been used. Citrate concentrations: (a) $1.05 \cdot 10^{-4}$ M, (b) $1 \cdot 10^{-4}$ M, (c) 10^{-3} M, (d) $3 \cdot 10^{-3}$ M, (e) $6 \cdot 10^{-3}$ M, (f) $1 \cdot 10^{-2}$ M.

closely to the CO_2 content of the outside air, which at the time of the year of the experiments (December) varies only with about 0.001 vol. % (P. HOLMGREN, personal communication).

Visible and ultraviolet spectra as a function of the degree of saturation of transferrin with Fe^{3+} or Cu^{2+}

To a series of tubes containing 1.0 ml of 0.13 mM apotransferrin in 0.04 M $NaHCO_3$, increasing amounts of either 3.80 mM Fe^{3+}–citrate or 4.00 mM Cu^{2+}–citrate were added with construction pipettes and the volume was made up to 1.1 ml

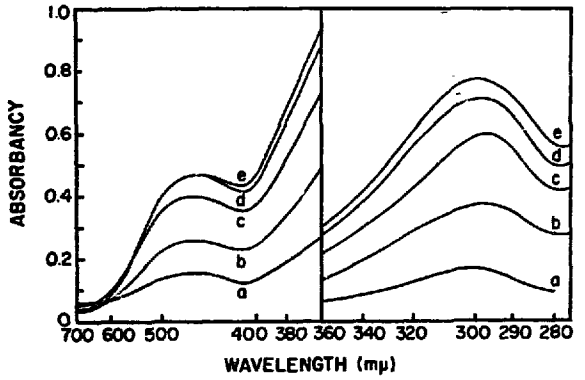

Fig. 2. Optical spectra of $1.2 \cdot 10^{-4}$ M transferrin with increasing concentrations of Fe^{3+}–citrate added. Concentrations of Fe^{3+}: (a) $0.68 \cdot 10^{-4}$ M, (b) $1.36 \cdot 10^{-4}$ M, (c) $2.04 \cdot 10^{-4}$ M, (d) $2.72 \cdot 10^{-4}$ M, (e) $3.40 \cdot 10^{-4}$ M. For measurements below 360 mμ, the solutions were diluted 3 times with 0.04 M $NaHCO_3$.

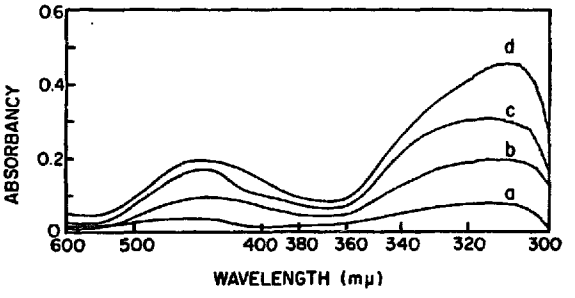

Fig. 3. Optical spectra of $1.2 \cdot 10^{-4}$ M transferrin with increasing concentrations of Cu^{2+}–citrate added. Concentrations of Cu^{2+}: (a) $0.72 \cdot 10^{-4}$ M, (b) $1.44 \cdot 10^{-4}$ M, (c) $2.16 \cdot 10^{-4}$ M, (d) $3.60 \cdot 10^{-4}$ M (light path, 0.4 cm).

with water. The solutions were allowed to stand overnight at 4°. Their spectra, recorded with an equivalent concentration of apotransferrin in the reference cell, are shown in Figs. 2 and 3. The increase in absorption at lower wavelengths at the highest metal concentrations is due to the absorption by the excess citrate chelates. There is a linear relationship between the absorbancy and the concentration of metal added up to saturation at all wavelengths except the highest ones; this is shown at the wavelengths of maximum visible absorption by experiments illustrated in Fig. 4. The molar extinction coefficient, on the basis of metal concentration, as read from this graph is $2.5 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ at 470 mμ for Fe^{3+}–transferrin.

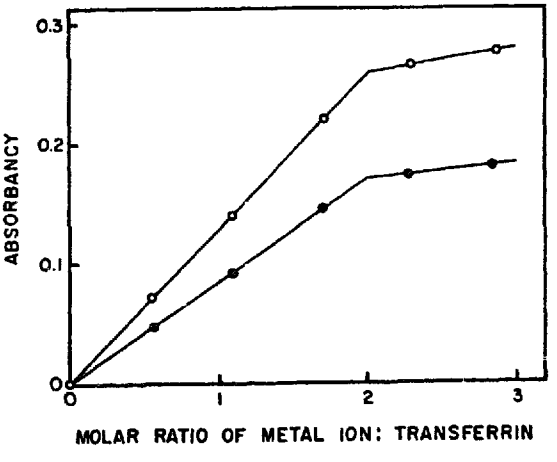

Fig. 4. Increase in absorbancy at 470 mμ for Fe^{3+} (○) and at 440 mμ for Cu^{2+} (●) when increasing amounts of the citrate chelates are added to apotransferrin ($5.2 \cdot 10^{-5}$ M).

A spectrum of Cu^{2+}–transferrin, extended to 1000 mμ, is shown in Fig. 5 and reveals a second, less intense absorption maximum at 670 mμ (extinction coefficient about 350 $M^{-1} \cdot cm^{-1}$ on the basis of Cu^{2+} concentration; the extinction coefficient at 440 mμ is $2.3 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$).

The effect of urea on the visible absorption of Fe^{3+}–transferrin is shown in Fig. 6; no appreciable further decrease occurred if the solutions were allowed to stand for an additional 2 h.

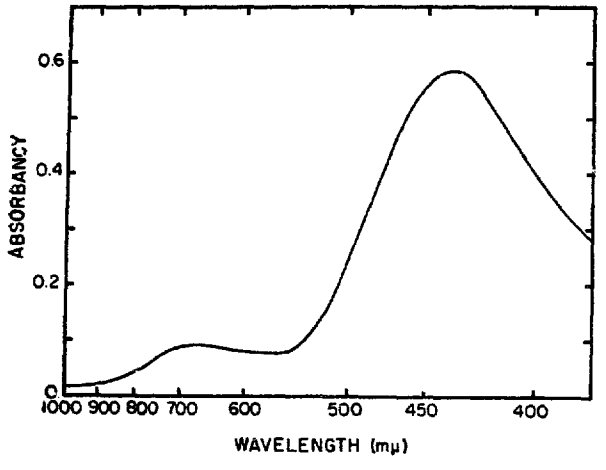

Fig. 5. Optical spectrum of Cu^{2+}–transferrin ($6.4 \cdot 10^{-4}$ M Cu^{2+}) in 0.15 M $NaHCO_3$ and 2.3 mM sodium citrate (light path, 0.4 cm).

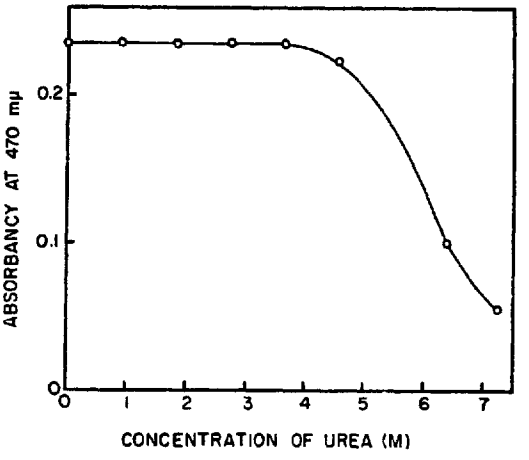

Fig. 6. The effect of urea in the absorbancy at 470 mμ of Fe^{3+}–transferrin (about $6 \cdot 10^{-5}$ M) in 0.25 M phosphate buffer with this buffer as blank.

ESR spectra

The spectrum of a solution of Fe^{3+}-saturated transferrin, frozen to 77° K, is shown in Fig. 7. It consists of a three-component part around $g = 4.14$ and a low-field part around $g = 8.9$. Both preparations used had identical spectra. The lines around $g = 4.14$ are also obtained from samples with molar ratios of Fe^{3+}:transferrin at least down to 0.5 (*cf.* titration experiments below) and also from a sample containing, on the average, 1 Cu^{2+} and 1 Fe^{3+} per protein molecule. In addition to the lines seen in Fig. 7, some samples show weak lines on the wings of the main line.

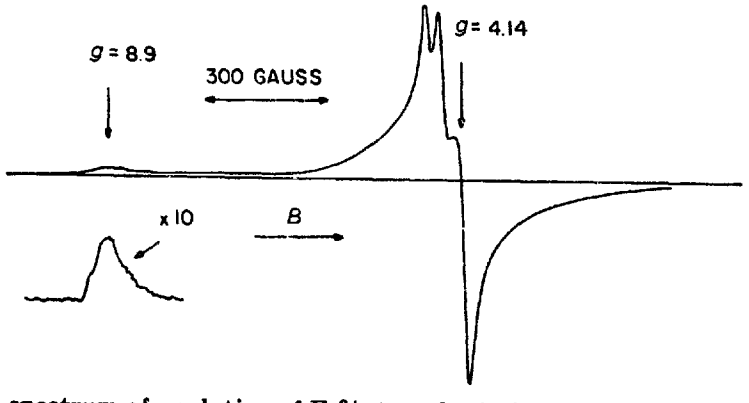

Fig. 7. ESR 3-cm spectrum of a solution of Fe^{3+}–transferrin frozen to 77° K. The arrow marked *B* indicates the direction of increasing magnetic field, in this as well as in Figs. 9–11. In the lower left of the figure part of the spectrum around $g = 8.9$ has been recorded with 10 times greater amplification.

These are thought to be due to non-specifically bound metal ions. No other lines were found. They would have been detected if they had a width not considerably greater than the observed lines, a g-value >0.85 and an amplitude $>0.5\%$ of that of the main line.

The spectrum of samples of freeze-dried Fe^{3+}-saturated transferrin had the same general appearance at 77° K, only slightly less resolved. At room temperature the spectrum of the powder had a width of about 130 gauss and showed no resolved structure.

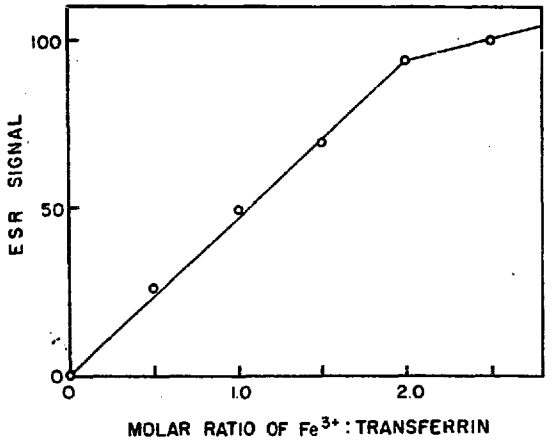

Fig. 8. Amplitude in arbitrary units of the ESR 3-cm signal around $g = 4.14$ of frozen solutions of transferrin with increasing amounts of Fe^{3+}–citrate added. The transferrin concentration was $1.2 \cdot 10^{-4}$ M.

In one experiment we obtained a rough estimate of the intensity of the main line by comparing it with the signal from a frozen solution of Cu^{2+}–histidine[16,17]. The ratio of the areas under the absorption curves (from the first moments) was $I^{Fe}_{obs}/I^{Cu}_{obs} = 0.7$ when normalized to the same metal concentration. The solutions with transferrin saturated to different degrees with Fe^{3+} used in the study of visible and ultraviolet spectra (see above) were also investigated with ESR. The shape of the signal was independent of the amount of Fe^{3+} and the amplitude of the signal then was taken as a measure of the Fe^{3+} content of the samples. The results are shown in Fig. 8. The excess Fe^{3+} at high metal concentrations is present as the citrate complex, which gives a very weak signal at $g = 4.14$ (see below). The sample treated with 7.25 M urea (*cf.* Fig. 6) was also investigated in the ESR apparatus. It was found that the signal had decreased to about 9% as compared to the untreated protein.

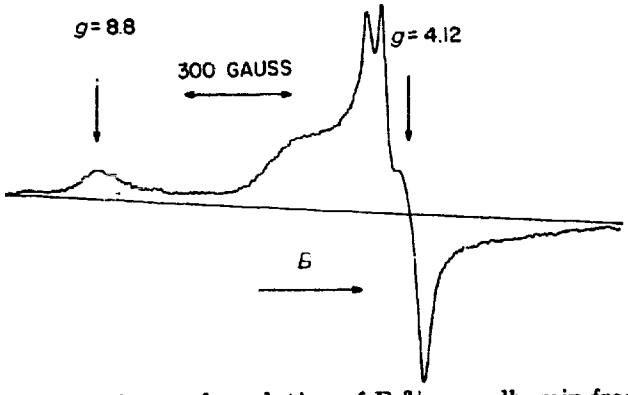

Fig. 9. ESR 3-cm spectrum of a solution of Fe^{3+}–conalbumin frozen to 77° K.

In the spectrum of conalbumin saturated with Fe^{3+} (Fig. 9) the main line around $g = 4.1$ has, within the experimental accuracy, the same g-value and splittings as that of the corresponding line of transferrin. However, on the low-field side there is a strong shoulder, and the $g = 8.8$ line is also much stronger than the corresponding $g = 8.9$ line of Fe^{3+}-saturated transferrin.

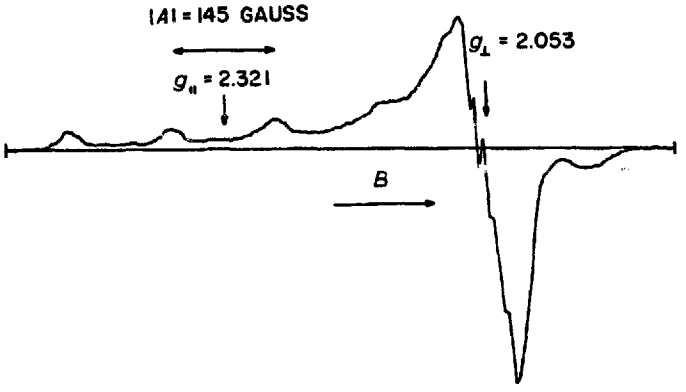

Fig. 10. ESR 3-cm spectrum of a frozen solution of Cu^{2+}–transferrin.

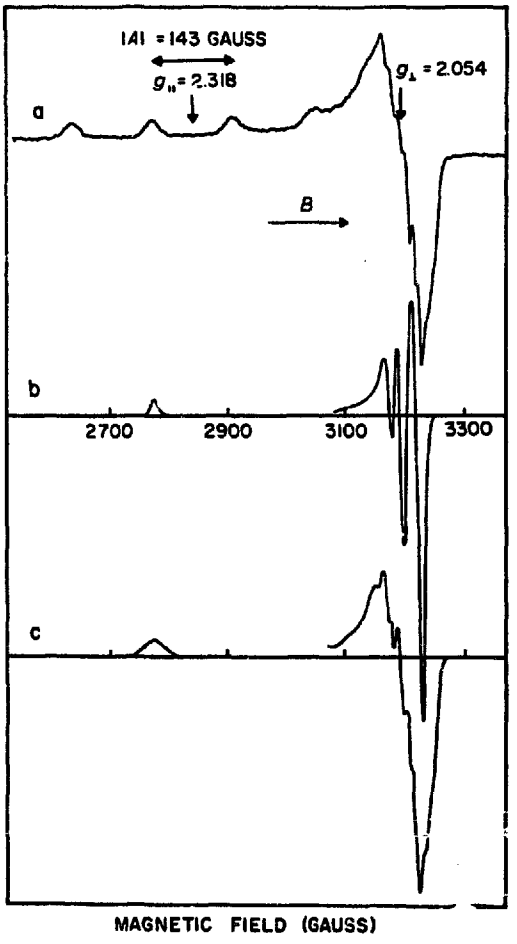

Fig. 11. (a) Experimental ESR 3-cm spectrum of a frozen solution of Cu^{2+}–conalbumin. (b) Part of the ESR spectrum calculated with $g_{\parallel} = 2.318$, $g_{\perp} = 2.054$, $|A| = 143$ gauss, $|B| = 12$ gauss and the width 13 gauss. The line shape is gaussian. (c) Part of the ESR spectrum calculated with the same parameters as in (b) but with isotropic hyperfine interaction of 12 gauss from 2 N nuclei included.

A number of low-molecular-weight complexes of Fe^{3+} in water solutions frozen to 77° K were investigated. All of them, EDTA at pH = 3 and 7.5, *N*-hydroxyethylethylenediaminetriacetic acid, phenol, *o*-dihydroxybenzene, imidazole, citrate and glycine, had a line at $g = 4.1$–4.3. This line was in all cases broader and had less amplitude than the transferrin and conalbumin signals with, if any, a less resolved structure. In particular, the EDTA complex had a rather large amplitude at $g = 4$ and no signal at $g = 2$, while the *o*-dihydroxybenzene complex had a weaker signal at $g = 4$ and a broad signal at $g = 2$ with high integrated intensity. Also the amplitude of the citrate signal was only about 3 % of that of the proteins with the same Fe^{3+} concentration.

Cu^{2+}-saturated transferrin (Fig. 10), as well as transferrin with less amounts of Cu^{2+}, shows in frozen solutions spectra typical of Cu^{2+} complexes[16] with $g_{\|} = 2.321$, $g_{\perp} = 2.053$ and $|A| = 145$ gauss. The extra hump at the highest fields is not present in all samples and is most likely due to non-specifically bound Cu^{2+}. In the resonance close to $g_{\perp}$ there is an additional splitting into at least 7 lines with an average separation of about 13 gauss. This extra structure is also visible in the spectrum of the sample of $Cu^{2+} - Fe^{3+}$ (1:1)–transferrin but not in a sample of Fe^{3+}-saturated transferrin to which Cu^{2+} had been added.

Essentially the same spectrum as from Cu^{2+}-saturated transferrin is obtained from conalbumin saturated with Cu^{2+} (Fig. 11a), although the details of the extra structure around $g_{\perp}$ are slightly different.

DISCUSSION

The liberation of 2.8–2.9 H^+ on the binding of Fe^{3+} to apotransferrin is a further indication of the similarity to conalbumin[7]. The small deviation from an integral value (3) may be attributed to the increase in negative charge of the protein on complex formation (see Eqns. 1, 2), as discussed by WARNER AND WEBER[7]. The fact that the same number of H^+ are liberated between pH 7.5 and 8.9 shows that no groups with pK values between 7 and 9 are involved in metal binding; instead the pK values must be well above 9, as in conalbumin[7]. On this basis, together with spectral evidence, WARNER AND WEBER[7] suggested that 3 phenolic groups are involved. Our ESR data, as will be discussed later, indicate that the metals also are coordinated to at least 2 N atoms.

The equilibrium data in Table II show that the binding of Fe^{3+} to transferrin may well be described as coordination to two equivalent and independent sites (see ref. 1). The average value of log K_1 (excluding the value at $3 \cdot 10^{-2}$ M citrate, where the binding is too low to be accurately measured) is 2.96, giving a log K_2 of 2.36. This is in contrast to the behavior of conalbumin, where WARNER AND WEBER could only account for their binding data by assuming that $K_2 \gg K_1$. While the accuracy of our data is not sufficient to claim that the difference between K_1 and K_2 involves only a statistical factor ($K_1 = 4\,K_2$; see ref. 1), the plots in Fig. 1 show that any possible deviation must be put within very narrow limits ($0.6 < R = 4K_2/K_1 < 6$).

DAVIS *et al.*[4], on the basis of dialysis experiments with Fe^{3+}-saturated transferrin at pH 7.5 in the presence of EDTA, claim that $K_2 \ll K_1$. However, according to the binding constants obtained here, almost no binding should be obtained at the pH and EDTA concentrations used, indicating that true equilibrium was never attained.

At pH about 9, binding should occur but the rate of dissociation is extremely slow (see RESULTS). The equilibrium is more rapidly approached in the other direction but still requires several weeks, as shown in Table I. As the rate is so low, one easily gets the impression that equilibrium has been reached if measurements are taken with intervals of a few days. Measurements far from equilibrium can only be fitted to the equilibrium equations by assuming $R < 1$, as indicated in Table I. However, as equilibrium is approached, the apparent value of R increases. It should be noted that when the data are fitted to the equilibrium equations by adjustment of R, $\log K_1$ becomes almost unaffected by the time of dialysis, which is equivalent to saying that the concentration of molecules with 2 Fe^{3+} can be neglected in the initial stages. The fact that a higher value of $\log K_1$ is found here than from the data of Table II is reasonable, since the negative charge on the protein must increase considerably when the pH is changed from about 6.5 to 9 (*cf.* ref. 1).

It is interesting to compare the absolute values of K_1 and K_2 found here with those found by WARNER AND WEBER[7] for conalbumin at about the same pH (6–7). If, for conalbumin, $K_2 = 100\, K_1$ (see ref. 7), $\log K_1 = 0.1$ and $\log K_2 = 2.1$. Smaller values of R cannot account for the data, while the assumption of larger values would lead to a decrease in K_1 and an increase in K_2. Thus, K_1 for conalbumin is smaller than that for transferrin, while K_2 may be very similar in value. As the ESR data (see below), as well as earlier results[1,5,6], provide strong evidence that Fe^{3+} complexes of transferrin and conalbumin are chemically identical, this would indicate that the interaction observed in conalbumin involves a barrier to the first metal ion bound rather than a strengthening of the binding of the second one.

The sharp $g = 4.14$ signal from transferrin and conalbumin as shown in Figs. 7 and 9 is, with great certainty, due to Fe as the signal is obtained from both proteins, is proportional to the amount of Fe bound (*cf.* titration experiments) and has an intensity (see below) which excludes the possibility that it is due to impurities. As the signal can be observed at room temperature without very much broadening, we think that the signal arises from Fe^{3+} and not from Fe^{2+}. Further evidence for this assumption of the valence state is obtained from measurements of the static susceptibility by EHRENBERG AND LAURELL[18]. They showed that the susceptibility corresponded to an effective magnetic moment of 6.08 ± 0.01 Bohr magnetons. This is higher than what one expects from high spin $S = 2$ Fe^{2+} (4.9–5.64 Bohr magnetons[19]) and more close to the theoretical susceptibility for high spin $S = 5/2$ Fe^{3+} (5.92 Bohr magnetons). The measured susceptibility is even higher than this value and therefore a possible ferromagnetic type of interaction between the 2 Fe^{3+} ions on a molecule was discussed[18], although not found probable. For transferrin, where the binding constants are rather equal, we have at low molar ratios of Fe^{3+}: transferrin an appreciable concentration of protein with only 1 Fe^{3+}, and therefore by the titration experiments we have a possibility to see whether the spectral properties of such molecules are different from those of Fe^{3+}-saturated transferrin. For instance assuming $R = 6$, which is the maximum value consistent with equilibrium data (*cf.* Fig. 1) and a molar ratio of Fe^{3+}: transferrin of 0.5, 47 % of all Fe^{3+} is bound to transferrin with only 1 Fe^{3+}. Now, in our titration experiments, the ESR and optical spectra have the same shape when the Fe^{3+} concentration is lowered and the intensities are proportional to the concentration (Figs. 4 and 8). Thus, any strong interaction, such as a ferromagnetic exchange interaction, which could lead to an increased suscepti-

bility, can be ruled out as such interaction certainly would strongly modify at least the ESR spectrum. Also, strong magnetic dipole–dipole coupling can be excluded. From the ESR spectra we estimate that possible dipolar broadening is less than say 30 gauss, which in turn would suggest a distance between the two ions greater than 9 Å as calculated from the formula given by BLOEMBERGEN *et al.*[20]. By similar argument we find that the details of the ESR resonance structure at $g = 4.14$ are not due to any other type of interaction such as a modification of the site of the first Fe^{3+} when the second Fe^{3+} goes on.

The above discussion about possible interaction was given for transferrin. For conalbumin WARNER and WEBER showed that $K_2 \gg K_1$ and thus the metal ions have a strong tendency to attach to this protein in pairs. This makes it much more difficult to get information about molecules with only 1 Fe^{3+}. Now, the spectral properties of transferrin and conalbumin are rather similar, the only significant difference being the strong $g = 8.9$ line and the shoulder in the ESR spectrum of conalbumin which is not found in transferrin. Although this difference is not understood, we think that the above conclusions about possible interactions in transferrin are valid also for conalbumin.

Another question of interest is whether Fe^{3+} in the two sites has the same ESR signal, which does not directly follow from our experiment. However, if for instance the obtained spectrum is a superposition of two resonances due to two different sites, then the binding strength of the two sites must be similar. This is an improbable situation and in the following discussion we assume that the main signal with its details is due to one kind of Fe^{3+} only.

We have tried to fit the experimental data to a spin-Hamiltonian of the usual form

$$H = g\beta\vec{B}\cdot\vec{S} + D\left[S_z^2 - \frac{1}{3}S(S+1)\right] + E(S_x^2 - S_y^2)$$

where the g-factor is assumed isotropic and equal to 2.00 for the high-spin Fe^{3+} ion. In ionic crystals usually the first term, which takes account of the interaction with the laboratory field B, is dominant and the other terms can be treated as perturbations. This leads to a spectrum consisting of five lines symmetrically displaced around $g = 2$. In most cases investigated, the D term, representing the axially symmetric crystal–field interaction, is greater than the E term, which has to be included when the symmetry is less than axial. To explain the results from high-spin ferric hemoglobin, one must assume that D is much greater than $g\beta B$ and E. This yields the observed spectrum with the appearant g-value ranging from 6 to 2. CASTNER *et al.*[21] have observed that the narrow and strong $g = 4.266$ signal from Fe in glass can be explained by taking the E term as the dominant term in the spin-Hamiltonian. This then predicts an isotropic line at $g = 30/7 = 4.286$ plus weaker lines at $g = 9.678$, 0.857 and 0.607. We believe that the spin-Hamiltonian also in our case is characterized by a large E term although we meet with some difficulty in explaining results obtained at a wavelength of 1.25 cm, as discussed by two of us (R.A. and T.V.) in the APPENDIX. A single-crystal study would give much more information about the spin-Hamiltonian and therefore we are trying to make sufficiently large crystals. The low-molecular-weight Fe^{3+} complexes which we have investigated also have lines at g-values 4.1–4.3 and similar lines have been found in substances

of biological interest (see, for example, refs. 22–24). Some of these results might also have their explanation in a Hamiltonian with a large E term.

Now one might ask which geometrical configuration can give rise to an E term larger than the other terms in the spin-Hamiltonian. There does not exist a unique solution and two possibilities are discussed by CASTNER *et al.*[21]. One possibility is a tetrahedral arrangement where the charges at the corners of the tetrahedron are equal in pairs. The other is a configuration with six identical neighbours at the same distance from the metal ion, four in a rectangle with the Fe in its center and two on a line through the Fe at right angles to the plane of the rectangle.

The observed intensity I of the sharp line around $g = 4.14$ can be related to the number N of the metal ions present in a given volume through the relation (*cf.* ref. 25):

$$I = \text{const.} \cdot N g_{\perp}^2 \tau p$$

where for transferrin $g_{\perp} = 4.2$ and $\tau = 1$ and for Cu^{2+}–histidine[26] $g_{\perp} = 2.058$ and $\tau = 1.06$. p is a population factor which can be taken as 1 for Cu^{2+} and 1/3 for Fe^{3+} when $kT \gg E$ (*cf.* APPENDIX). With these assumptions, we get $I^{Fe}/I^{Cu} = 1.3$. The deviation from the observed ratio (0.70) is probably within the experimental accuracy. The intensity measurements definitely show that the signal cannot be due to any metal impurity.

The Cu^{2+}–transferrin and conalbumin signals show in their low-field part the hyperfine lines typical for Cu^{2+} originating from the interaction with the Cu nucleus. The hyperfine constant $|A| = 143$–145 gauss $= 0.0155$–0.0157 cm^{-1} has a value which is normal to Cu^{2+} complexes[16]. The constant $4/7\ a'^2 + \kappa$ in Table I of ref. 16 then has a value of 0.78. In order to get a value of a^2 in the same table, we need the wavelength of the appropriate *d–d* transition. Very likely, this is the absorption maximum at 670 mμ $= 14900$ cm^{-1} although this has an extinction coefficient in the higher range for *d–d* transitions. The value of a^2 then becomes 0.71 and we see that the Cu^{2+}–transferrin complex has ESR properties similar for example to Cu^{2+}–EDTA. The very strong absorption maximum at 440 mμ is believed to be a charge-transfer band (used in the ESR calculations it would give $a^2 = 1.08$, which is too high). In some Cu^{2+}–complexes this charge-transfer band might be shifted to still longer wavelength and thus overlap the *d–d* transition, which is perhaps the case for ceruloplasmin and laccase.

The analysis of the ESR data in ref. 16 (*cf.* also ref. 27) was based on the assumption of tetragonal symmetry. Tetrahedral distortions would modify this analysis[28], but the N hyperfine structure (see below) makes large distortions of this kind improbable in our case as tetrahedral distortions tend to reduce the overlap of the unpaired "hole" with the ligands. The spectrum is nicely consistent with the spectrum calculated on the basis of axial symmetry (Fig. 11c) so the crystal field symmetry around Cu^{2+} must be quite different from that of the Fe^{3+} complex.

The additional structure in the high-field part is thought to be due to hyperfine structure from N nuclei. Such a structure has been reported in a number of cases[16,22,29,30]. The obtained resonance is usually complicated but in the case of the phthalocyanine complex[29] where there are about 17 lines in the "perpendicular" resonance it has been possible to account for all the details of the structure by numerical calculations of the resonance line[26]. We have, using the same methods, tried to reconstruct the experimental spectrum from Cu^{2+}–conalbumin and the result is shown

in Fig. 11. The agreement as seen is rather good. The calculations using an isotropic N hyperfine structure agree with the experimental observation that this hyperfine structure is most easily seen at the perpendicular direction. The structure around $g_\perp$ is, even in the absence of N hyperfine structure, fairly complicated (Fig. 11b), and it is of course not easy to determine from the observed spectrum how many N nuclei interact just by counting the number of lines. Assuming axial symmetry, interaction with only 1 N nucleus cannot explain the experimental spectrum. Even if deviations from axial symmetry are taken into account, we are unable to reproduce the spectrum with the assumption that only 1 N nucleus interacts. Furthermore, as the N hyperfine structure is expected to be mainly isotropic both for theoretical reasons and earlier experimental evidences[26,31], the width in the "parallel" resonance should be greater than the experimental width if as many as 4 N nuclei interact equally. Thus, most likely 2 or 3 N nuclei give rise to this extra structure.

The numerical calculations of the resonance lines show that the resolution of the N hyperfine structure is considerably reduced if the width is increased by as little as 5 gauss. The fact that the N hyperfine structure is seen also from a sample of $Fe^{3+}-Cu^{2+}$ (1:1)-transferrin again illustrates the absence of any strong magnetic coupling between ions at the two sites of the protein.

The fact that denaturation destroys the specific binding of Fe^{3+} in transferrin, as shown by the parallel loss of color and ESR signal, indicates that chelate formation with the protein is involved, which was first pointed out for conalbumin by WARNER[32]. This is indeed necessary to account for the high affinity of the specific binding sites. As the proteins specifically bind 2 ions of Fe^{3+} or Cu^{2+} with high affinity (see, for example, ref. 1), the same two sites are undoubtedly involved in the binding of both metals. This is demonstrated by our finding that the specific Cu^{2+}-transferrin signal is absent when Cu^{2+} is added to protein previously saturated with Fe^{3+}. However, the fact that binding of Cu^{2+} leads to the liberation of 2 H^+ only[7], while Fe^{3+} binding leads to the release of 3 H^+, as shown also here, suggests that one less H^+-releasing ligand is involved in the coordination of Cu^{2+}. This may be related to the differences in symmetry of the Fe^{3+} and Cu^{2+} complexes indicated by the ESR studies.

The N hyperfine structure in the Cu^{2+} complexes provide quite conclusive evidence that the metal is bonded to N atoms in at least two ligands. Since the H^+ released are derived from phenolic groups, as shown by titrimetric studies on conalbumin[33], these N-containing ligands must be in their unprotonated form even at the lowest pH (7.5) of our titration experiments. The only groups satisfying this condition are imidazole groups of histidine. It is thus suggested that each chelating site involves three phenolic and two imidazole groups of the protein. This conclusion is also supported by recent determinations of difference titration curves between Fe^{3+}-free and Fe^{3+}-saturated transferrin[34].

It should be noted that attempts to determine the chemical nature of protein chelating sites by correlations with the properties of model complexes must be carried out with caution. It is true that WARNER AND WEBER[7] found that the visible spectra of small complexes containing phenolic hydroxyl groups are related to those of the conalbumin complexes. However, when these same compounds are studied also by ESR, they certainly are found to be no more similar to transferrin or conalbumin than, for example, EDTA complexes.

In order to compare the strength of binding of the metals in transferrin with that

in other chelates, it is necessary to know the association constant for the first metal ion of the protein ion formed, if the phenolic groups involved in binding were fully dissociated. This constant should equal K_1 (Eqn. 1) divided by the third power of the dissociation constant for a single phenolic group. If the pK of the phenolic groups is taken as 11 due to the strong electrostatic effect at the chelating site (*cf.* refs. 33, 34), the association constant becomes about 10^{36}. This constant cannot be directly compared to association constants as usually defined due to the involvement of HCO_3^- in the reaction, but its high value indicates a stability greater than that of most Fe^{3+} chelates known[14].

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APPENDIX

The Fe^{3+}–transferrin ESR spectrum

The main line in the 3-cm ESR spectrum of Fe^{3+}–transferrin (Fig. 7) can be interpreted as a nearly isotropic line with g-values 4.08, 4.27 and 4.41. In a 1.25-cm spectrometer, designed by Dr. R. PETTERSON and one of us (T.V.) and operating with a frequency-stabilized klystron and with 400 cycles/sec field modulation, the same compound gave at 77° K a single line with $g = 4.45$ and a width of about 260 gauss. As compared to a Cu^{2+}–histidine solution, the integrated absorption of this line was approximately the same as at 3 cm.

The appropriate spin-Hamiltonian was given in the preceding discussion (fourth-order terms in the spin variables neglected). CASTNER *et al.*[21] have shown that if the E term is the largest and with the D term as a perturbation to first order we get three g-values symmetrically displaced around $g = 30/7 = 4.286$. This does not fit very well to our 3-cm data and certainly not to the 1.25-cm result. Therefore, we calculated the effect of the magnetic field term to third order (the second order gives no contribution to the g-values) and together with the effect of the D term[21] we get:

$$g_{x,y} = \frac{30}{7} \mp \frac{120}{49}\cdot\frac{D}{E} - \frac{1620}{2401}\left(\frac{\beta B}{E}\right)^2 \qquad (18)$$

$$g_z = \frac{30}{7} - \frac{2880}{2401}\cdot\left(\frac{\beta B}{E}\right)^2 \qquad (19)$$

Taking $D/E = 0.067$ and $\beta B/E = 0.18$ we get $g_x = 4.10$, $g_z = 4.25$ and $g_y = 4.43$ in reasonable agreement with the experimental results at 3 cm. Thus we obtain $E = 0.40$ cm^{-1}, and the condition $kT \gg E$ used in the estimation of the intensity is well fulfilled at 77° K.

Obviously, Eqns. 18 and 19 cannot account for the 1.25-cm result, and therefore exact diagonalization was performed. However, we could not find a suitable transition around $g = 4.45$, because from the high experimental intensity we require it to be nearly isotropic. This is illustrated in Fig. 12 which shows the result of the diagonalization for the case $D = 0$. The only isotropic transition with $E > 0$ occurs for very

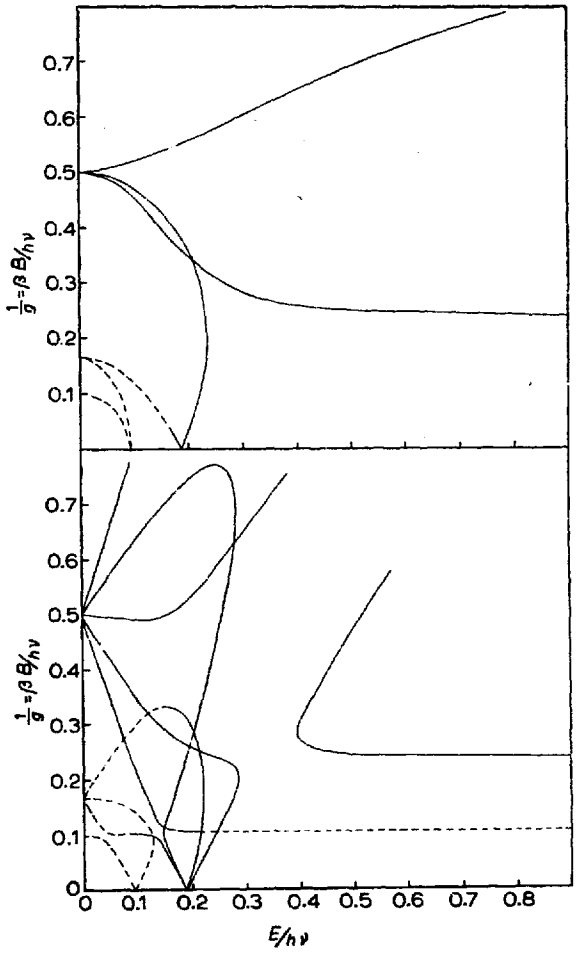

Fig. 12. ESR transitions obtained by diagonalizing the spin-Hamiltonian with $D = 0$ and different values of E. The upper part shows the case when the magnetic field is parallel to the z-axis and the lower part when the field is parallel to x- and y-axis. Dashed lines indicate transitions with small transition probabilities.

large E and corresponds to $g = 4.2$ and from Fig. 12 we see that the g-values for this transition become smaller when $E/h\nu$ is decreased. Elucidation of the 1.25-cm result must therefore await single-crystal studies. We also investigated a glass giving the signal reported by CASTNER *et al.*[21] in the 1.25-cm apparatus and obtained a signal with g about 4.20, which can be well accounted for by Eqns. 17 and 18.

The model proposed by CASTNER *et al.*[21] predicts a second anisotropic transition with the g-values 9.7, 0.86 and 0.61. In the powder spectrum peaks should appear at these g-values but with small amplitudes due to the large anisotropy. We estimate that the amplitude of the $g = 9.7$ line should be only about 1/150 of that of the main line. The observed line at $g = 8.9$ thus appear to have too high intensity (as does the low-field line in glass[21]) and can most likely be interpreted as an impurity in the

same way as the corresponding line in the conalbumin spectrum. The $g = 0.86$ line should have higher intensity than the $g = 8.9$ line but we have not been able to detect it in transferrin (also not in glass).

Thus, although by using a spin-Hamiltonian as described by CASTNER *et al.* we can account for the main features of the spectrum, there are some experimental facts that we cannot explain on the basis of the same theory.

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